Population structure and biofilm formation of *Pseudomonas aeruginosa* isolated from patients with severe burn wounds at Tygerberg Hospital.

by
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Abstract

Pseudomonas aeruginosa is a common opportunistic pathogen which is responsible for more than 11% of nosocomial infections including urinary tract infections (UTI's), bacteraemia, pneumonia and soft tissue infections. Little is known about *P. aeruginosa* associated infections in burn wound patients in South Africa, and in particular at Tygerberg hospital. Burn wound patients are highly vulnerable to infections due to natural defence destruction. *P. aeruginosa* has the ability to form a biofilm and cause persistent biofilm associated infections. The biofilm acts as a protective layer defending organisms against the environment, host immune system and antibiotic treatment. *P. aeruginosa* infections have a mortality rate of 40-50% in burn wound patients.

This study aimed to determine the population structure of *P. aeruginosa* isolated from the burns unit and burns ICU in comparison to isolates from other wards at Tygerberg hospital, to investigate their ability to form biofilms and to determine the impact of various antibiotics on biofilm formation. *P. aeruginosa* isolates from blood cultures, swabs and tissue specimens from adult and paediatric patients at Tygerberg hospital were collected from February 2015 to March 2016. Forty isolates from the burns unit and 40 isolates from outside the burns unit were used for the study. Multiple locus variable number tandem repeat analysis (MLVA) was used for strain typing. Biofilm formation was assessed by crystal violet staining. The strength of biofilm formation of the isolates was determined after a 12h incubation period and the effects of varying concentrations of four different classes of antibiotic on biofilm formation was determined over a 24 hour period.

Forty two different MLVA types were described, of which ten were assigned to two or more isolates. Thirty two MLVA patterns were unique to a single isolate. MLVA type 1 was the most abundant MLVA type; 60% of the isolates from the burns unit and burns ICU were type 1. The predominance of a single MLVA type within the burns unit implies nosocomial transmission within the burns unit. Greater diversity was observed outside the burns unit. *P. aeruginosa* appeared to form multiple biofilm formation patterns. Three distinct patterns of biofilm formation could be described after 10 hours incubation. These patterns did not correlate with MLVA type. The effect of exposure to four antibiotics (cefepime, ciprofloxacin, imipenem, and gentamicin) on biofilm formation over time was shown to differ between organisms with early and late onset biofilm formation patterns, but is not predicted by MLVA type. The mechanisms of action of the antibiotics also did not seem to predict the response since two antibiotics with the same mechanism of action (cefepime and imipenem) had different biofilm formation patterns.

Increased knowledge of the *P. aeruginosa* population structure and biofilm forming ability in this patient group, and enhanced understanding of the effect of antibiotic treatment on biofilm formation may enable improvements in transmission prevention, the selection and use of antibiotics for treatment and, ultimately, improve patient outcome.

Abstrak

Pseudomonas aeruginosa is ‘n algemene opportunistiese patogeen wat verantwoordelik is vir meer as 11% van hospitaalinfeksies wat urienweginfeksies, bakteremie, longontsteking en sagteweefsel infeksies insluit. Daar is min inligting beskikbaar rondom *P. aeruginosa* infeksies in brandwondpatiënte in Suid Afrika, spesifiek in Tygerberg hospital. Brandwondpatiënte is hoogs vatbaar vir infeksies as gevolg van die vernietiging van hul natuurlike verdedigingstelsel. *P. aeruginosa* het die vermoë om ‘n biofilm te vorm en ‘n voortdurende biofilmgeassosieerde infeksie te veroorsaak. Die biofilm tree op as ‘n beskermingslaag wat die organismes beskerm teen die omgewing, die gasheer se immuunstelsel en antibiotiese behandeling. *P. aeruginosa* is verantwoordelik vir ‘n 40-50% sterftesyfer in patiënte met brandwonde.

Hierdie studie is daarop gemik om die bevolkingstruktuur van *P. aeruginosa*, geisoleer uit die brandwondeenheid en brandwond intensiewesorgeenheid te vergelyk met isolate van ander eenhede in Tygerberg Hospitaal ten opsigte van hul vermoë om biofilms te vorm en die effek van antibiotika op biofilmvorming. *P. aeruginosa* isolate was ingesamel van bloedkulture, deppers en weefsel monsters van volwasse en pediatriese patiënte in Tygerberg hospital oor die tydperk van Februarie 2015 tot Maart 2016. Veertig isolate vanuit die brandwondeenheid en buite die brandwondeenheid onderskeidelik was gebruik in die studie. Multi lokus veranderlike aantal tandem herhaling ontleding (MLVA) was uitgevoer om stamtipering te doen. Die vorming van die biofilms was bepaal met kristalvioletkleuring na ‘n 12-uur inkuberingsperiode, terwyl die effek wat vier antibiotika met verskillende konsentrasies en meganismes van werking oor ‘n 24-uur inkubasieperiode bepaal was.

Twee-en-veertig verskillende MLVA tipes was geidentifiseer waarvan tien van die tipes twee of meer organisme besit. Twee-en-dertig MLVA patrone was uniek en het slegs een organisme besit. MLVA tipe 1 was die volopste; sowat 60% van brandwondeenheid en brandwond intensiewesorgeenheid isolate het aan MLVA tipe 1 behoort. Die oorheersing van hierdie MLVA tipe binne die brandwondeenheid/intensiewesorgeenheid impliseer dat daar wel oordrag binne-in die eenheid plaasvind. Daar was meer stamdiversiteit buite die brandwond eenheid. *P. aeruginosa* het drie verskillende biofilm patrone gevorm na 10 ure van inkubering waarvan daar geen ooreenstemming was tussen verskillende MLVA tipes nie. Die invloed van vier verskillende antibiotikas (cefepime, ciprofloxacin, imipenem en gentamicin) op die vorming van ‘n biofilm het getoon dat die aanvanklike hoeveelheid biofilm wat ‘n organisme vorm ‘n groot invloed op die werking van antibiotika het en dat daar ook geen ooreenkoms is tussen die biofilmvorming van organismes van dieselfde MLVA tipe. Die meganismse van aksie het ook geen merkwaardige impak getoon nie aangesien twee antibiotikas wat dieselfde aksie toon (cefepime and imipenem), biofilmvorming verskillend geaffekteer het.

Verhoogde kennis van die *P. aeruginosa* bevolkingstruktuur en biofilmvormingsvermoë in hierdie groep pasiënte en ‘n verbeterde begrip van wat die effek van antibiotikabehandeling op biofilms is mag help om die uitkomste van gereelde oordrag en kliniese behandeling te verbeter.

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List of abbreviations.

AFLP	Amplified fragment length polymorphism
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	Concentration
C. albicans	*Candida albicans*
c-di-GMP	cyclic diguanylate
CI	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
CVP	central venous catheter
DNA	Deoxyribonucleic acid*E. coli* *Escherichia coli*
eDNA	extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EPS	extrapolysaccharide matrix
FDA assay	Fluorescein diacetate hydrolysis
GacS	global activator of antibiotic and cyanide synthesis
GM	Gentamicin
h	hour
HCl	hydrogen chloride
ICU	Intensive care unit
IP	Imipenem
IPC	Infection prevention and control unit
IS	Insertion sequence
kb	kilo-base pair
LecA	Lectin A
LecB	Lectin B
Mbp	Megabase pair
MDR	Multidrug resistance
mg	milligram

MH	Mueller Hinton
MIC	Minimum inhibitory concentration
ml	millilitre
MLST	Multi-locus sequence typing
MLVA	Multiple locus variable number tandem repeat analysis
ms	minisatellite
NA	No amplification
NCBI	National Centre for Biotechnology Information
NHLS	National Health Laboratory Service
NICD	National Institute of Communicable Diseases
nm	nanometre
P. aeruginosa	*Pseudomonas aeruginosa*
PA14	*Pseudomonas aeruginosa* 14
PAK	*Pseudomonas aeruginosa* K
PAO1	*Pseudomonas aeruginosa* O1
PCR	Polymerase chain reaction
Pel	Pellicle
PFGE	Pulsed field gel electrophoresis
pH	Potential of hydrogen
PIA	polysaccharide intercellular adhesion
PM	Cefepime
(p)ppGpp	guanosine tetra- and penta-phosphate
PQS	*Pseudomonas* quinolone signal
P. acnes	*Propionibacterium acnes*
Psl	polysaccharide synthesis locus
QS	Quorum sensing
RAPD	Random amplified polymorphic DNA assays
RFLP	Restriction fragment length polymorphism DNA analysis
RND	resistance-nodulation-cell division
rpm	revolutions per minute
S. aureus	*Staphylococcus aureus*
TBA	Tryptose blood agar

TBE	Tris/Borate/EDTA
Tm	Melting temperature
rRNA	ribosomal ribonucleic acid
tRNA	transfer ribonucleic acid
TSB	Tryptic soy broth
V	Volume
VNTR	Variable number tandem repeat
˚C	degrees Celsius
β	beta
µl	Microlitre
µg	Microgram

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Chapter 1

Chapter 1 – Literature review

1.1 Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium which was first discovered in 1882 by the chemist Carle Gessard.[1][2] This organism, which has a genome size of 5.2 to 7.1 Mbp, can be acquired from the environment and can be found in multiple habitats including soil, marine habitats and commonly in plants.[3][4] The organism is known to cause opportunistic infections which are often health-care associated. *P. aeruginosa* can cause chronic and lethal infections amongst immune-compromised individuals and results in more than 11% of nosocomial infections.[4][5] Infections can include urinary tract infections (often associated with urinary catheters), bacteraemia, respiratory infections (pneumonia) commonly found in cystic fibrosis patients and soft tissue infections linked to burn wounds, as well as burns sepsis that causes high morbidity and potential mortality.[3][6]

Patients with severe burn wounds are prone to bacterial infections due to the physical destruction of the skin barrier, allowing colonising organisms within the sweat glands and hair follicles to cause infection. The majority of burns patient infections are due to Gram negative organisms, specifically *Acinetobacter*, *P. aeruginosa*, and *Klebsiella pneumoniae. P. aeruginosa* is one of the leading organisms causing infections in patients with burn wounds and is a major problem in hospitals due to patient to patient transmission.[7][8] *P. aeruginosa* was found to be responsible for 17.7% and 12.3% of infections in the ICU burn wards and common burn ward respectively in a study done in China.[8] A study has also shown that *P. aeruginosa* is responsible for the majority of burns patient infections in the United States.[7] The mortality rate of burn wound patients with *P. aeruginosa* infections can be up to 40-50%.[7]

It is often difficult to treat and eliminate infections caused by *P. aeruginosa* due to its intrinsic resistance; meaning a natural resistance to a few broad spectrum antibiotics which are not able to enter the cell due to the low permeability of the outer membrane of the organism. This, in conjunction with efflux pump regulation, aids in antibiotic resistance.[9] *P. aeruginosa* also has the ability to form biofilms in a variety of environments and to develop resistance to antibiotics and disinfectants. Biofilms, which are bacterial cells grouped together and surrounded by an extrapolysaccharide matrix, are commonly found on wound tissue, body surfaces, lungs, as well as abiotic surfaces such as medical devices namely ventilators, catheters, joint and organ replacement parts.[10] Organisms within a biofilm have been shown to be less sensitive and some totally resistant to antibiotics and also to the immune system.[11][12]

Developing drug resistance is a major problem in the clinical environment, especially in *P. aeruginosa* where increasing resistance can lead to multidrug resistance (MDR).[13] Drug resistance, together with biofilm formation, can result in recurring infections which are nearly impossible to treat. Research has shown that factors contributing to MDR include using the incorrect antibiotic and/or concentration of drugs as well as environmental stress.[14–16] A study revealed that MDR has been shown to emerge more easily during antibiotic therapy when treating with a single antibiotic (4.4%) than when using combination therapy (3.1%).[17] The same study also revealed that MDR infections are more likely to occur in the ICU setting when a patient is on excessive fluoroquinolone treatment such as ciprofloxacin. To avoid the development of MDR strains one needs to apply suitable infection control methods and avoid unnecessary use of broad spectrum antibiotics.[16]

There is a need for the rapid identification, treatment and prevention of transmission of *P. aeruginosa* in hospitals. This organism is a rapidly developing problem due to biofilm formation and the development of MDR. Patients with burn wounds are more susceptible to infection and *P. aeruginosa* is frequently the most abundant isolate isolated from the burns unit.[18]

1.2 P. aeruginosa strain typing

Molecular typing methods are used to distinguish between bacterial species and/or between different strains of the same species and have been expanding due to developing technologies.[19] Typing methods such as serotyping have long been used for *P. aeruginosa* strains but are known to be less successful when typing mucoid strains which are commonly found in patients with pulmonary infections. Molecular typing methods for the analysis of *P. aeruginosa* isolates provide higher discrimination compared to other phenotypic tests and are also highly reproducible.[20] Molecular typing methods can be easy, inexpensive and rapid tools for molecular epidemiological analysis and outbreak investigations. Common molecular techniques that are being used include pulsed field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), ribotyping, restriction fragment length polymorphism DNA analysis (RFLP), random amplified polymorphic DNA assays (RAPD), amplified fragment length polymorphism (AFLP) and multiple locus variable number tandem repeat analysis (MLVA).[21][22] Strain typing of *P. aeruginosa* isolates from burn wounds has shown that the majority of isolates from the same ward are of the same strain. A study found that 83% of patients acquired a *P. aeruginosa* infection during hospitalisation within a burns ward while two distinct genotypes were responsible for 60% of the burn wound patient colonization.[23]

1.2.1 Pulsed field gel electrophoresis)

PFGE is classified as the gold standard for strain typing since the discriminatory power is very high and it is also a inexpensive method. However, the method remains laborious and has low reproducibility as results are often difficult to compare to other databases from other studies.[24] PFGE works on the basis of restriction endonuclease digestion of the entire genome with restriction enzymes such as SpeI and XbaI, after which the DNA fragments are separated on an agarose gel by means of a changing electrical current.[25] The changing electrical current allows the separation of large DNA fragments by changing the direction of the flow and helping large fragments to sieve through the pores. The fragmented bands can be visualized generating a unique pattern for each species or strain.[26] A limitation of PFGE is that it has low resolution when it comes to distinguishing similar band sizes. PFGE is becoming less frequently used because PCR and sequencing based methods are becoming easier, inexpensive and more accessible.

1.2.2 Multi-locus sequence typing

MLST involves the amplification and sequencing of 450 to 500bp fragments of up to seven housekeeping genes. Each allele, which is a variant form of a gene, has a unique sequence. The specific alleles for each of the genes can be combined to generate a specific MLST profile. Loci commonly used for MLST of *P. aeruginosa* include *acsA, aroE, guaA, mutL, noD, ppsA, and trpE.*[2][25] MLST is very reproducible and the results are easy to compare to other databases such as http://pubmlst.org and www.mlst.net since the method is universal, however MLST is very expensive, laborious and time consuming.[26]

1.2.3 Ribotyping

Ribotyping focuses on the 16S, 23S and 5S ribosomal RNA genes which are conserved regions in all bacteria. Ribotyping works on the basis of restriction endonuclease digestion of the entire genome. The DNA fragments can then be separated by means of gel electrophoresis according to size and the fragments that contain a piece of the ribosomal operon are transferred and visualised by southern blotting through hybridisation with a radiolabelled ribosomal operon probe.[27][28]

1.2.4 Restriction fragment length polymorphism DNA analysis

RFLP involves the use of restriction enzymes such as *Eco*RI to generate DNA fragments which are separated by gel electrophoresis based on size. Southern blotting followed by hybridisation with specific probes, is used to identify sequence variation between strains. These fragments sizes vary between organisms. The fragment will be classified as a RLFP when the size differs between individuals. This method has high reproducibility and discriminatory power, but is time consuming and can be expensive.[29][30]

1.2.5 Random amplified polymorphic DNA assays

RAPD assays amplify a number of fragments using a single pair of non-specific primers that are roughly 10bp long. Amplification is performed at a low annealing temperature so that mismatch pairing can occur. Amplicon sizes normally range between 0.1 kb and 3 kb. Amplicons are separated and visualised using gel electrophoresis. RAPD has lower discriminatory power than PFGE but is an easy and cheap alternative. The reproducibility of this method remains low due to sensitivity to different reagents and machines as well as the low melting temperatures used during amplification.[26][31]

1.2.6 Amplified fragment length polymorphism .

This method works on the basis of cutting genomic DNA with *Eco*RI or *Tru*9I restriction enzymes followed by PCR after adapters have been added to one end of the cut fragments.[32] The fragments containing the adapters will be amplified with primers corresponding to the adapters. Fragments are labelled with fluorescent PCR primers for easier detection and visualisation with the aid of an automated DNA sequencer. AFLP can assist in distinguishing between strains and determining the corresponding genetic relatedness. AFLP is reproducible with high discriminatory power but the method is laborious and expensive.[26]

1.2.7 Multiple locus variable number tandem repeat analysis .

Multiple locus variable number tandem repeat analysis (MLVA) is a genotyping method using variable number tandem repeats (VNTRs). This method can be performed at low cost, produces a lot of information, delivers high discriminatory power as well as reproducibility and can be used for the identification of a number of strains from the same species.[33] It was initially used for human DNA fingerprinting and then developed for use in bacterial genomes. This method is based on the principle that each strain contains a different number of sequence repeats at specific loci. The number of repeats can be determined by the size of the amplified product (allele) which can be compared to other strains and enables unique

identification.[34] The repeats can be amplified by attaching locus specific primers which flank the repeat region.[20] VNTRs include microsatellites (smaller than 9bp) as well as minisatellites (larger than 9bp).[35] *P. aeruginosa* has a number of VNTRs which can be combined and used for unique strain identification.

1.3 Antibiotic resistance in P. aeruginosa.

MDR *P. aeruginosa* is a major developing problem since it causes persisting infections in hospitals and high rates of mortality. Organisms use different mechanisms to counter antibiotic activity and antibiotic resistance can be classified in three types namely intrinsic, acquired and adaptive resistance.[36] Intrinsic resistance can be explained as the organism's natural resistance mechanisms without any previous exposure to antibiotics. Intrinsic resistance mechanisms include the semi-permeable outer membrane of Gram negative bacteria which slows down the entry of small hydrophilic antibiotics such as β-lactams and quinolones, efflux pumps which actively pump antibiotic out of the cell and the production of intrinsic periplasmic β-lactamases.[36] Acquired resistance is derived from exposure to antibiotics which results in selection of organisms with chromosomal mutations which mediate antibiotic resistance.[37] Acquired resistance can also be gained through genetic elements such as plasmids, transposons, interposons and integrons by horizontal gene transfer. Gram negative bacteria in particular use several acquired resistance mechanisms to counter the action of antibiotic therapy (Figure 1.1). Adaptive resistance is gained from environmental (physical and chemical) changes and growth circumstances of the bacteria which will then trigger reversible regulatory responses in the cell.[36],[38] Factors such as sub MIC concentrations of antibiotics, pH, rapid temperature changes, DNA stress, cations and nutrient deficiency are all inducers of adaptive resistance.[38] Sub inhibitory concentrations of ciprofloxacin are known to cause gene dysregulation.[39] Heat shock can induce aminoglycoside resistance while DNA stress induces fluoroquinolone resistance.[40]

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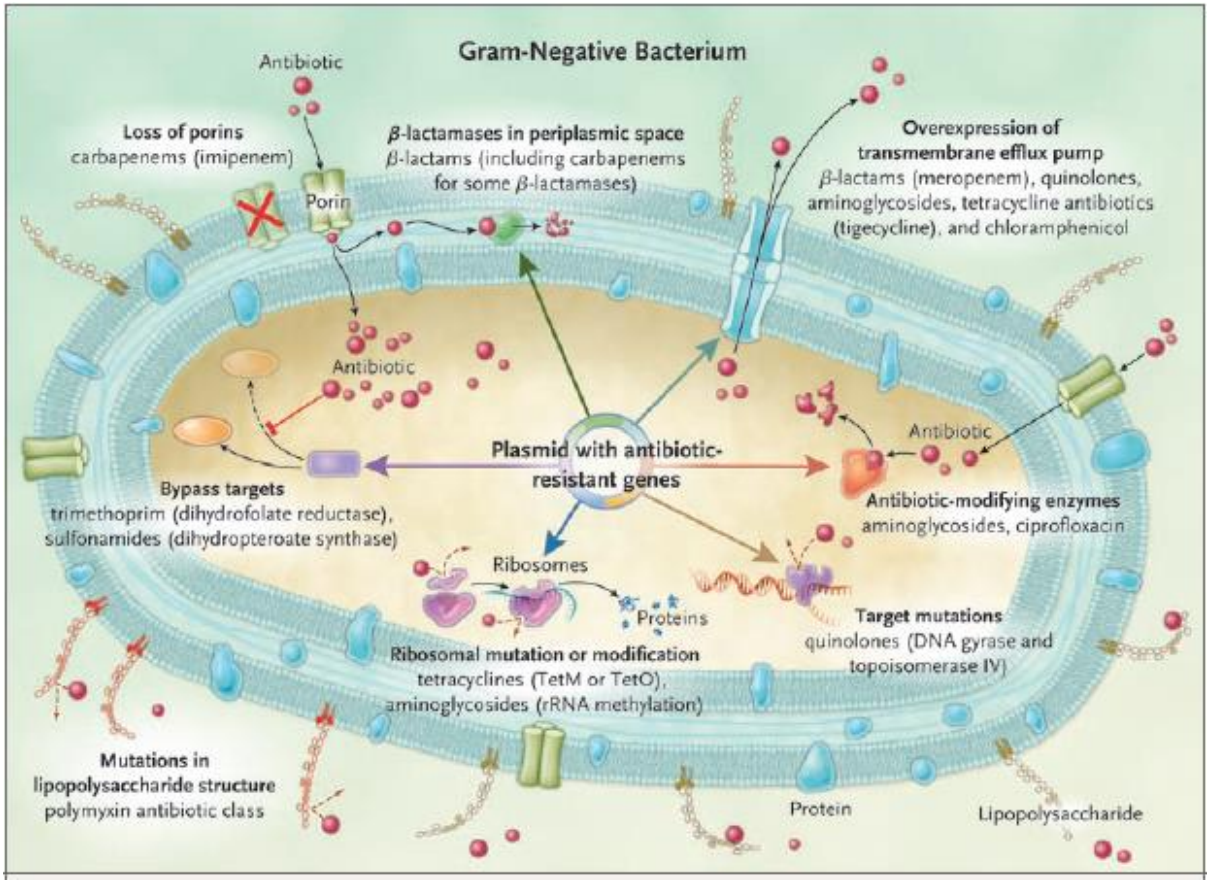

Figure 1.1: Acquired resistance mechanisms in Gram negative bacteria. Gram negative bacteria use at least eight mechanisms, mediated by antibiotic resistance gene containing plasmids as well as chromosomal mutations, to counter the effects of antibiotics. Resistance mechanisms include porin loss, production of β-lactamases, efflux pump overexpression, modifying antibiotics, target mutations, ribosomal mutations, lipopolysaccharide mutations and metabolic bypass. Source: Peleg *et al.,* (2010).[41]

1.3.1 P. aeruginosa antibiotic resistance mechanisms.

P. aeruginosa uses a number of resistance mechanisms to counter antibiotic effects (Table 1.1). Antibiotics such as β-lactams, namely penicillins, cephalosporins, carbapenems and monobactams, and other major antimicrobials such as aminoglycosides, fluoroquinolones

and polymyxins are generally suitable for the treatment of *P. aeruginosa* but could become ineffective after mutations occur or resistance mechanisms are acquired.[16] Common resistance mechanisms of *P. aeruginosa* include efflux pump regulation, mutations and modified enzymes.[42]

Table 1.1: *P. aeruginosa* mechanisms of resistance. *P. aeruginosa* can acquire several mechanisms to counter antibiotic effects. Source: Hirsch *et al.,* (2011).[42]

Resistance mechanism/mutation	**Components involved**	**Antibiotic classes/agent affected**
Overexpression of RND-type multidrug efflux pump	MexAB-OprM MexCD-OprJ MexEF-OprN	Macrolides, aminoglycosides, sulphonamides, fluoroquinolones, tetracyclines, β-lactams
Porin deletions	OprD	imipenem, meropenem
β-lactamases	PSE-1, PSE-4 AmpC Metallo-β-lactamases	Penicillins, Third generation cephalosporins, piperacillin, carbapenems
Aminoglycoside modifying enzymes	Acetyltransferases Nucleotidyltransferases Phosphotransferases	Aminoglycosides
16S rRNA methylase	*rmtA, rmtB, armA* genes	Aminoglycosides
Quinolone resistance determining region	*gyrA, gyrB, parC, parE*	Fluoroquinolones

P. aeruginosa contains multidrug efflux systems (MexAB-OprM and MexXY-OprM) which together with inactivating enzymes pump antibiotics such as fluoroquinolones, penicillins, cephalosporins, macrolides and sulphonamides across the Gram negative membrane and simultaneously degrade them.[43] The up-regulation of MexAB-OprM can result in these antibiotics becoming ineffective. Mutations in *oprD* or its regulatory regions result in the down regulation or loss of the OprD outer membrane porin, preventing uptake of carbapenems and resulting in resistance, specifically to imipenem and meropenem.[44][45] The absence of the OprD membrane porin in combination with overexpression of efflux pumps results in increased resistance to some carbapenems.[9]

Enzymes such the β-lactamases, PSE-1, PSE-4, AmpC and metallo-β-lactamases, break down β-lactam antibiotics such as penicillins, cephalosporins and carbapenems. Overexpression of the AmpC β-lactamase can result in resistance to mainly penicillin as well as cephalosporins.[46] Aminoglycoside modifying enzymes inactivate aminoglycosides by catalysing either the acetylation of an amino group or the adenylation or phosphorylation of a hydroxyl group in the antibiotic. 16S rRNA methylases play a significant role in countering antibiotic activity by inhibiting the activity of aminoglycosides. Aminoglycosides normally

interfere with protein synthesis by binding the 30S ribosomal subunits.[42] Mutations occurring in topoisomerases II and IV can lead to fluoroquinolone resistance.[47]

1.4 P. aeruginosa biofilms.

Bacteria such as *P. aeruginosa* can grow either in a planktonic, free floating form or as a biofilm attached to a surface. Biofilms are biologically active bacterial cells which are grouped together and surrounded by an extrapolysaccharide matrix (EPS).[48] The EPS makes up 75-90% of the biofilm and keeps the cells together and attached to a substrate, while the cells, which can consist of one or more bacterial species, make up 10-25% of the biofilm.[49][50] Biofilms serve as a barrier which delays or even prevents antibiotics, other biocides, cationic antimicrobials and antimicrobial peptides from entering and affecting the organisms; and protects against environmental factors. The biofilm protects organisms from harsh conditions such as extreme dryness and against oxidation.[51] Within a biofilm, communication between the cells can occur and impacts the regulation of expression of virulence factors and helps the organism survive in a nutrient deficient environment.[50]

Biofilms were first described by Antonie van Leuwenhoek in 1674 after taking scrapes from a tooth and describing aggregates of cells on the surface. It has been found that 99% of bacteria will be in a biofilm state at a certain point in their life cycle.[50] Biofilms can cause harmful side effects and commonly affect humans in a number of ways, for example food spoilage, corrosion, malodours, infections and pipe blockages. In hospitals nosocomial infections are commonly caused by instruments, drips, catheters and ventilators which are contaminated with biofilms.[50] Biofilms are also commonly associated with recurring diseases such as periodontal disease, endocarditis and osteomyelitis.[12] *P. aeruginosa* infections are common in burn wound patients and while this organism is associated with biofilm formation there is still evidence lacking as to whether biofilms are common in these patients. Apart from the negative effects of biofilms, they can also be useful in industry. Biofilms are being used in bioremediation, a process that involves using the biofilm to remove contaminants such as oil spills and purifying waste water.

1.4.1 Biofilm structure

The presence of a substrate as well as microbes is necessary for biofilm formation. The EPS of a biofilm consists of biomolecules (metabolites), exopolysaccharides, extracellular DNA (eDNA), lipids and a polypeptide mixture and forms the structure of the biofilm.[52][53] *P. aeruginosa* can produce three types of polysaccharides namely alginate, pellicle (Pel) and polysaccharide synthesis locus (Psl) polysaccharide.[54] At least one of the two main

polysaccharides, Psl polysaccharide and Pel, is necessary for the first structural developmental stages of a biofilm, while alginate is not essential for biofilm development.[55]

The *psl* operon (polysaccharide synthesis locus) contains 15 coding genes that are involved in the production of Psl polysaccharide, which contributes to the attachment of cells to other cells as well as to surfaces, and especially to mucin surfaces found in airways (Figure 1.2).[54] Psl polysaccharide contributes to the maintenance of a biofilm after it has formed by supporting the structure. Psl polysaccharide is not a vital component for biofilm formation in all strains, except for strains PAO1 and ZK2870.[56]

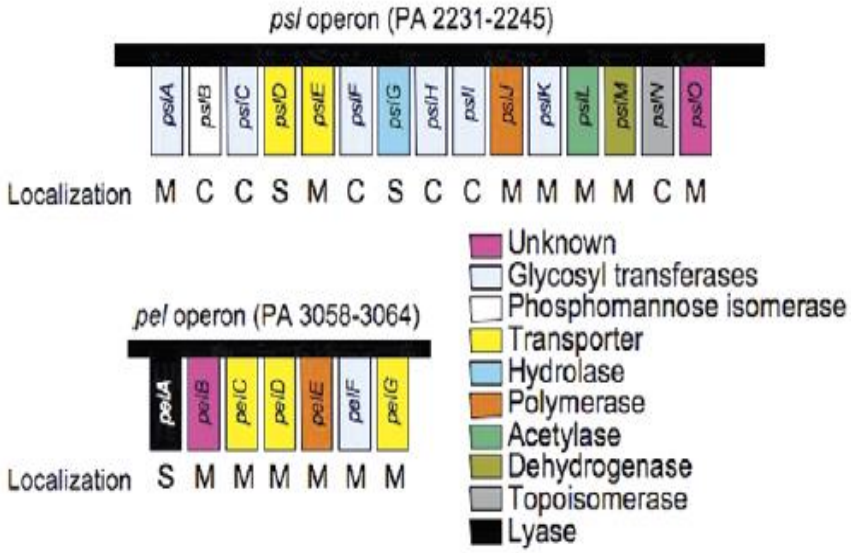

Figure 1.2: The *psl* and *pel* operons. Each operon contains different genes coding for enzymes which assist in biofilm formation. The enzymes being produced are localised in the following areas of the cell: M-membrane, C-cytoplasm, S-secreted. Source: Ryder *et al.,* (2007)[54].

The *pel* operon contains 7 coding genes and plays an important role in the biofilm structure, especially in the PA14 and PAK *Pseudomonas* strains since they don't produce Psl polysaccharide (Figure 1.2).[54] The glucose rich matrix polysaccharide, pellicle, is produced by enzymes encoded by the *pel* locus. Pel polysaccharide production is regulated by *c-di-GMP, a* monophosphate messenger.[57] The *P. aeruginosa strain* PAO1 is known to possess both the *pel* and *psl* operons.[56]

Alginate is an acetylated polymer made up of mannuronic and guluronic acid which is overproduced as a result of a mutations in the *mucA* gene.[58] These mutations induce the regulation, therefore inducing expression of the anti-sigma factor AlsT, which is an essential factor for the production of alginate, thereby enhancing the expression of the alginate producing operon.[58] Alginate is not essential for biofilm formation and some strains such as PAO1 and PA14 produce minimal amounts. Some *P. aeruginosa* strains have the ability to grow either in a mucoid or non-mucoid state. These two states differ in the composition of their polysaccharide matrix; the mucoid state resulting from overproduction of alginate.[59]

eDNA plays an important role in the biofilm matrix and is necessary during the initial formation of a biofilm.[60] *P. aeruginosa* is known to release eDNA and it is speculated that it is released through vesical formation rather than cell death.[61] In the absence of eDNA, biofilms are easily affected by detergents such as sodium dodecyl sulfate. eDNA also aids in the twitching motility for the enlargement of a biofilm by maintaining coherent cell alignments. eDNA can be used as a source of nutrients for bacteria during starvation and can aid in cell to cell connections.[62]

1.4.2 Biofilm development

According to Garret et al.,(2008)[50] and Rasamiravaka et al.,(2015)[63] there are three important stages for the initial development of a biofilm (Figure 1.3), starting with free cells attaching to a surface forming a thin film (biotic or abiotic) in a process called adhesion after approximately 2 hours. Secondly, free floating cells will cohere irreversibly to these cells in a process called cohesion, 8 hours after the initial inoculation. Thirdly, micro colonies will arise by forming polymer bridges between each other, which occurs 14 hours after the initial inoculation. These three stages form the first two phases of a biofilm, namely the lag (step one and two) and exponential phases (step 3). After these initial biofilm formation steps, stationary phase will be reached when the rate of cells being formed is equal to the amount of cells dying. During the stationary phase of a biofilm the cells will start communicating through quorum sensing where auto inducers are produced for the development of unique gene expression changes which contribute to antibiotic and biocide resistance. The final phase of the biofilm cycle is the death phase (breakdown of biofilm) which results in the release of cells into planktonic growth for the formation of new biofilms on other substrates.[50]

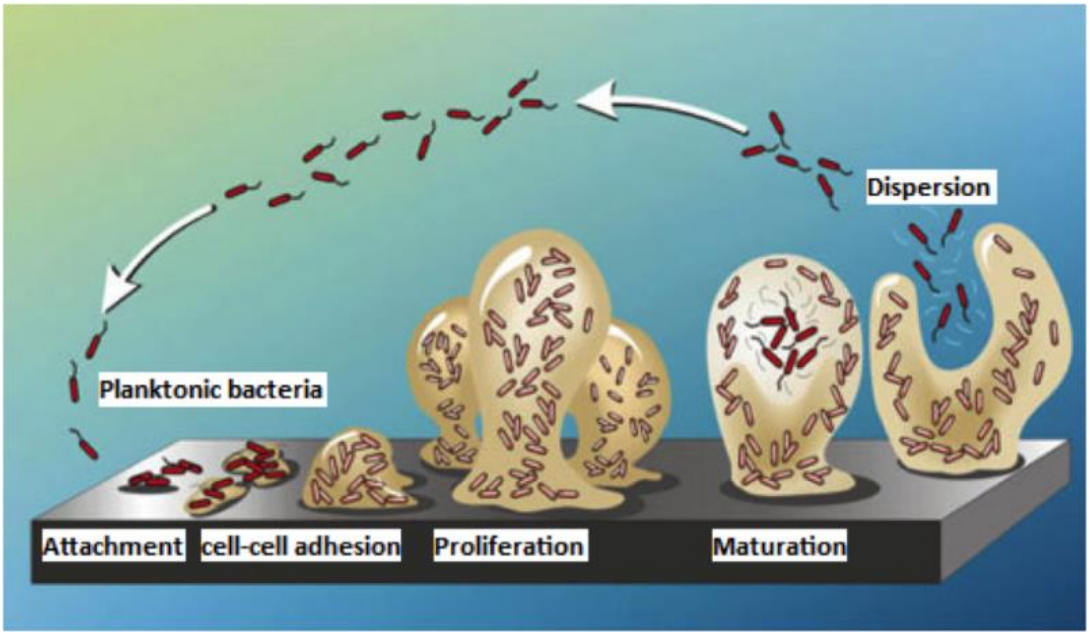

Figure 1.3: Biofilm life Cycle. 1. Free floating cells (planktonic cells) will attach to a surface (adhesion). 2. Cell to cell cohesion will occur. 3. Cells will proliferate on the surface and form a biofilm by producing an extracellular polymeric substance. 4. After the biofilm has matured biofilm growth will come to a halt, the cell will die off and some cells will disperse back to free floating cells (stationary and death phases).

Source: http://mpkb.org/home/pathogenesis/microbiota/biofilm.[64]

During the first stage of biofilm formation a conditioning layer is formed. This layer acts as the foundation of the biofilm. Other molecules or particles can attach to the surface and form part of the conditioning layer. This layer will aid in the attachment of the bacteria and carry the necessary nutrients for biofilm formation and growth. Cells will attach to the conditioning layer either reversibly or irreversibly. Planktonic cells that attach reversibly usually attach with weak forces such as van der Waals forces, steric interactions and electrostatic interactions. Some cells that are bound reversibly will bind irreversibly by countering the repellent forces using their flagella, fimbriae or pili, staying attached to the surface.[50]

As the attachment stage progresses the attached cells will divide and differentiate. The new cells being formed will grow outwards and upwards to form clusters.[65] This process will allow the biofilm to form a mushroom-like structure. It is hypothesized that this structure allows nutrients to be transferred to the bottom of the biofilm.[56]

An exponential growth phase will be reached where the cell population will increase rapidly. Stronger bonds between cells will be initiated by the cells through the release of polysaccharide intercellular adhesion (PIA) polymers as well as the formation of cationic interactions.[66]

After the rapid growth phase of the biofilm the cells will go into the stationary phase where the number of cells being formed will be equal to the amount of cells dying in the biofilm. Cells will start to disperse with the help of alginate lyase which will break down the EPS.[67] This process will aid in cells being released into a planktonic state to attach to new surfaces and to start a new biofilm. The genes coding for the flagellum are also up regulated to enhance cell dispersion.

Biofilm formation is controlled by physical, chemical and biological processes.[50] Numerous environmental conditions will affect the formation, structure and maintenance of a biofilm. When the pH of the environment differs greatly from the pH of the cells it affects the function of the membrane proton motors and causes a passive influx of the protons and can result in biocidal effects, affecting the bacteria and altering biofilm formation. A rapid change in environmental pH will cause more damage than a slow change since it has been shown that cells adapt to this change by synthesising and adjusting the necessary proteins for counter action.[68]

The correct temperature is necessary for the organism to increase its nutrient intake to allow rapid growth and biofilm formation.[68] Temperature is also important for enzyme activity which can be linked to cell development and biofilm formation. Temperatures lower or higher than the optimal required temperature will result in little to no growth since this will slow down or stop enzyme activity as well as metabolic activity.[50] Temperature also has an effect on compounds found close to the cell or in the cells that are involved in cell development. In a previous study it has been shown that a lower temperature can reduce the likelihood of cells binding to a surface which is mainly due to the molecule (surface polymer) that is responsible for the attachment of the cell to a surface being reduced, while the temperature can also result in a smaller surface area for attachment.[50] Organisms may also have variable numbers of flagella at different temperatures. According to a study by Herald *et al*., (1988), bacteria such as *Listeria monocytogenes* have one flagellum at 35˚C while the number of flagella increases as the temperature decreases, for example at 10˚C the bacteria had several flagella.[69] Studies have also shown that higher temperatures increase the adherence of the organisms to a substrate making it more difficult for biofilms to be removed.[50]

1.4.3 Biofilm regulation

Several systems regulate EPS production and thereby biofilm formation; the most important being the quorum sensing (QS) systems (Figure 1.4).[50][70] Quorum sensing is involved in cell to cell communication by producing signal molecules which regulate the production of virulence factors and help the organism with motility and biofilm formation.[71] There are three main QS systems found in *P. aeruginosa* namely *las*, *rhl* and *Pseudomonas* quinolone signal *system (*PQS).[72] The *las* and *rhl* systems are responsible for the production of two signalling molecules, *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butanoyl-L-homoserine which play an important role in the formation of biofilms as well as the expression of virulence factors.[55],[63] Signalling molecules are produced as the population expands, and bind to target sites of transcriptional activators such as LasR or RhlR to regulate gene expression.[73][72] A third system, the PQS system is responsible for the formation of the *Pseudomonas* quinolone signal and is actively involved with the auto inducer molecules. Two additional two component systems, GacS/GacA and RetS/LadS are also involved in biofilm formation and growth. The two-component systems play an important role in the structure of a biofilm, virulence factor production and the overall fitness of the organism (Figure 1.4). These systems work on the basis of membrane associated sensor histidine kinases which track signals and changes in the environment of the organism. A stimulus will result in phosphorylation of the histidine kinase residue which will be carried over to the cytoplasmic response regulator, which can then regulate the expression of various genes. Stimuli affecting this mechanism include factors such as acyl-homoserine lactones (quorum signals), nutrients, and antibiotics.[74] An additional monophosphate messenger, *c-di-GMP,* is important for further biofilm formation enhancement by inducing Pel and alginate production. Each system influences the biofilm in a unique way.

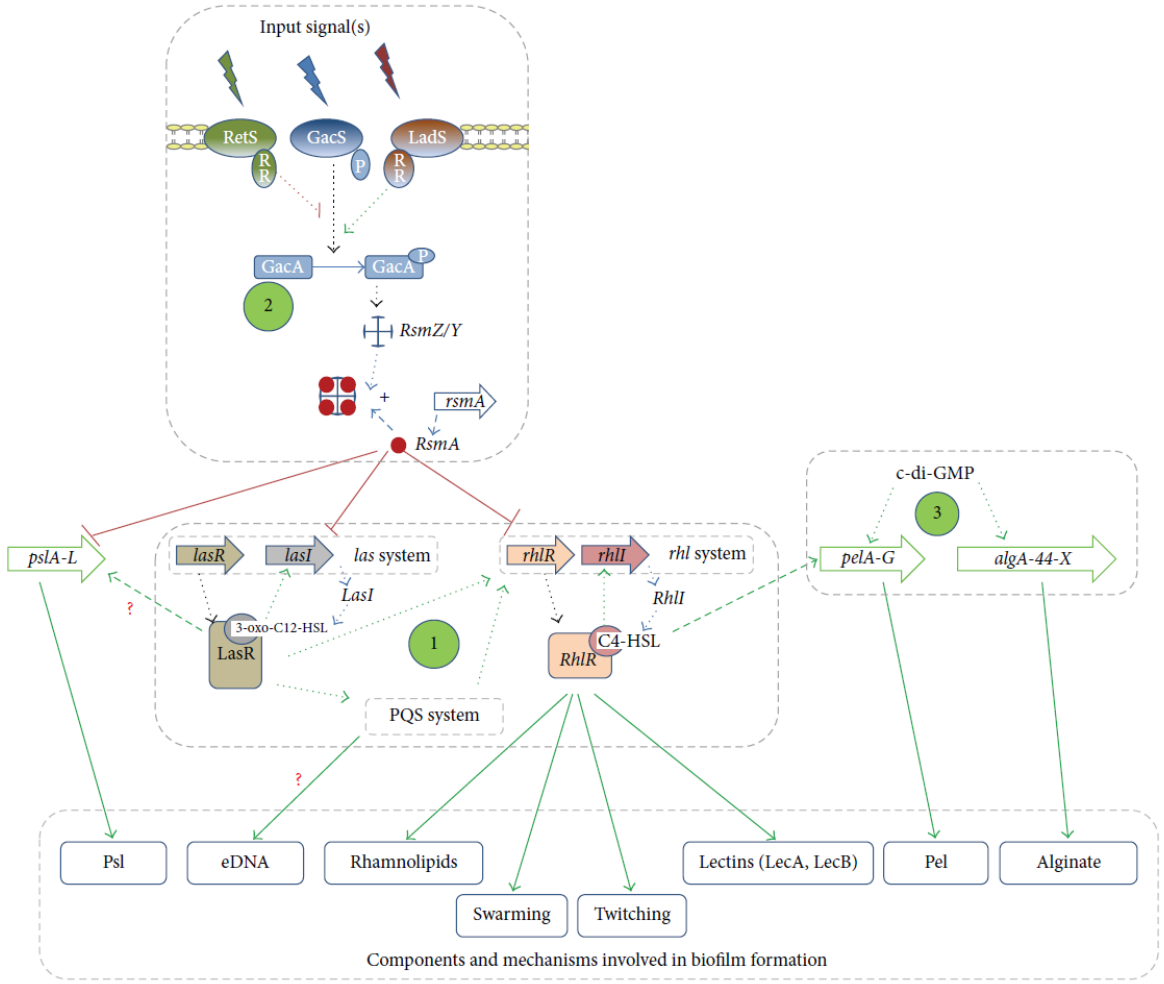

Figure 1.4: The regulation of the *P. aeruginosa* biofilm. The formation of a biofilm is regulated by a number of regulatory genes which are responsible for the production of products and processes that are actively involved in biofilm formation. 1. Quorum sensing systems. 2. Two component systems. 3. Extra-polysaccharide production c-di-GMP regulation. Source: Rasamaravika *et al.,* (2015).[63]

1.4.3.1 las system.

The *las* system is made up of two transcriptional activator proteins namely LasR and LasI. LasR is a cognate regulator gene while LasI is responsible for the production of the auto inducer signalling molecule *N*-(3-oxododecanoyl)-L-homoserine lactone. When the signalling molecules bind to the transcriptional activator LasR, it will activate expression of genes such as *lasB, lasA, apr and toxA,* which are responsible for the production of virulence factors.[75] Without the presence of the *las* system a biofilm will appear flat and minimal changes will

occur in the biofilm; while detergents will have a bigger impact on biofilm dispersal.[76] The *rhl* and *PQS* systems are also positively regulated by the *las* system.

1.4.3.2 rhl system.

The *rhl* system consists of two transcriptional activator proteins namely RhlR and RhlI. RhlR is a cognate regulator gene while RhlI is responsible for the production of the auto inducer signalling molecule *N*-butanoyl-L-homoserine.[77] As seen in Figure 1.4, the *rhl* system directly influences the biosynthesis of Pel polysaccharides by enhancing their production.[63] The swarming of cells and cell to cell contact is regulated by the *rhl* system for organized surface translocation.[78] Without the ability to move, biofilms will form unstable cell structures making the biofilm weak and sensitive to environmental stress or treatment. The *rhl* system is also responsible for the production of glycolipids called rhamnolipids. These glycolipids have several responsibilities during biofilm formation. Rhamnolipids are hypothesised to be responsible for the formation of micro colonies in biofilms, they are responsible for controlling cell to cell adhesion and cell to surface adhesion to form channels in the biofilm for molecules to "flow". Mushroom shaped structures are formed with the help of these glycolipids which also help with the release and dispersion of cells from the biofilm. The production of two lectins is also regulated by the *rhl* system. These are LecA and LecB which are cytotoxic virulence factors.[79]

1.4.3.3 PQS system

A third system, the PQS system, produces *Pseudomonas* quinolone signalling molecule (2-heptyl-3-hydroxy-4-quinolone) and primarily interacts with the auto inducer, acyl homoserine lactones.[71] The products formed by the *pqsABCD* and *phsH* genes aid in the production of PQS. The regulator, known as PqsR is essential for the production of PQS and regulates a number of genes active in this process.[80] This system is also responsible for the release of eDNA during biofilm development. The PQS molecule regulates a number of virulence factors of *P. aeruginosa.* PQS is commonly produced in the lungs of patients with cystic fibrosis. Studies have shown that PQS is actively involved in signalling between the *las* and *rhl* systems.[81]

1.4.3.4. GacS/GacA *system*

The GacS/GacA system is known to regulate virulence factor production and biofilm formation and is also an important regulator of quorum sensing in *P. aeruginosa*.[82] This system works as follows: the GacS (hybrid sensor kinase) will transfer a phosphate group over to the GacA regulator; which upregulates the small regulatory RNAs *RsmZ and RsmY*

which will bind to the RNA binding regulatory protein RsmA which aids in regulating the *psl* locus. The production of the autoinducers *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butanoyl-L-homoserine is also influenced by the *GacS/GacA* system. The system can inhibit the production of these acyl-homoserine lactones (acyl - AHL), thereby inhibiting quorum sensing via the *las* and *rhl* systems.

1.4.3.5 RetS/LadS *system*

The RetS/LadS histidine kinases have an effect on the activity of GacS by regulating its phosphorylation and thereby influencing the production of the exopolysaccharide Psl.[83] RetS on its own will inhibit biofilm formation while LadS will counter this effect by inhibiting the activity of RetS. It has been shown that the RetS/LadS system regulates the expression of genes necessary for the organism to grow and colonize causing an acute infection; or developing a biofilm for a more sustainable infection.[74] This system not only regulates the production of polysaccharides Pel and Psl polysaccharide, but also regulates genes expressing virulence factors and involved in motility.

1.4.3.6 c-di-GMP

The production of Pel polysaccharide is regulated by the monophosphate messenger *c-di-GMP* (bis-(3'-5')-cyclic dimeric guanosine monophosphate) together with the *pel* operon. *c-di-GMP* is produced by diguanylate cyclases (Figure 1.4). The polysaccharide alginate and Pel will be synthesised when *c-di-GMP* induces their production. This process occurs when *c-di-GMP* binds to proteins PelD or Alg44, which possess receptors for the monophosphate messenger, resulting in the production of individual polysaccharides and enhancing biofilm formation.[57] *c-di-GMP* is also known to improve the movement of bacterial cells which aids in the dispersion of the biofilm cells.[84]

1.4.4 Gene expression in P. aeruginosa biofilms

Gene expression profiles of bacteria in a biofilm differ significantly from those in planktonic growth, which contributes to antibiotic resistance, evasion of immune responses and expression of virulence factors.[11][36] Proteins active within a biofilm population undergo up and down regulation as antibiotics are introduced to the environment to counter the action of the antibiotic.[85] In *P. aeruginosa*, genes coding for the flagella or pili undergo down regulation when cells are in a biofilm, since they are not of use.[11] Further studies have shown that in a biofilm, genes such as *tolA* are actively expressed and can result in aminoglycoside resistance.[12] Cytochrome c oxidase activity is also repressed and can result

in reduced sensitivity to aminoglycosides.[86] Wild type *P. aeruginosa* strains contain the *rpoS* genes, which contributes to transcription by regulating the RNA polymerase *sigma* subunit. This gene is a known stress response regulator which aids in regulating genes to handle environmental stresses while it also plays a role in the formation and development of a biofilm.[11][87] Cellular stress, such as amino acid, carbon, nitrogen, phosphorus and iron shortages, as well as temperature stress will trigger the production of the alarmone, guanosine tetra- and penta-phosphate (p)ppGpp by the enzymes RelA and SpoT.[88] (p)ppGpp is responsible for mediating a stringent response which alters the expression of various genes and contributes to the formation of a biofilm and also to help the cell adapt from a growth phase to a survival phase.[71][36]

1.5 Clinical impact of biofilms in *P. aeruginosa*

Multidrug resistant *P. aeruginosa* is a rapidly developing problem in the clinical environment due to the spread of MDR organisms between patients, especially in the ICU. MDR is defined as resistance to one or more agents within at least three classes of antibiotics including aminoglycosides, penicillins, cephalosporins, carbapenems and fluoroquinolones.[42][89] Biofilms are important for the protection of the organism, promote persistence and provide an advantage to the organism's wellbeing. A biofilm will protect the organism from the environment as well as physical and chemical factors such as antibiotic treatment and the host's immune system.[12] Research has shown that organisms within a biofilm are more resistant to antibiotics. Resistance occurs at a hundred or even a thousand times the minimum inhibitory concentration (MIC) when compared to planktonic growth.[90] Biofilms show high resistance to antimicrobials which can be attributed to altered gene expression, external stress and unique biofilm structures.[36] Different strategies are being used to overcome the resistance mechanism of biofilms. This includes preventing the attachment of the organism to a surface, breaking down and disrupting a biofilm to assist the entry of antibiotics and preventing the maturation of the biofilm.[91][12]

Biofilm forming strains can commonly be found on burn wounds and in cystic fibrosis patients. *P. aeruginosa* infections are found to be more difficult to treat and eradicate with antibiotics as the disease progresses which may be due to the development of a biofilm.[34] *P. aeruginosa* biofilms found in cystic fibrosis patients are impossible to fully eradicate during a pulmonary infection, even with aggressive antibiotic therapy which will only aid in slowing down the development of the disease.[92] Polysaccarides such as alginate have been proven to lead to the development of resistance against tobramycin. It is also speculated that alginate can protect organisms against environmental factors such as oxidative stress and the immune system.[93] During inflammation in the lungs of cystic fibrosis patients alginate is

released and can protect the organisms from phagocytes.[94] Alginate, which can result in mucoid growth *in vivo* also aids in the resistance of the cell to the host's immune system.[95][54] Previous studies have shown that alginate plays a significant role in biofilm antibiotic resistance as the excessive production of alginate results in major changes to the morphological structure of the biofilm.[54][58] Treating mucoid *Pseudomonas aeruginosa* with alginate lyase to assist alginate breakdown results in increased sensitivity to gentamicin.[58]

Although some bacteria appear to be resistant to antibiotics when growing in a biofilms, they have been shown to become susceptible when leaving the biofilm, suggesting that mechanisms other than mutations contribute to antibiotic resistance in a biofilms.[96] There are three main hypotheses that have been described for the antibiotic resistance of bacteria in biofilms. The first hypothesis involves the prevention of the antibiotic from entering the biofilm matrix or slowing down the process; such as for aminoglycosides. However penetration of certain antibiotics such as fluoroquinolones will not be altered by the structure of the biofilm.[12] There is indeed a limitation on this mechanism since the amount of antimicrobial binding proteins is limited and will thus result in further penetration of the antimicrobial when all binding proteins are occupied.[12] Secondly, the microorganisms may change or remove molecules or processes present in the environment which can influence the antibiotic activity. Factors such as pH,[97] oxygen[98], and the osmotic environment[99] can directly affect antibiotic susceptibility. When an organism stops growing due to insufficient nutrients or substrates, antibiotics can lose their effectiveness since some antibiotics such as penicillin only target growing organisms.[100] The third hypothesis describes cells that form a spore-like state with a decrease in metabolic activity and growth and can persist after antibiotic treatment since they are not actively targeted.[96][101] This can also be explained by nutrients not diffusing to the innermost part of the biofilm due to the outer organisms consuming it and none reaching the persister cells which become metabolically inactive.[36]

According to Livermore *et al.,*(2002)[16] changes in multiple factors such as the up regulation of efflux pumps, down regulation of OprD as well as impermeability to aminoglycosides can increase resistance to multiple antibiotics. *P. aeruginosa* is known to cause high morbidity related persisting nosocomial infections due to developing multidrug resistance and biofilm formation in immunocompromised patients.

1.6 Problem statement

Biofilm forming organisms cause persisting infections within the healthcare setting. In the clinical setting biofilm formation commonly occurs on wound tissue, body surfaces, lungs, as well as medical devices namely ventilators, catheters, joint and organ replacement parts.[10] Biofilms play a significant role in antibiotic resistance influencing the effects of antibiotic activity in different ways such as preventing penetration of antibiotics and reaching the cells within the biofilm.

1.7 Aims & Objectives

The aim of the study is to determine the population structure of *P.* aeruginosa in tygerberg hospital and to investigate the biofilm formation of thesis isolates and study the impact of common gram negative antibiotics on the formation of a biofilm.

Objectives

1. To determine the population structure of *P. aeruginosa* isolates from patients from the burns ward/ICU at Tygerberg Hospital in comparison to isolates from outside these wards.

2. To determine the biofilm formation ability of these isolates.

3. To determine the effect of common Gram negative antibiotics on the biofilm formation abilities of these isolates.

The study will enable us to determine whether transmission occurs within the clinical setting which will allow the implementation of the necessary control measurements, if required. An improved understanding of the biofilm formatting ability of these isolates and the impact of antibiotic treatment on the formation of biofilms may lead to improvements in the clinical treatment of *P. aeruginose* infections and thereby their outcome.

Chapter 2

Chapter 2 – Strain typing

2.1 Introduction

Nosocomial infections play a major role in morbidity and mortality and commonly result in the spread of MDR organisms. *Pseudomonas aeruginosa* is responsible for 10% of nosocomial infections and is ranked the 4th most abundant pathogen acquired in hospitals.[102] Spread within wards, between immune compromised individuals and patients receiving chemotherapy, occurs regularly in the ICU (intensive care unit) and burns wards. *P. aeruginosa* is commonly associated with a range of infections and the risk of developing a *P. aeruginosa* infection increases as the length of stay increases.[103]

The aim of this study was to determine the population structure of *P. aeruginosa* isolated from patients from the burns unit/ICU (burns unit and burns ICU) and to compare it to other *P. aeruginosa* isolates from Tygerberg Hospital.

The population structure was determined using MLVA (multi locus variable number tandem repeat analysis). MLVA works on the basis of amplifying variable number tandem repeats (VNTRs) targeting polymorphic tandem repeat loci. *P. aeruginosa* is known to be tandem repeat rich.[20] The polymorphic tandem repeats were previously identified using a program for strain comparison, developed by Denoeud and Vergnaud to identify intergenic sequences which are tandemly duplicated and which can be found multiple times throughout the genome..[34] Primers were designed to flank the 5' and 3' VNTR tandem repeat regions to amplify the tandem repeat. As the number of tandem repeats at each locus can differ between different strains, different allele sizes result in different product sizes which can be visualized by means of gel electrophoresis, as seen in figure 2.1. The allele sizes can then be combined to create an MLVA pattern by assigning an allele repeat number to the different allele sizes and generating a repeat pattern specific to an MLVA type.[20] MLVA typing data can be added to the MLVA genotyping database MLVAbank for Microbes Genotyping for comparison between different studies. The database contains MLVA typing information of different organisms, including *P. aeruginosa,* from all over the world, and with all the described allele sizes.

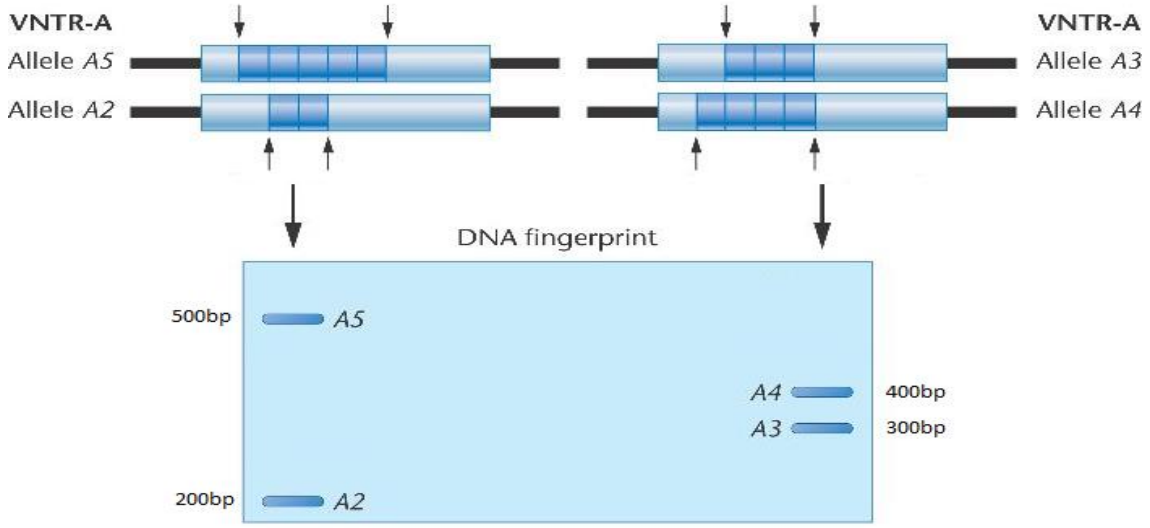

Figure 2.1: Representation of variable number tandem repeats at a specific locus. Each allele can have a different number of repeats which will result in a different size which can be visualized by means of gel electrophoresis. Source: Chen (2008).[104]

Previous studies have shown that although MLVA typing is relatively stable, it has some drawbacks. It has been documented that some loci do not amplify in certain strains. This could be explained by the addition, deletion or even mispairing of repeats at a specific VNTR locus (partially or entirely) and occurs more frequently with microsatellites.[34] Repeat sizes larger than those previously described (more than 1.5kb) have been identified and may be explained by the presence of an IS element (insertion sequence) in the tandem repeat.[34] An IS element is a transposable element which normally codes for proteins aiding in the movement of the element within a genome, thus regulating transposition.[105] However, MLVA is an easy and rapid molecular strain typing method for the identification of related strains within a community. Compared to other molecular methods, it is relatively inexpensive and robust and has a high inter-laboratory reproducibility.[33]

Accurate typing is necessary to determine the epidemiology of *P. aeruginosa* in Tygerberg Hospital. Identifying the strain types will help to describe transmission events and allow the implementation of precautions to avoid nosocomial transmission; which will assist in infection control in the hospital. Strain typing can also help to identify differences in virulence properties, such as biofilm formation, as described in chapters 3 and 4.

2.2 Materials and methods

2.2.1 Sample collection and storage

P. aeruginosa isolates were collected from the National Health Laboratory Service (NHLS) microbiology laboratory at Tygerberg Hospital (South Africa). Isolates were collected from February 2015 to March 2016 and included all *P. aeruginosa* isolates from blood cultures, pus swabs, tissue samples and aspirates from patients admitted to Tygerberg Hospital. Duplicate isolates from the same patient were not included.

Species identification and antibiotic susceptibility testing were done by the NHLS routine diagnostic laboratory using the VITEK-2 automated platform (Biomerieux, France) in conjunction with disk diffusion susceptibility testing and other routine tests and interpreted according to the Clinical and Laboratory Standard Institute (CLSI) guidelines[106], as part of routine diagnostic procedures. Isolates were stored in Microbank™ tubes (Pro-Lab diagnostics) at -80˚C, according to the manufacturer's protocol.

A total of 40 *P. aeruginosa* isolates obtained from patients within the burns unit/ICU and 40 *P. aeruginosa* isolates from patients outside the burns unit/ICU were used for this study. All isolates from patients admitted to the burns unit/ICU were included. Selected isolates from outside the burns unit/ICU, representing all sample types over the full collection period, were included. The *P. aeruginosa* PAO1 (Harvard) strain was obtained from the National Institute of Communicable Diseases (NICD) and used as a control strain as the whole genome sequence and MLVA typing results for this strain are available.

2.2.2 Bacterial culture conditions

A Microbank bead vial containing stored culture at -80˚C was thawed and streaked out on TBA agar (tryptose blood agar, NHLS Greenpoint Media Laboratory) and incubated aerobically overnight at 37 ˚C.

2.2.3 DNA extraction

Crude DNA extraction was performed on all isolates. A loop full of a pure overnight culture was suspended in 300 µl of dH_2O in an Eppendorf tube and vortexed for 30 seconds. The sample was placed in a heating block at 95 ˚C for 30 minutes and then placed directly at -80 ˚C for 30 minutes. The samples were thawed and centrifuged in a Spectrafuge 24D centrifuge (Labnet) at maximum speed, 15 600 x g (13 000 rpm), for 10 minutes to remove cell debris. DNA extracts were stored at -20 ˚C.

2.2.4 MLVA strain typing

2.2.4.1 MLVA PCR

MLVA analysis was done using 13 VNTR loci (ms77, ms127, ms142, ms172, ms211, ms212, ms213, ms214, ms215, ms216, ms217, ms222 and ms223) using specific primer sets (Table 2.1) as described by Vu-Thien et al., (2007).[34] PCR was performed using KAPA Taq ReadyMix PCR Kit (KAPA Biosystems) according to manufacturer's conditions, with the exception of using KAPA2G Robust HotStart ReadyMix PCR kit (KAPA Biosystems) for ms213. The PCR master mix was made up as follows: 7.5µl KAPA Taq ReadyMix/KAPA2G Robust HotStart ReadyMix, 0.6µl of each primer (50pmol/µl) (2µM per reaction), 1µl template DNA and 5.9µl dH_2O in a 15µl reaction. The PCR assays were performed on the Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific) or the ProFlex PCR system (Thermo Fisher Scientific). The cycling conditions used were as follows: an initial denaturation of 3 minutes at 95 ˚C, followed by 35 cycles of denaturation of 30 seconds at 95 ˚C, annealing for 30 seconds at 55/60/62˚C and extension for 1 minute at 72˚C, followed by a final extension of 1 minute at 72 ˚C and hold at 4˚C until downstream analysis. Different annealing temperatures were used for different primer sets, as shown in Table 2.1.

Table 2.1: MLVA PCR and sequencing primers. The repeat unit size of each VNTR locus, the number of possible alleles at each locus and the repeat number of each allele, and the expected *P. aeruginosa* PAO1 product size are indicated. Adapted from Vu-thien *et al.*, (2007)[34] and Youenou *et al.,* (2014).[24]

VNTR locus	Primer name	Primer sequence	Tm ˚C	Repeat unit size (bp)	No. of alleles (possible allele repeat numbers)	PAO1 product size (bp)
ms77	ms77F ms77R	GCGTCATGGTCTGCATGTC TATACCCTCTTCGCCCAGTC	60	39	8(1-6; 1,5; 2,5)	442
ms127	ms127F ms127R	CTCGGAGTCTCTGCCAACTC GGCAGGACAGGATCTCGAC	60	15	2(8-9)	210
ms142	ms142F ms142R	AGCAGTGCCAGTTGATGTTG GTGGGGCGAAGGAGTGAG	60	115	10(1-7; 12; 2,5; 3,5)	890
ms172	ms172F ms172R	GGATTCTCTCGCACGAGGT TACGTGACCTGACGTTGGTG	60	54	7(8-13; 8,5)	789
ms211	ms211F ms211R	ACAAGCGCCAGCCGAACCTGT CTTCGAACAGGTGCTGACCGC	60	101	9(2-9; 1,5)	663
ms212	ms212F ms212R	TGCTGGTCGACTACTTCGGCAA ACTACGAGAACGACCCGGTGTT	60	40	10(3-9; 11-12; 14)	522
ms213	ms213F ms213R	CTGGGCAAGTGTTGGTGGATC TGGCGTACTCCGAGCTGATG	62	103	12(0-7; 9; 10; 4,5)	640
ms214	ms214F ms214R	AAACGCTGTTCGCCAACCTCTA CCATCATCCTCCTACTGGGTT	55	115	8(2-6; 12; 2,5; 5,5)	426
ms215	ms215F ms215R	GACGAAACCCGTCGCGAACA CTGTACAACGCCGAGCCGTA	55	129	8(1-7; 2,5)	765
ms216	ms216F ms216R	ACTACTACGTCGAACACGCCA GATCGAAGACAAGAACCTCG	55	113	5(1-5)	543
ms217	ms217F ms217R	TTCTGGCTGTCGCGACTGAT GAACAGCGTCTTTTCCTCGC	55	109	8(1-6; 9; 1,5)	606
ms222	ms222F ms222R	AGAGGTGCTTAACGACGGAT TGCAGTTCTGCGAGGAAGGCG	55	101	9(1-7; 1,5; 5,5)	390
ms223	ms223F ms223R	TTGGCAATATGCCGGTTCGC TGAGCTGATCGCCTACTGG	55	106	7(2-7; 1,5)	454

2.2.4.2 Gel electrophoresis

Gel electrophoresis was performed to determine the sizes of the VNTRs for each strain. Electrophoresis was performed on 20cm, 2% 1xTBE (89 mM Tris, 89 mM borate, 2.5mM Ethylenediaminetetraacetic acid gels. Amplicons were mixed with Novel Juice (GeneDireX) for DNA visualization at a ratio of 5:1 and run for 4 hours at 110 volts. The gels were visualised on the Alliance 2.7 (UViTec) optic analysis system. A 50bp DNA ladder (New England Biolabs) was used to determine the product sizes.

2.2.4.3 MLVA genotyping

The amplicon sizes were used to determine the allele sizes and to deduce the number of repeats at each locus, as described by Vu-thien *et al.,* (2007)[34] and Youenou *et al.,* (2014)[24] (Table 2.2). The bands were separated sufficiently for accurate size determination when compared to the DNA ladder and previously described allele sizes. The repeat number of the allele at each locus was combined in the order ms77, ms127, ms142, ms172, ms211, ms212, ms214, ms215, ms216, ms217, ms222, ms223, ms213 to create an MLVA pattern which was used to manually define an arbitrarily numbered MLVA type.

MLVA data with arbitrarily numbered MLVA type information were added to the MLVA genotyping database MLVAbank for Microbes Genotyping (http://mlva.u-psud.fr/mlvav4/genotyping/). Clustering of the MLVA types was analysed using MLVAbank to construct a Newick dendrogram (Fredslund, 2006).[107]

Table 2.2: Thirteen VNTR loci allele band sizes[24]. Each VNTR locus contains a different number of tandem repeats which results in a different size allele. Red – PAO1 allele sizes and repeat numbers. Adapted from Vu-thien *et al.,* (2007)[34] and Youenou *et al.,* (2014).[24]

VNTR locus	Repeat size	Number of repeats	Allele size
ms77	39	1	325
		2	364
		3	403
		4	442
		5	481
		6	520
		1.5	344
		2.5	384
ms127	15	8	210
		9	225
ms142	115	1	201
		2	316
		3	431
		4	546
		5	661
		6	776
		7	890
		12	1465
		2.5	373
		3.5	488
ms172	54	8	627
		9	681
		10	681
		11	735
		12	789
		13	843
		8.5	654
ms211	101	2	360
		3	461
		4	562
		5	663
		6	764
		7	865
		8	966
		9	1067
		1.5	310
ms212	40	3	284
		4	324
		5	364
		6	404
		7	444
		8	484
		9	522
		11	602
		12	642
		14	722

VNTR locus	Repeat size	Number of repeats	Allele size
ms214	115	2	311
		3	426
		4	540
		5	655
		6	770
		12	1345
		2,5	368
		5.5	712
ms215	129	1	378
		2	507
		3	636
		4	765
		5	894
		6	1023
		7	1152
		2.5	571
ms216	113	1	315
		2	428
		3	543
		4	656
		5	769
ms217	109	1	497
		2	606
		3	715
		4	824
		5	933
		6	1042
		9	1369
		1.5	551
ms222	101	1	289
		2	390
		3	491
		4	592
		5	693
		6	794
		7	895
		1.5	339
		5.5	743
ms223	106	2	242
		3	348
		4	454
		5	560
		6	666
		7	772
		1.5	295

VNTR locus	Repeat size	Number of repeats	Allele size
ms213	103	0	125
		1	228
		2	331
		3	434
		4	537
		5	640
		6	743
		7	846
		9	1052
		10	1155
		4.5	588
		5.5	691

2.2.4.4 VNTR sequence analysis

Amplicons which were of allele sizes that were not described in the literature were sent to Inqaba biotech™ for Sanger sequencing. The sequences were analysed using BioEdit Sequence Alignment Editor (Hall, 1999)[108] to determine the allele size and repeat number. Certain sequences were blasted using the NCBI (National Centre for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) server (https://www.ncbi.nlm.nih.gov/) for the identification of insertion sequences.

2.2.5 Ethics

Ethical approval was obtained from the Health Research Ethics Committee of Stellenbosch University. Ethics reference: (S15/02/019) (A4 Appendix).

2.3 Results

2.3.1 Isolate collection

A total of 80 *P. aeruginosa* isolates were typed using MLVA typing. Forty isolates were obtained from patients within the burns unit and the burns ICU. Samples were collected from a number of sites including CVC (central venous catheter) tips, wound/pus swabs, sputum, blood cultures and tissue. Another forty samples were isolated from patients hospitalized in wards other than the burns unit and burns ICU at Tygerberg Hospital. These included patients with pneumonia, sepsis, cystic fibrosis, abscesses and surgical site infections. The samples were categorised into three specimen types; Respiratory, Swab/tissue and Blood/Other specimens, and into three ward types; Paediatric, Surgery and Medical (Table 2.3). Respiratory specimens included any samples from the respiratory tract such as aspirates, sputum and washings. Swabs and tissue specimens included pus, fluid and tissue biopsies from wounds. Blood and other specimens included blood cultures and CVC tips.

Table 2.3: Specimen types and ward types of non-burn isolates. Ward information was not available for nine of the isolates.

Specimen type	No. of Samples	Ward type, n
Respiratory	12	Paediatric, 2
		Surgery, 2
		Medical, 5
Swab/Tissue	21	Paediatric, 2
		Surgery, 13
		Medical, 2
Blood/Other	7	Paediatric, 0
		Surgery, 3
		Medical, 2
Total	**40**	

2.3.2 MLVA typing

MLVA typing was done on the 80 *P. aeruginosa* isolates. The allele sizes and repeat numbers of 13 loci were determined for each isolate. For example, the allele sizes for all 13 VNTR loci of control strain PAO1 are represented in figure 2.2. The allele repeat number of each locus was determined and then combined to form the MLVA type. The assigned MLVA pattern for PAO1 was 4,8,12,7,5,9,3,4,3,2,2,4,5.

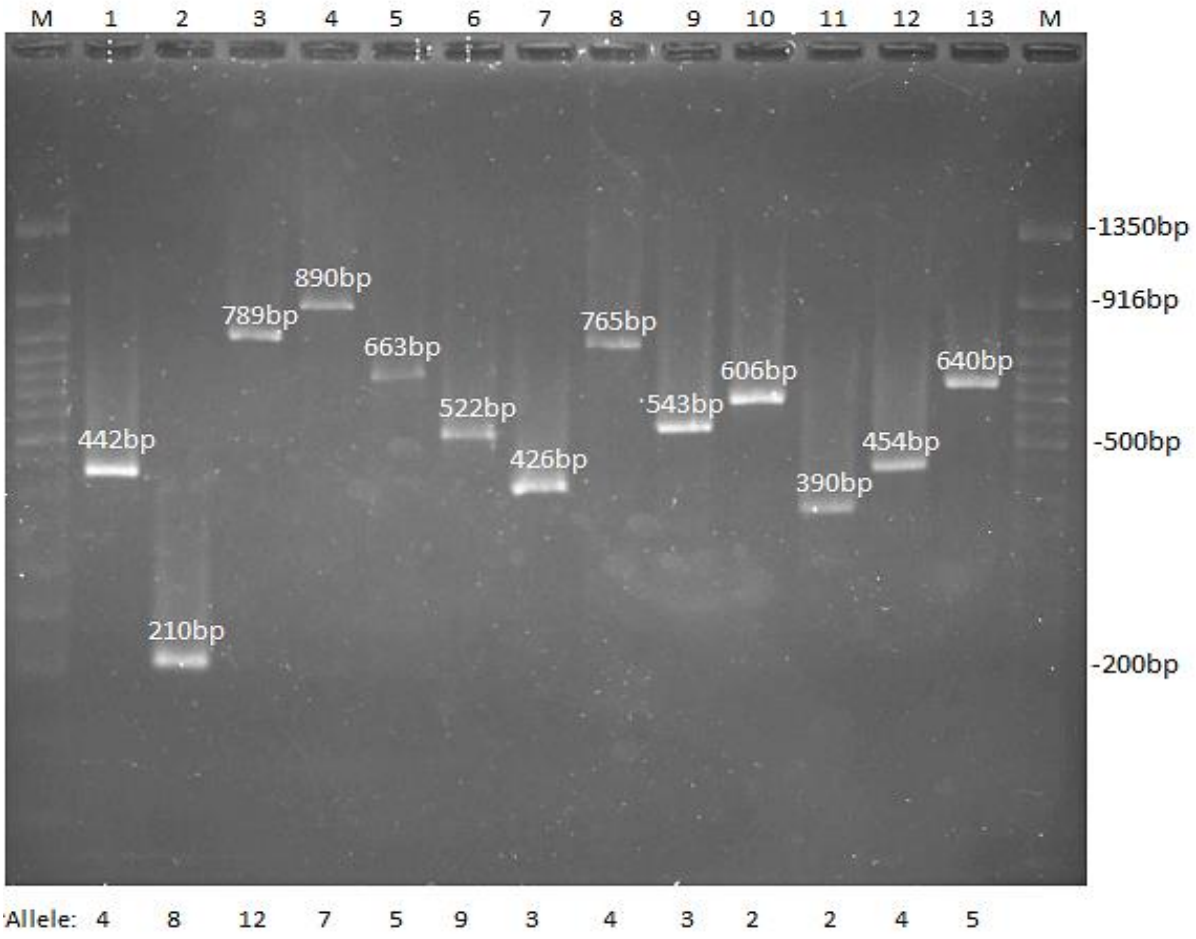

Figure 2.2: Gel electrophoresis of PAO1 with 13 VNTR loci. M-Molecular marker (DNA ladder 1350bp), 1-13: VNTR loci ms77, ms127, ms172, ms142, ms211, ms212, ms214, ms215, ms216, ms217, ms222, ms223, ms213.

Certain loci showed amplification of band sizes that are not described in the literature or could not be differentiated successfully from other alleles since the size differences were too small to be visualized with gel electrophoresis. Several amplicons appeared to be slightly bigger than other corresponding alleles on the gel and representative amplicons of these alleles were sent for sequencing to confirm the size of the alleles. Sequencing of ms172 from TBP 36, ms212 from TBP 57 and ms217 from TBP 6 confirmed the previously described allele sizes (Appendix Table A1 and Table A2) of 843bp, 602bp and 497bp,

respectively, allowing the correct VNTR repeat numbers to be assigned (Table 2.4). The apparent difference in size that was seen on the gel may be due to the gel melting because of the long run and rising temperature, which resulted in the bands shifting slightly. This can be overcome by reducing the voltage and extending the run time to keep the buffer temperature low.

Table 2.4: New and confirmed allele sizes and repeat numbers determined. N – Newly described allele sizes. C – Confirmed allele sizes.

Locus (representative isolate sequenced)	Size (bp)	Allele repeat number
ms172 (TBP 36)	843	13 (C)
ms212 (TBP 57)	602	11 (C)
ms217 (TBP 6)	497	1 (C)
ms172 (TBP 33)	897	14 (N)
ms212 (TBP 24)	147	0.5 (N)
ms215 (TBP 3)	281	0.25 (N)
ms215 (TBP 69)	66	None
ms216 (TBP 69)	196	0 (N)
ms223 (TBP 6)	205	1.125 (N)

Previously undescribed allele sizes were obtained for a number of loci. The representative loci that were sequenced included ms172 from TBP 33, ms212 from TBP 24, ms215 from TBP 3 and TBP 69, ms216 from TBP 69, and ms223 from TBP 6 (Table 2.4). For TBP 3 a 281bp product was obtained for locus ms215 which correlates with a repeat unit number of 0.25 and was detected in 35 isolates. Other previously undescribed VNTR product sizes included an 897bp product for ms172 from TBP 33, a 66bp product for ms215 from TBP 69, a 147bp product for ms212 from 4 isolates, a 196bp product for ms216 from TBP 69 and a 205bp product for ms223 from 2 isolates. A new allele number was assigned to each new allele size (Table 2.4). Studies have previously shown the presence of half repeat sizes;[24][34] for example, if a repeat size is 54bp (ms172) a repeat of 27bp was identified. Here we describe the presence of 0.25 and 0.125 repeat sizes.

An allele repeat number could not be assigned to locus ms215 for isolate TBP 69 and numerous other loci also failed to amplify in this isolate (Appendix Table A2). 16S rRNA PCR and sequencing was performed on the isolate to confirm its identity and it was found to be *Acinetobacter baumannii* with 99% sequence homology with published sequences and therefore excluded from further analysis. Failed amplification, noted as NA (No amplification) in Table A1 and A2 (Appendix), also occurred for some loci in some other isolates. This

occurred more frequently in non-burn isolates than in burn isolates. Only four isolates from the burns unit/ICU had missing amplification at one locus; while five non-burn isolates failed to amplify between one and three loci.

Two isolates, TBP 40 and TBP 46, produced products of 1300 – 1500bp for VNTR loci ms216 and ms223 respectively. This phenomenon is known to occur in *P. aeruginosa* MLVA typing and is a result of an insertion sequence present within the locus. The PCR amplicons were sequenced and the IS elements in both were identified as the IS110 family transposase.

2.3.3 MLVA analysis (Burn and non-burn isolates)

Isolates from patients in the burns unit and burns ICU were compared to each other as well as to non-burn patient isolates. MLVA analysis identified forty two different MLVA types amongst the 79 isolates (TBP 69 excluded) (Table 2.5). Ten of the MLVA types were shared by two or more isolates, while the remaining 32 types were represented by a single isolate and had unique MLVA patterns.

Table 2.5. MLVA typing results of *P. aeruginosa* isolates from patients in the burns unit/ICU and non-burns wards at Tygerberg hospital. A total of thirteen VNTR loci were used to create an MLVA pattern for each isolate, which represents an MLVA type. Unique MLVA types represent MLVA patterns which were only identified in one isolate.

MLVA type	MLVA pattern	Burns unit (n)	Non-burns (n)	Total
1	3,9,13,1,3,9,4,0.25, 1,5,1,4,6	24	1	25
2	3,9,13,1,7,0.5,4,0.25,1,5,1,4,6	4	0	4
3	2.5,8,11,4,8,11,4,1,2,3,1,2,1	2	0	2
4	2.5,8,12,2,3,8,5,6,1,4,1,2,4	0	2	2
5	2,8,10,1,2,6,4,1,1,4,2,2,3	1	1	2
6	4,9,13,1,3,9,4,0.25,1,5,1,5.5	3	0	3
7	2,8,11,5,4,6,2,1,2,2,1.5,2,5	0	2	2
8	2,8,11,4,8,11,4,1,2,3,1,2,1	0	3	3
9	2,8,12,4,8,9,2,4,2,4,3,3,2	0	2	2
10	3,9,13,1,3,3,4,0.25,1,5,1,4,6	0	2	2
Unique	Varying patterns	6	26	32
Total		40	39	79

MLVA types 1, 2, 3, 5 and 6 were identified in isolates from the burns unit/ICU. Sixty percent (24 /40) of the isolates from the burns units/ICU belonged to MLVA type 1. The second most abundant MLVA type, MLVA type 2, was identified in four isolates, while two MLVA type 3 isolates and three MLVA type 6 isolates were identified. Six of the forty burns isolates had a unique pattern which did not correspond to any other isolate. MLVA types 1, 4, 5, 7, 8, 9 and

10 were identified amongst the non-burn isolates (Table 2.5). Only one of the non-burn isolates was MLVA type 1. One MLVA type, type 5, had isolates belonging to patients in the burns unit/ICU and outside of the burn unit. Two MLVA type 4 isolates were identified, both of which were from young patients (<7years) with cystic fibrosis. Twenty six of the non-burn isolates had unique MLVA types, and no more than three non-burn isolates clustered within an MLVA type. The results show that non-burn isolates are much more diverse than those from the burns unit and burns ICU.

2.3.4 MLVA clustering analysis

MLVA data was added to MLVAbank for Microbes Genotyping (http://bacterial-genotyping.igmors.u-psud.fr/). A dendrogram was constructed to represent the clustering of the different MLVA types using 13 loci and 10 loci respectively, to determine how the number of loci analysed influences the assignment and clustering of MLVA types (Figures 2.3 and 2.4). Clusters are seen localized on one branch. Some type 1 isolates, such as TBP 15 and TBP 26, failed amplification at locus ms214, and TBP 18 failed amplification at ms127. These loci were therefore excluded from the phylogenetic analysis.

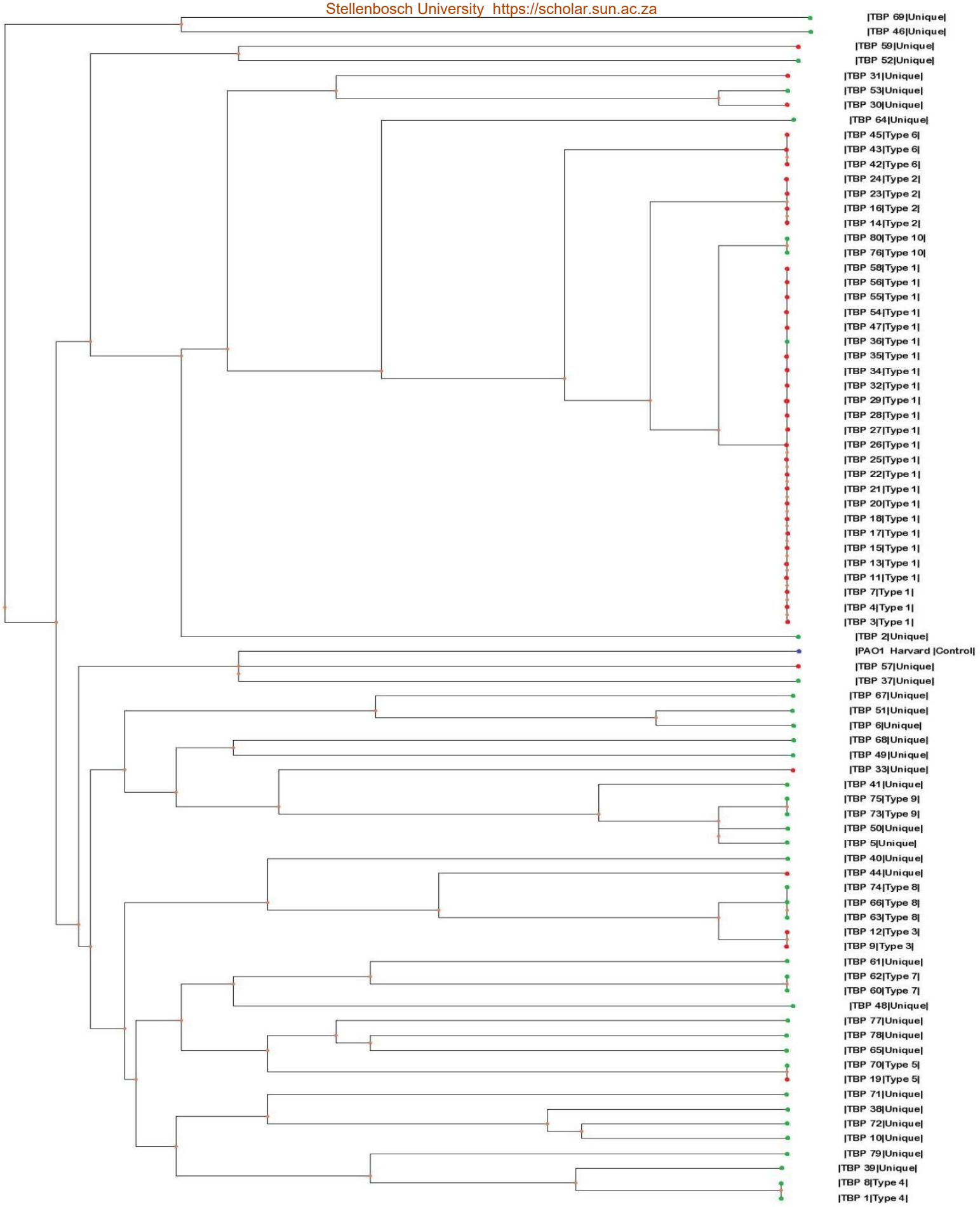

Figure 2.3: Dendrogram showing MLVA type clustering using 13 VNTR loci. Red: Burn isolates, Green: Non-burn isolate, Blue: PAO1 control.

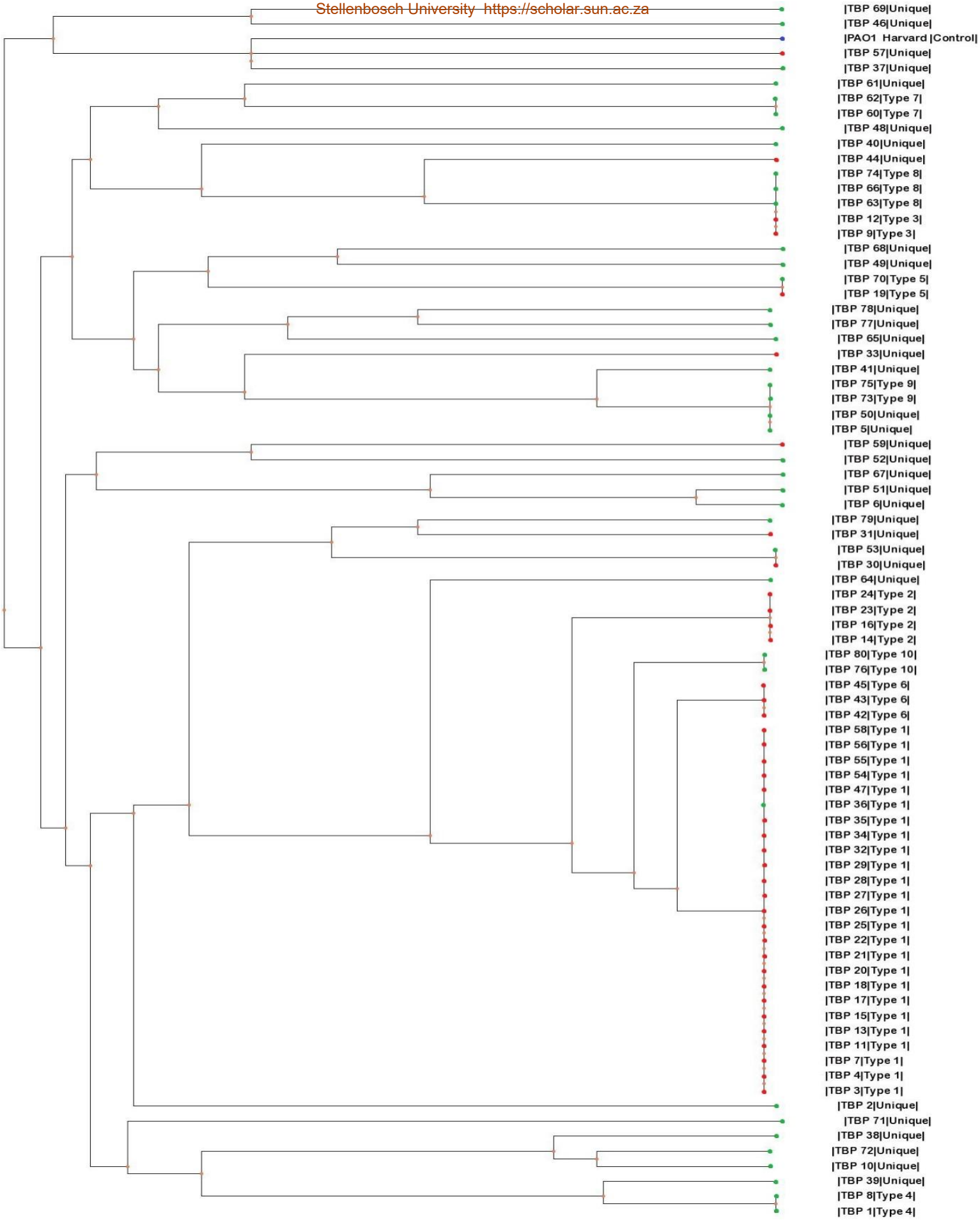

Figure 2.4: Dendrogram showing MLVA type clustering using 10 VNTR loci. Red: Burn isolates, Green: Non-burn isolate, Blue: PAO1 control.

The clustering of the MLVA Type 1 isolates is clear on the dendrograms when using 13 loci, as is the clustering of the other 9 MLVA types with more than one isolate. MLVA type 3 and MLVA type 8 cluster together, and differ with only one allele size difference at locus ms77. TBP 30 and TBP 53 (both unique MLVA types) also cluster together, and differ only at locus ms77. Two non-burn isolates, TBP 5 and TBP 50 (classified as unique strains), also differ from each other with one allele repeat at locus ms77; and also differ from type 9 isolates only at locus ms77. Overall, locus ms77 appears to be responsible for most of the single differences that are found between MLVA types.

When using only 10 loci (excluding ms77, ms127 and ms172), as previously used by Vu-thien *et al.*, (2007)[34] MLVA types 3 and 8 form one MLVA type; two additional isolates, TBP 5 and TBP 50 which were unique types, are included in MLVA type 9; and TBP 30 and TBP 53 are classified as the same MLVA type. The number of clusters remained the same but the number of unique MLVA patterns was reduced from 32 to 29. It appears that reducing the number of VNTR loci reduces the discrimination of MLVA typing considerably in this isolate collection compared to the study by Onteniente *et al.*,(2003)[20] which did not lose discrimination. This is most likely due to the fact that ms77 is excluded and appeared to be responsible for most of the differences between MLVA types in our population.

2.4 Discussion

This study investigated the population structure of *P. aeruginosa* isolates obtained from patients at Tygerberg Hospital, who were either admitted to the burns unit and burns ICU or the other wards within the hospital. *P. aeruginosa* is a major health problem in Tygerberg Hospital, especially in terms of nosocomial infections and particularly in the burns unit and burns unit ICU which accounted for 38% of isolates collected. A total of 80 isolates were typed using thirteen VNTR locus MLVA analysis. The pathogen *P. aeruginosa* has at least 201 tandem repeats within its genome, of which several are VNTRs.[20] These VNTRs are polymorphic, meaning that there is more than one allele present within a population. Forty two different MLVA types were described of which ten were most abundant and were found in more than one isolate. There were twenty four type 1, four type 2, two type 3, two type 4, two type 5, three type 6, two type 7, three type 8, two type 9 and two type 10 isolates. The rest of the isolates (32/80) were all unique MLVA types from single patient isolates. Five different MLVA types with one or more isolates were described for burn isolates and seven different types with one or more isolates for non-burn isolates. MLVA type 5 included both a burn and non-burn isolate.

Majority of the isolates belonged to MLVA type 1 (25/80), all of which were isolated from patients in the burns unit/ICU, except for one isolate. The same was seen for MLVA type 2,

the second most abundant type, of which the isolates were all obtained from patients in the burns unit/ICU. This is an indication that organisms are regularly being spread from patient to patient within the burns unit/ICU. Transmission could be due to non-sterile methods, environmental contamination or equipment being shared between patients causing patient to patient transmission. This information has been communicated to the infection prevention control unit (IPC) at Tygerberg Hospital to address the issue. The IPC is actively involved in protecting patients and staff members from spreading infectious diseases within the hospital setting. Outbreaks of infectious diseases can occur when proper infection prevention control methods are not in place and can cause major morbidity and mortality.

None of the non-burn isolates that had the same MLVA type were isolated from patients within the same ward. Looking at non-burn isolates, the MLVA type clusters were very small compared to the burn isolates with a maximum of three isolates belonging to any MLVA type. This indicates that very little patient to patient transmission occurs within or between wards.

Organisms with similar MLVA patterns which differ at a single locus may be genetically related or share a recent common ancestor. However, sequencing results have shown that diversity at a specific locus can be very high and that these alleles of the same size may have unique sequences.[20] According to Le Fleche *et al.,*(2002) MLVA does contain phylogenetic information which can be useful to determine the ancestral relationship of some organisms.[109] A single change in one locus can also imply that it is the same strain but a different variant. Locus ms77 was shown to vary the most in our study. Clustering MLVA types can be difficult since only one change or deletion in the MLVA pattern or at a specific locus can result in a big change in the structure of a dendrogram. This can result in a branch shift out of a cluster giving a different representation of the isolate. For more accurate strain identification MLVA typing can be confirmed with PFGE or a sequence-based typing method. In some isolates there were failed attempts at amplifying certain loci; which could be due to deletions, complete or partial absence of the locus or mispairing of the allele.[20][34] This has also been found in a study conducted by Onteniente *at al.,*(2003)[20] where they found that 12% of their isolates failed amplification at one or more loci. The specific reason for these deletions and mispairing is still unclear.

The amplification of some loci resulted in products greater than 1.3kb in size. According to Vu-Thien *et al.,*(2007)[34] this can be explained by an IS (insertion sequence) element. Insertion sequences have been identified in more than one locus; particularly ms216 and ms223, the same loci that showed the presence of IS elements in our study. The sequences of these products were aligned on the NCBI website and an IS110 insertion sequence was

identified. IS elements are partial DNA sequences known as transposons with the ability to move from one place to another within the genome of the organism. Environmental stress can induce transposition causing mutant strains to emerge.[105][110]

In our study, thirteen loci were used for typing the isolates. According to Onteniente *et al.,*(2003)[20] it is possible to reduce the number of loci for typing to eight loci without losing any discriminatory power in the assay. Vu-Thien *et al.,*(2007)[34] showed that reducing the number of VNTRs to ten, using ms142, ms211, ms212, ms213, ms214, ms215, ms216, ms217, ms222, and ms223, can yield a much easier and robust assay. When reducing the number of VNTR loci to ten in our study, we found that a few unique isolates appeared to join existing MLVA types; MLVA type 3 and 8 merged to form one type, MLVA type 9 received two more unique MLVA type isolates and two unique MLVA type isolates, TBP 30 and TBP 53, that were previously defined as very similar MLVA types, were defined as the same MLVA type. This indicates that using 10 loci in our setting lowers the discrimination of MLVA typing, compared to using 13 loci.

Two loci, namely ms207 and ms209, were not included in the MLVA typing assay as difference in base pairs between the alleles are too small (6bp repeat unit) to discriminate by normal gel electrophoresis. Alternative methods such as sequencing, polyacrylamide gel electrophoresis or even capillary electrophoresis are required, which remain expensive and time consuming.[20][34] This is a limitation to our study since these loci have been shown to be the more polymorphic loci within the *P. aeruginosa* genome and to increase the discriminatory index of MLVA typing; however studies still tend to exclude these loci.[34]

Studies have shown that the discriminatory power of MLVA is much higher than ribotyping as well as serotyping.[34][20] In a study by Onteniente *et al*.,(2003) when testing 89 isolates, MLVA discriminated 72 different genotypes while ribotyping and serotyping only discriminated 46 and 12 respectively.[20] MLVA was also shown to be much faster compared to the gold standard PFGE and can generate the same discrimination as PFGE when using up to only nine loci.[97] A study by Vu-thien *et al.,*(2007)[34] showed that including other organisms (not *P. aeruginosa*) in the MLVA assay using *P. aeruginosa* specific primer sets resulted in loci not amplifying or producing a faint band; whereas *P. aeruginosa* will produce a distinct solid band. In our study, one isolate had multiple loci which failed amplification and was confirmed not to be *P. aeruginosa.* This indicates that the assay has a high specificity for *P. aeruginosa*.

MLVA was shown to be a very robust molecular typing method for *P. aeruginosa.* This genotyping method could be enhanced by sequencing the products while combining fluorochromes and using loci which have different sizes for PCR. This would enable the genotyping of a single isolate with only one run.[34] The alternative, PFGE, which is the gold standard for strain typing, has been shown to be labour intensive and expensive. MLVA shows high inter laboratory reproducibility and discrimination and is relatively inexpensive but also remains somewhat labour intensive and time consuming for clinical strain typing.

2.5 Conclusion

The study of the population structure of *P. aeruginosa* at Tygerberg Hospital identified a number of clones within the burns unit and burns ICU, and specifically one MLVA type which accounted for 60% of *P. aeruginosa* infections in these wards; which is an indication of nosocomial transmission. There is more strain diversity found outside these wards. This could be explained due to less patient to patient transmission occurring within other wards compared to the burns/ICU ward. However this analysis is limited by our study design, as only 40 randomly selected isolates were selected from the entire Tygerberg Hospital, to span the collection period. Therefore it is not possible to rule out more transmission between patients outside of the burns unit/ICU. Focussing on just one ward and isolating all *P. aeruginosa* organisms from that specific ward may reveal different results. There is a need for better infection prevention control in the burns unit and burns ICU and medical staff need to be informed and trained accordingly. Accurate typing methods such as MLVA need to be implemented to understand the epidemiology of the organism for early identification of outbreaks and rapid treatment to prevent further transmission. MLVA is easy to perform withhigh discriminatory power and reproducibility and was shown to be a promising method for future strain typing and outbreak investigations in *P. aeruginosa*.

Chapter 3

Chapter 3 – Biofilm analysis

3.1 Introduction

Biofilms consist of cells attached to a surface and also to each other, surrounded by an extra-polymeric substance which helps the organism by providing protection against antibiotic agents and biocides by preventing them from diffusing into the biofilm and also protects against the host's immune system.[111] *P. aeruginosa* is renowned for developing multidrug resistance and persistent chronic infections due to biofilm formation.[48] Biofilms commonly occur on medical devices, open wounds and respiratory tracts. The prevention of spread and treatment of persistent infections become more difficult due to biofilm formation and multidrug resistance.

Several studies have shown that *P. aeruginosa* is linked to burn wound infections causing high mortality rates and that *P. aeruginosa* is one of the most frequent organisms isolated from this setting.[8][7] These isolates are also potential biofilm formers and often result in biofilm related sepsis.[112] Patients with severe burn wounds stand a higher chance of developing an infection due to the destruction of the skin's natural defence barrier and other defences.[112] Sepsis among patients in burn ICUs has also been reported to be caused by *P. aeruginosa* in up to 14-20% of cases.[4] Burn wounds may be free of any infective organisms but are prone to infections due to organisms within sweat glands and hair follicles that are able to colonize the wound.

There are several different ways in which biofilms can be quantified. Different methods such as the Syto9, FDA, resazurin, XTT and dimethylmethylene blue assay are commonly used but can be expensive and time consuming. In this study biofilm assays were performed using crystal violet staining in 96 well micro titre plates, which is a commonly used method due to the reproducibility, time efficiency and the low cost involved. The aim of this study was to determine the biofilm formation ability of isolates from the burns ward/ICU and outside the burns ward at Tygerberg hospital. The biofilm forming ability of isolates from patients with burn wounds and isolates from non-burn associated infections were compared and differences in the capacity of different strain lineages, as identified by MLVA typing, to form biofilms was also assessed.

3.2 Materials and methods

3.2.1 Isolate selection

The clinical isolates described in Chapter 2 were used in the biofilm assays. A control strain, PAO1 Harvard (obtained from the National Institute of Communicable Diseases (NICD)) was included in all biofilm assays since it is a known biofilm former.[112]

3.2.2 Biofilm time-point assay

Biofilm analysis was done using the micro titre plate method, as previously described by O'Toole (2011)[113]. Glass broth bottles containing 10 ml TSB (Tryptic Soy Broth, Sigma), that contains sufficient glucose for cell growth, were inoculated with a microbank bead of the relevant isolate and incubated for 24 hours at 37˚C, with continuous shaking at 180 rpm, in a Labcon O_2 shaker incubator (Lasec). A 1:200 dilution of the overnight growth was made in fresh TSB media in a 1.5ml Eppendorf tube and vortexed for 5 seconds to ensure even suspension.

A micro titre flat well plate (Corning Costar Cell culture plates, 3595) (tissue culture treated for easy cell attachment) was inoculated with 200µl of the diluted cell suspension. Plates were incubated for varying durations (2h, 5h, 8h, 10h or 12h) at 37˚C, without shaking. After incubation, the wells were washed 3 times with millipore dH_2O to remove any loose cells. Any biofilm formed would be left behind in the well, and was heat fixed for 1 hour at 60˚C on a heating block (Accublock, Labnet). The wells were then treated with 200µl of 0.1 % crystal violet (Sigma) for 10 minutes to stain the formed biofilm. The plate was washed 3 times with dH_2O to remove the excess stain and air dried at 37˚C. Finally, 200 µl of 30% acetic acid was added to the wells and left for 10 minutes to dissolve the crystal violet stain to enable consistent absorbance measurements. The absorbance values of the wells were read in a micro titre plate reader (Biotek, Synergy HT) at 595nm. Higher absorbance values indicate more biofilm material in the well, and thus a 'stronger' biofilm forming organism.

The assay was performed in both biological and technical triplicate. Biological replicates were done using inoculations of three different microbank beads to control for biological variation between different samples of the organism. Technical replicates were done by inoculating three wells with the same inoculation which controls for human error. These replicates are necessary to determine the reproducibility of the assays. Average absorbance values and standard deviations were calculated for each time point.

Initial optimisation of the assay, using the 5 different incubation times described above, was performed on a convenience sample of 12 of the clinical isolates (TBP 24-36) as well as the control strain, PAO1. These thirteen isolates were selected to determine the optimal

incubation time for biofilm formation. Based on the optimisation assay, the biofilm formation of all 79 isolates (excluding TBP 69 - non burns) was tested using the above methodology after incubation of the plates for 12 hours.

3.3 Results

3.3.1 Optimisation of the biofilm formation time-point assay

Thirteen isolates were selected to perform a time point assay to select an ideal incubation time to assess biofilm formation. These isolates included TBP 24-35 which were isolated from patients in the burns unit/ICU and TBP 36 from a different ward (Figure 3.1).

Three distinct biofilm formation patterns were observed, as shown in Figure 3.1. Most isolates showed some increase in biofilm formation over time; this pattern of increasing biofilm could be classified in three categories characterised by a small, moderate or rapid increase. Isolates TBP 25 and TBP 26 showed a small increase in biofilm formation, with a maximum absorbance of <0.25 after 12 hours; the majority of the isolates showed a moderate increase in biofilm formation with increasing incubation time (TBP 24, 27, 28, 29, 30 and 33); while TBP 34 showed a rapid increase over time, with an increase in absorbance of more than a 0.5 between each time point. The second pattern, observed in TBP 32 shows no noticeable increase over time. The third pattern showed a rapid increase in biofilm formation in the first 2-5h followed by a decrease in biofilm formation which may indicate that the biofilm was starting to disperse (TBP 31 and PAO1). Isolates TBP35 and 36 showed a similar increase over the first 8 -10h and appeared to be either on the verge of dispersing or increasing by 12h.

The results of the optimisation showed that measuring biofilm formation at either 10 or 12 hours would reliably differentiate between different biofilm forming abilities, although one would not be able to differentiate between isolates that have ongoing biofilm formation compared to those which may be dispersing after 5 hours (e.g. TBP 31 and PA01). Therefore, given the technical challenges associated with performing biofilm assays on all 79 isolates at multiple time points, we elected to perform the assay on the collection of 79 isolates at a single time point at 12 hours.

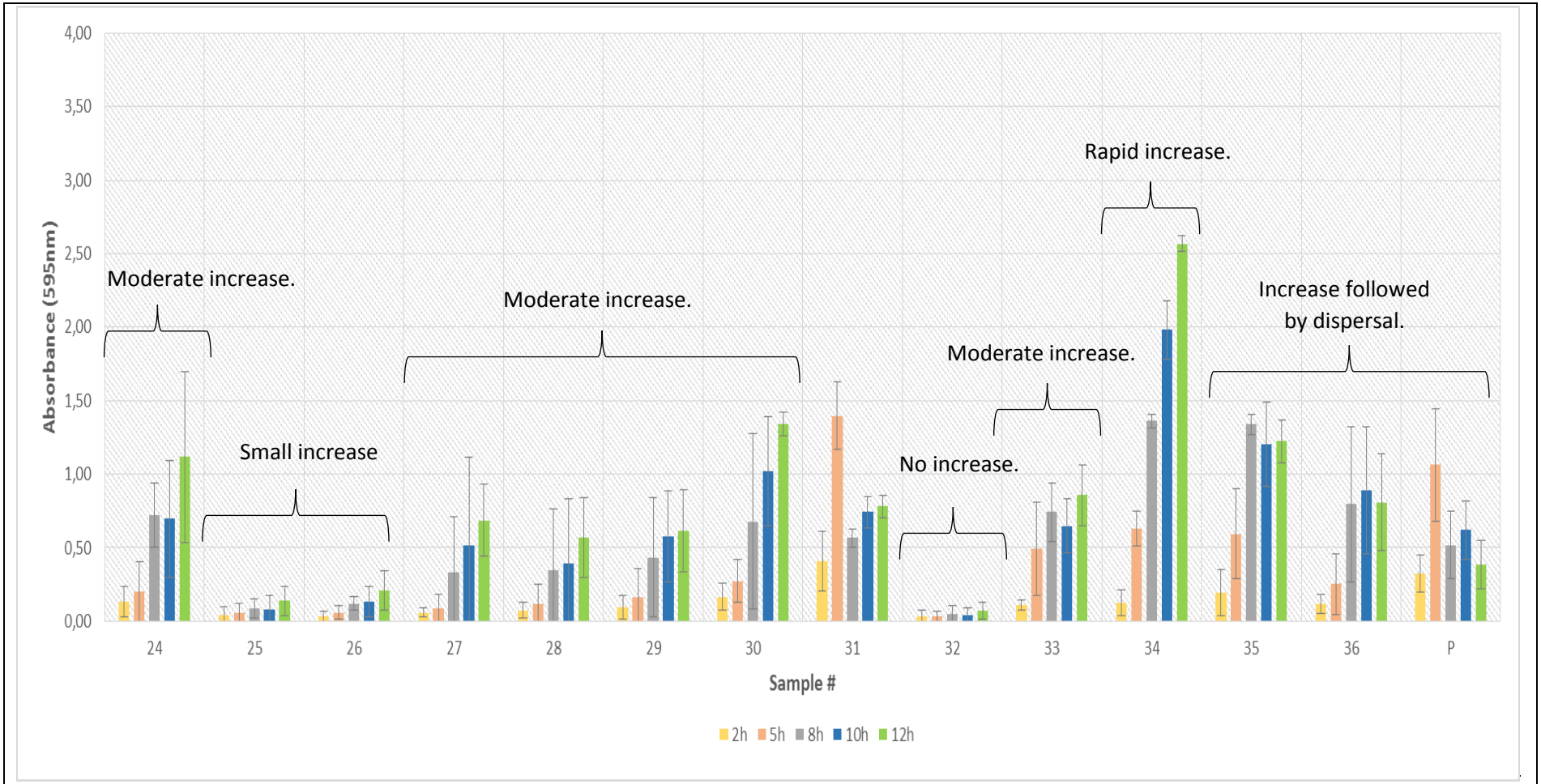

Figure 3.1: Biofilm formation time point assay. Biofilm formation was measured at 5 time points over 12 hours (2, 5, 8, 10, 12h). 13 isolates (TBP 24-36) were analysed and three distinct formation patterns described; increase in biofilm formation, no noticeable increase in biofilm formation and increase followed by dispersal. Absorbance values were measured at 595nm. P – Control strain (PAO1)

3.3.2 Biofilm formation of isolates from different clinical settings

Based on the results of the time point assay further biofilm analysis was carried out on all 79 isolates after a 12 hour stationary incubation. The control strain PAO1 is a moderate biofilm former according to our study and formed biofilms with an average absorbance value which ranges from 0.25 to 0.5. Based on this, for the purposes of this study, we defined strong biofilm formers as having an absorbance of more than 0.5, moderate biofilm formers as having an absorbance of 0.25-0.5 and weak to non-biofilm formers as having an absorbance value of less than 0.25 at an incubation time of 12 hours.

For both the isolates from patients in the burns unit and from other patients, there was a wide range of biofilm formation (Figure 3.2 and 3.3). There was more variation in biofilm forming ability among burns unit/ICU isolates than in non-burn isolates. Amongst the burn isolates, 55% (22 out of 40) were strong biofilm formers with the strongest biofilm former showing an absorbance of 3.64 (Table 3.1), 40% of the isolates (16 out of 40) showed weak to no biofilm formation, while only 5% (2 out of 40) were moderate biofilm formers. The majority of the non-burn isolates, 72% (28 out of 39), are strong biofilm formers; with the strongest biofilm former reaching an absorbance value of 1.63. Only 13% (5 out of 39) of the isolates showed weak to no biofilm formation (Table 3.1). Overall, strong biofilm formers were more common amongst non-burn isolates.

Table 3.1: Biofilm forming abilities of isolates from the burns unit/ICU compared to isolates collected from outside the burns unit.

Biofilm formation categories	**Burns unit/ICU: n, (%)**	**Other wards: n, (%)**
Weak biofilm (<0,25)	16, (40%)	5, (13%)
Moderate biofilm (0,25 – 0,5)	2, (5%)	6, (15%)
Strong biofilm (>0,5)	22, (55%)	28, (72%)

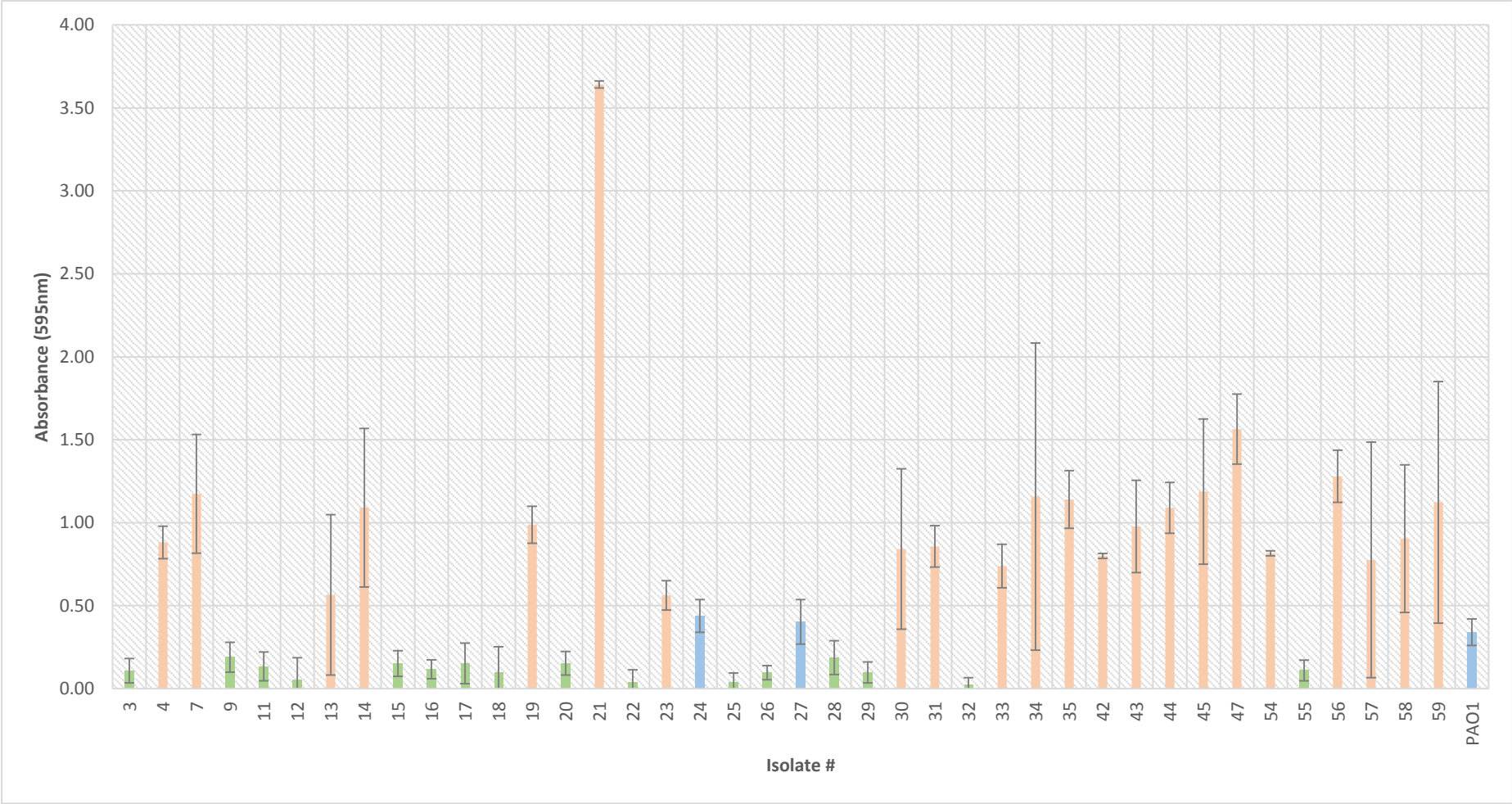

Figure 3.2: Biofilm formation 12h assay. Biofilm formation of 40 isolates from the burns unit and burns ICU after an incubation time of 12 hours. Absorbance values were measured at 595nm. PAO1 – Control strain. Orange: Strong biofilm former (>0.5), Blue: Moderate biofilm former (0.25-0.5), Green: Weak biofilm former (<0.25).

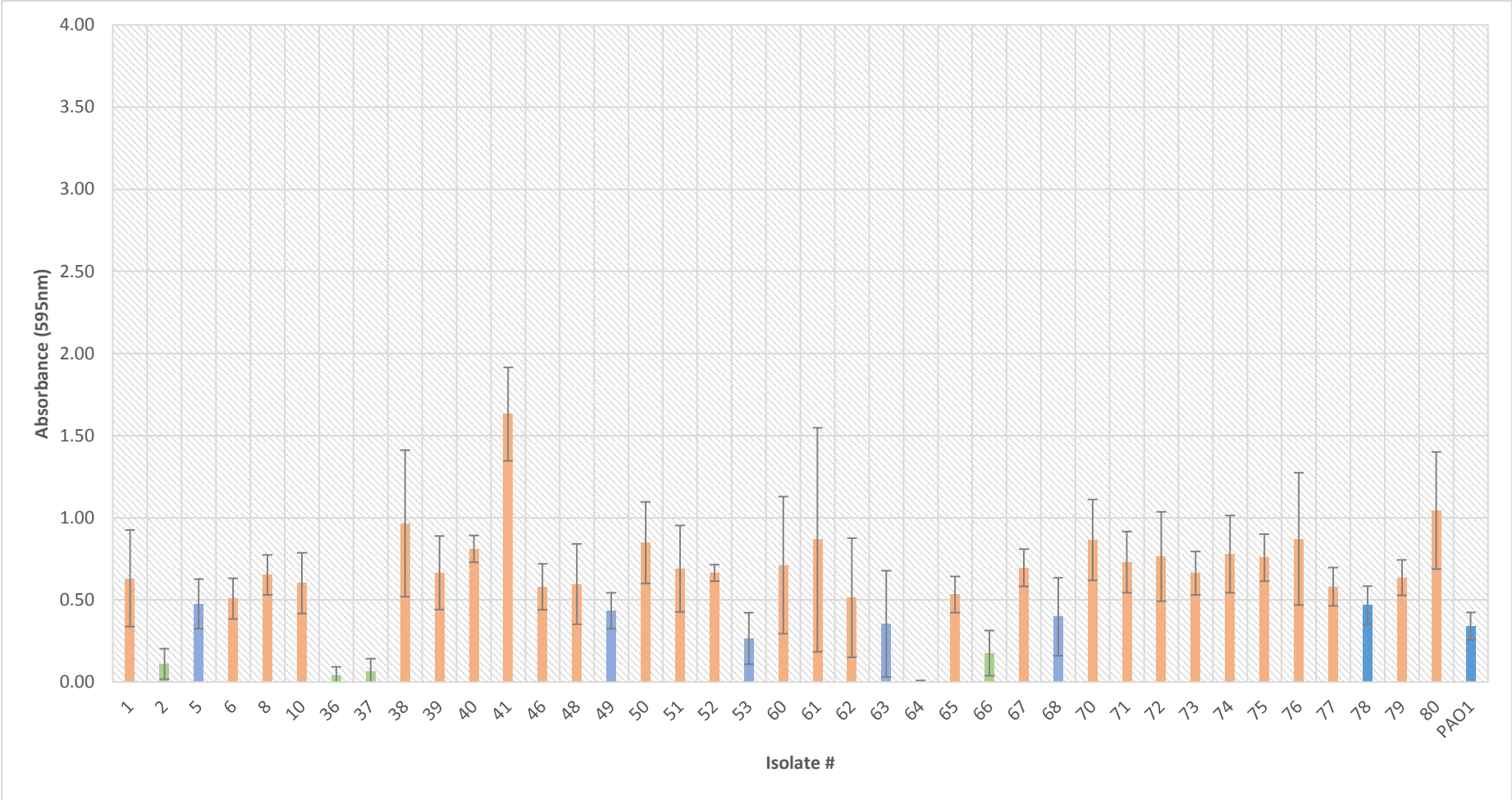

Figure 3.3: Biofilm formation 12h assay. Biofilm formation of 39 non-burn isolates after an incubation time of 12 hours. Absorbance values were measured at 595nm. PAO1 – Control strain. Orange: Strong biofilm former (>0.5), Blue: Moderate biofilm former (0.25-0.5), Green: Weak biofilm former (<0.25).

3.3.3. Biofilm formation of isolates based on specimen type

The biofilm forming ability of isolates was further analysed based on the specimen type. Isolates were obtained from several sample types and categorised in four categories, wound/pus swabs and tissue, CVC (central venous catheter) tips, blood cultures and sputum. One isolate's data was not indicated on the patient information form. The biofilm forming ability of isolates from each specimen type from both the burns unit/ICU and outside the burns unit/ICU was determined (Tables 3.2 and 3.3 respectively). The majority of the burns unit/ICU isolates were obtained from wound/pus swabs and tissue (26 out of 40). Isolates from wounds/pus swabs, tissue and blood cultures had a range of strong, moderate and weak biofilm formation abilities. The majority of the isolates from CVC tips (67%) appeared to be weak biofilm formers.

Table 3.2: The biofilm formation ability of burns unit/ICU isolates classified according to specimen type.

Specimen type	Strong: n, (%)	Moderate: n, (%)	Weak: n, (%)
Wound/pus swabs/tissue (n=26)	16, (61.5%)	2, (7.7%)	8, (30.8%)
CVC tips (n=6)	2, (33%)	0	4, (67%)
Blood cultures (n=6)	3, (50%)	0	3, (50%)
Sputum (n=2)	0	0	2, (100%)

There were more isolates that form strong biofilms amongst the non-burn isolates. The majority of the isolates from wounds/pus swabs/tissue, CVC tips and sputum were strong biofilm formers (67%) (Table 3.3). Very few isolates appear to be weak biofilm formers; accounting for 33% of blood culture, 18% sputum isolates and 5% of wound isolates, but only 12.8% of isolates overall.

Table 3.3: The biofilm formation ability of non-burns unit isolates classified according to specimen type.

Specimen type	Strong: n, (%)	Moderate: n, (%)	Weak: n, (%)
Wound/pus swabs/tissue (n=21)	17, (81%)	3, (14%)	1, (5%)
CVC tips (n=1)	1, (100%)	0	0
Blood cultures (n=6)	2, (33%)	2, (33%)	2, (33%)
Sputum (n=11)	8, (73%)	1, (9%)	2, (18%)

We compared the burn unit/ICU specimen types to those of the non-burn specimen types. It appeared that the majority of the wound isolates from both the burns unit (61.5%) and non-burns units (81%) were strong biofilm formers. More isolates from the burns unit/ICU had a

lower biofilm forming capability as shown by high proportions of isolates from CVC tips (67%), blood cultures (50%), and sputum (100%) which formed weak biofilms.

When analysing the biofilm formation ability of all isolates, independent of the ward of origin, it is clear that the wound/pus swabs and tissue specimens were the most common specimens and these isolates were predominantly strong biofilm formers (Table 3.4). The majority of sputum isolates were also strong biofilm formers, while similar proportions of isolates from CVC tips and blood cultures were strong and weak biofilm formers.

Table 3.4: The biofilm formation ability of burns unit/ICU and non-burns unit isolates classified according to specimen type.

Specimen type	Strong: n, (%)	Moderate: n, (%)	Weak: n, (%)
Wound/pus swabs/tissue (n=47)	33, (70%)	5, (11%)	9, (19%)
CVC tips (n=7)	3, (43%)	0	4, (57%)
Blood cultures (n=12)	5, (42%)	2, (16%)	5, (42%)
Sputum (n=13)	9, (67%)	1, (8%)	3, (25%)

3.3.4 Biofilm formation based on strain type

Chapter 2 described the population structure of the isolates based on MLVA typing. The biofilm forming ability of the most abundant MLVA types (Types 1-10) were compared (Table 3.5, Figures 3.4 and 3.5). Isolates belonging to unique MLVA types were not included. The most predominant MLVA type was type 1 which included 25 isolates of which 24 were isolates from patients within the burns unit/ICU. Different isolates from MLVA type 1 showed different levels of biofilm formation (Figure 3.4). As seen in Table 3.5, 56% (14 / 25) of the type 1 isolates showed weak to no biofilm forming ability while 40% (10 / 25) showed strong biofilm forming ability.

Table 3.5: Biofilm forming abilities of different MLVA types

MLVA type	Clinical ward	Strong: n, (%)	Moderate: n, (%)	Weak: n, (%)
1 (n=25)	Burns/non-burn	10, (40%)	1, (4%)	14, (56%)
2 (n=4)	Burns	2, (50%)	1, (25%)	1, (25%)
3 (n=2)	Burns	0	0	2, (100%)
4 (n=2)	Non-burn	2, (100%)	0	0
5 (n=2)	Burns/Non-burn	2, (100%)	0	0
6 (n=3)	Burns	3, (100%)	0	0
7 (n=2)	Non-burn	2, (100%)	0	0
8 (n=3)	Non-burn	1, (33%)	1, (33%)	1, (33%)
9 (n=2)	Non-burn	2, (100%)	0	0
10 (n=2)	Non-burn	2, (100%)	0	0

This lack of consistency in biofilm forming ability was also evident in isolates from MLVA types 2 and 8 (Figure 3.5). Biofilm formation varied from weak biofilm formation to strong biofilm formation. Isolates from MLVA types 3-10 (excluding type 8) showed more similar biofilm formation within each MLVA type, however the numbers of isolates in each MLVA type were small. The two MLVA type 4 isolates appear to have the most similar biofilm formation ability with a difference in absorbance of only 0.02. Within the other MLVA types, the isolates had differences in absorbance values ranging from 0.10 to 0.20. All MLVA types contained strong biofilm formers except for MLVA type 3, which were all weak biofilm formers. These results suggest that the genetic background of the isolates, as determined by MLVA, does not predict the ability of the isolate to form a biofilm.

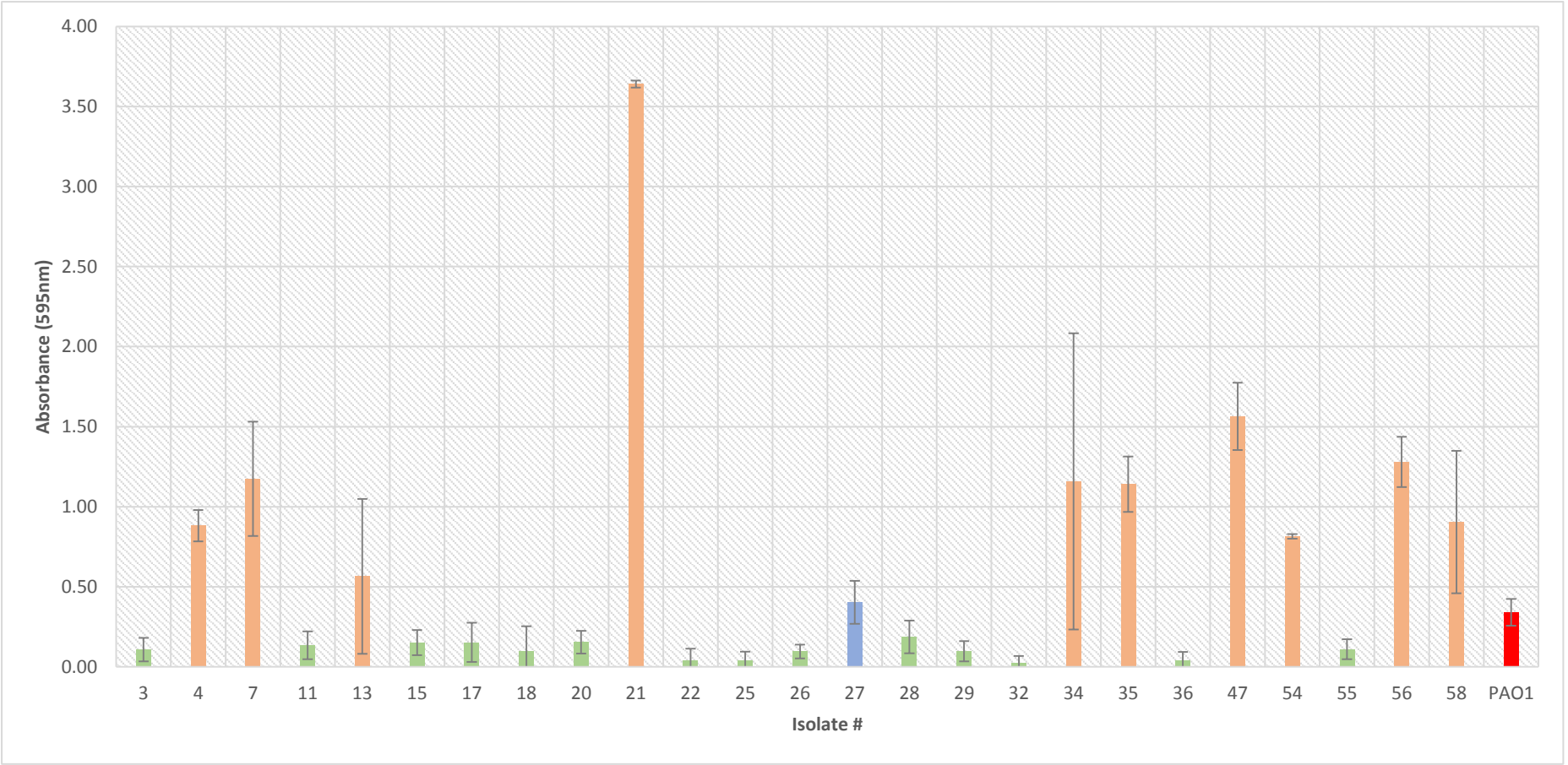

Figure 3.4: Biofilm formation of MLVA type 1 isolates. Biofilm formation was measured at an incubation time of 12 hours. All of the isolates were burn patient isolates except for TBP 36. Absorbance values were measured at 595nm. Orange: Strong biofilm former (>0.5), Blue: Moderate biofilm former (0.25-0.5), Green: Weak biofilm former (<0.25). PAO1 – Control strain.

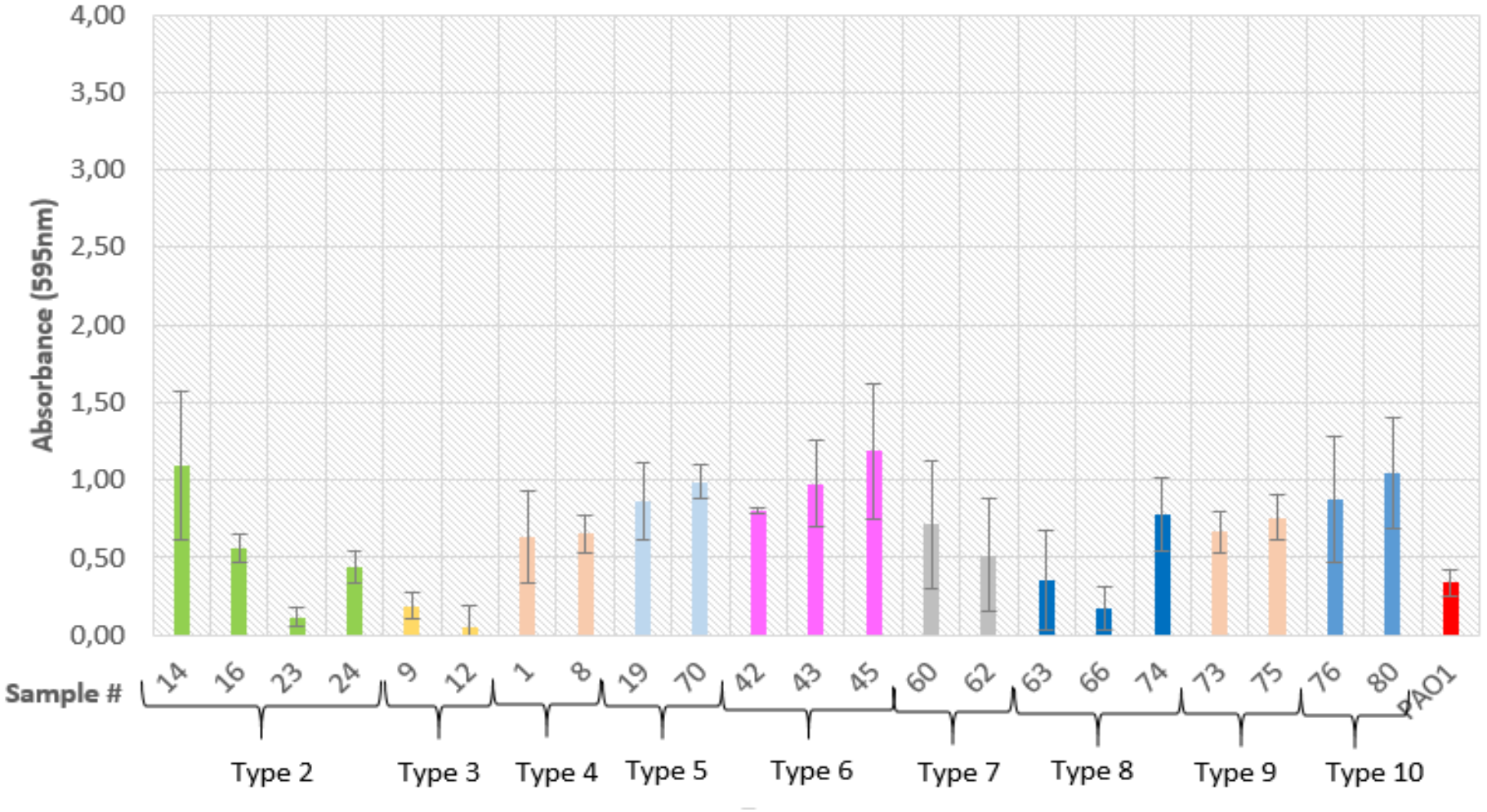

Figure 3.5: Biofilm formation of MLVA type 2-10 isolates. Biofilm formation was measured at an incubation time of 12 hours. PAO1 – Control strain. Absorbance values were measured at 595nm.

3.4 Discussion

This component of the study describes the biofilm forming ability of *P. aeruginosa* isolates from the burns ward/ICU and other wards at Tygerberg hospital, and compares the biofilm forming ability of isolates from different specimen types and MLVA types.

A time point assay with 2h, 5h, 8h, 10h, and 12h incubation times was performed on a subset of isolates to determine the optimal incubation time to assess biofilm formation and to determine the reproducibility of the assay. A 12 hour incubation period was selected since this time point differentiated strong from weak biofilm forming isolates. A decrease in biofilm formation was observed in some isolates after 8 hours, which could be an indication of the biofilm maturing resulting in the dispersion of planktonic cells, aided by proteins breaking down the extra polysaccharide matrix.[84] This enables the organism to form a new biofilm at other sites once the environment is suitable.

Biofilm assays were performed on all isolates at an incubation time of 12 hours. Forty percent of burns isolates were weak biofilm formers compared to only 13% of non-burns isolates; while the majority of non-burns isolates (72%) were strong biofilm formers compared to 55% of burns isolates. *P. aeruginosa* and other biofilm forming organisms such as *Staphylococcus epidermidis, Staphylococcus aureus* and *Escherichia coli* are commonly isolated from burn wound related infections, however biofilm formation has not yet been described in burn wounds[111]

Specimen type appeared to play a role in the biofilm forming abilities of the isolates. The majority of the isolates were from wounds, pus swabs and tissue and had strong biofilm forming abilities. Most sputum isolates were also strong biofilm formers, in contrast to blood culture and CVC tip isolates which were evenly distributed between strong and weak biofilm formation. Surprisingly, most isolates from CVC tips (particularly those from the burns unit/ICU) had weak biofilm forming ability. One would expect an organism from a prosthetic device to be a strong biofilm former since biofilms are known to grow on prosthetic devices such as orthopaedic implants, cardiac pacemakers and CVC tips.[111][114] The significance of these findings warrants further investigation, as the numbers of some specimen types were small.

The biofilm formation ability of different *P. aeruginosa* strains, as determined by MLVA analysis, was compared. Considerable variation in biofilm formation ability was observed between isolates within a MLVA type, however with the exception of a few outliers, there was no obvious difference between the different MLVA types. More variation was observed within MLVA type 1 which is likely due to the large number of isolates belonging to this type. The least variation in biofilm formation was observed within MLVA type 4, which are all non-

burns isolates. It is known that isolates within the same clone can be phenotypically diverse when forming biofilms, which could explain this phenomenon.[115][116] A study from Comeau *et al.,*(2001) has shown that organisms within a biofilm can undergo phase variation and exhibit different phenotypic traits and can switch between these phenotypes, which can affect biofilm formation.[117] Different regulator proteins are active at different stages of the biofilm cycle and have been speculated to have the biggest impact on biofilm variation. The GGDEF and EAL protein domains in these regulators regulate the signal molecule c-di-GMP affect bacterial adhesion and aggregation.[115] Phenotypic variants which have the ability to form stronger biofilms when there is a sudden environmental change have previously been isolated. It is speculated that these organisms possess numerous mechanisms, including phase variation, adaptive mutation and gene transfer, which result in reversible phenotypic changes.[115][118] During the irreversible attachment stage it appears that physical properties, such as motility, can change. The Las and Rhl quorum sensing systems start playing a role during the attachment stage and maturing stage respectively, resulting in upregulation of a numerous genes which can all have an effect on the formation of a biofilm.[119] It is also possible that MLVA typing does not provide sufficient discrimination amongst these isolates and that further proteomic studies or whole genome sequencing may help to identify additional phenotypic and genotypic variation between isolates.

A limitation of the study was that large standard deviations were observed between the biological triplicates of the biofilm assays. It is not clear why the same isolate would form biofilms of different strengths. It could be due to the organisms behaving differently due to small changes in the environment and/or cells undergoing physiological, metabolic and phenotypic changes. Variation due to technical factors can also be a factor. It has been shown that the reproducibility of biofilm formation readings in *P. aeruginosa* strains PAO1 and ATCC 9027 using crystal violet staining was poorer than for other organisms such as *Staphylococcus aureus, Propionibacterium acnes* and *Candida albicans* and varied much more between experiments.[112] This can be explained by *P. aeruginosa* having more water in the extra cellular matrix of the biofilms which makes it difficult for the biofilms to attach to the plate properly.[112] This may also explain the change in biofilm formation that occurs after 10-12h incubation when some of the isolates biofilms seem to weaken resulting in lower absorbance being measured. It is speculated that this is due to high water content of the polysaccharide matrix which *P. aeruginosa* is known for.[112]

A study focussing on the formation of biofilms and the quantification thereof has shown similar results of the same or similar strains showing totally different biofilm formation ability, not only in *P. aeruginosa* but also in other organisms such as *C. albicans.*[116] A study from Sauer *et al.,*(2002) found that the protein activity of cells within a biofilm at different stages of

development may differ significantly resulting in biofilm changes.[119] *P. aeruginosa* in particular undergoes five different physiologies throughout the different stages of biofilm development, which means that cells within a different stage of biofilm development will be physiologically different from other cells. The different stages include reversible attachment and irreversible attachment where more than 11 proteins are shown to be active; and maturation 1, maturation 2 and finally dispersion of the cells back to planktonic cells.[119] Another study revealed that *Pseudomonas putida* shows a change in gene expression and the products thereof within the first 6 hours of biofilm formation.[120] These factors can vary between strains and have an effect on the outcome of biofilm formation.

This study used a crude biofilm assay using crystal violet staining of the attached cells. Crystal violet staining and the concentration used has an impact on the biofilm formation measurements. A previous study using crystal violet showed that variation occurred within experiments as well as between experiments (technical and biological replicates).[112] Our biofilm formation assays showed lower absorbance measurements for PAO1 than were described by another study which used 0.5% crystal violet compared to our 0.1% crystal violet staining. Our crystal violet concentration was selected based on a method from O'toole (2011).[113] On the other hand, another study compared 0.1% crystal violet staining to 1.0% and found that higher variance was found with higher concentrations of crystal violet although there was no statistical difference in the biofilm measurement.[121] Crystal violet also only stains bacterial cells present in the well and not the extracellular polymer matrix and therefore an alternative should be used when this needs to be visualized.[122] Staining materials such as alcian blue, safranin or trypan blue can be used as alternative for staining the slimy layers. Another limitation of *in vitro* staining is that when staining biofilm formation of weak biofilm formers it tends to produce more false negatives compared to staining strong biofilm formers.[123] Crystal violet staining may not be the most effective method for the measurement of biofilm formation in *P. aeruginosa* since it can cause variability in the results. Alternative methods for biofilm quantification using 96 well micro titre plates can be implemented for more accurate and reproducible results. These include Syto9 assay, FDA assay (fluorescein diacetate), resazurin assay, XTT assay and dimethyl methylene blue assay. The crystal violet biofilm assay is a cheap and easy method for our setting compared to methods such as XTT and Syto9 assays which are laborious and also relatively expensive. Other more complex and more expensive methods which are not always available in the clinical environment include scanning electron microscopy, transmission electron microscopy, confocal laser microscopy and detecting genes that are present during biofilm formation.

Another observation that was made in our study was that the majority of the biofilm formed by some strong biofilm formers occurred at the top sides of the well, which was where most of the staining could be visualized before the stain was redissolved. This phenomenon was also observed in a study from Bendouah *et al.,*(2006) who performed biofilm forming assays of *P. aeruginosa* isolates isolated from patients with chronic sinusitis. This is expected since *P. aeruginosa* is an aerobic organism and also forms air-liquid interface biofilms.[123]

Another factor that plays a significant role in biofilm formation is the growth medium being used. TSB, which was used in this study, is recommended for these types of assays since it contains enough glucose for growth.[122] An alternative growth medium that can be used is BHI medium (Brain heart infusion) but may affect biofilm formation differently. There are other important factors which can also influence biofilm formation and contribute to variable biofilm formation results. These include the amount of washing steps being performed on the plate. According to Stepanovic *et al.,*(2007)[122] three to four washing steps are sufficient; and three were used in our study. Using the correct micro titre plate for biofilm growth is also important. It is necessary to use a plate that has been treated for tissue culture to make it possible for the biofilm to attach to the surface. When reading the absorbance of biofilm formation the stained plates first need to be resolubilized in acetic acid or alternatives such as ethanol or methanol which will yield the same results. In this study 30% acetic acid was used. Resolubilizing the stained wells also aids in the equal distribution of the stained cells which is necessary since the micro titre plate reader only reads the absorbance in the middle of the well. These were all aspects that were taken into consideration in this study.

3.5 Conclusion

Very little similarity was found between the biofilm formation of isolates from the same MLVA type, between the two different environments, namely the burns unit/ICU and non-burn wards, or between the different specimen types. Variation in the degree of biofilm forming ability was observed within the same MLVA types especially for the most abundant MLVA type 1.

Based on published evidence it is clear that biofilm formation of organisms is a developing problem occurring in the clinical setting. Knowing the biofilm characteristics of *P. aeruginosa* can enhance the effectiveness of treatment of chronic persisting infections and may lift the burden of mortality, especially in burn wards where transmission occurs on a regular basis. It is suspected that organisms, especially *P. aeruginosa,* that are isolated from burn units are often biofilm formers. Although biofilm formation has not yet been described in burn patient wounds, it is known that biofilm related wound sepsis does occur, and biofilm formation has been described in mouse model burn wounds.[111] Biofilm formation is more frequently seen in chronic wounds than acute wounds, which is expected since chronic wound infections persist for longer.

P. aeruginosa can be used as a model organism for describing biofilm formation *in vitro* as well as *in vivo*. Further studies can determine the susceptibility of biofilms to antibiotics and the formation thereof in the presence of antibiotics, as well as determining the factors that are responsible for biofilm variation of isolates from the same strain. Studies focussing on protein activity at different stages of the biofilm cycle can also be of value.

Chapter 4

Chapter 4 – Effects of antibiotics on biofilm formation

4.1 Introduction

Antibiotics work in different ways to inhibit growth or kill bacteria, and five different mechanisms of action have been described.[124] The most common mechanism, linked to penicillins, carbapenems and cephalosporins, is inhibiting cell wall synthesis by either breaking down peptidoglycan bonds or by inhibiting the formation of peptidoglycan. Secondly, antimicrobials may inhibit protein synthesis necessary for basic cell function by either affecting the function of ribosomes or tRNA, thus inhibiting translation. Antibiotics with this mechanism include aminoglycosides, tetracyclines and macrolides. The third mechanism, exhibited by lipopeptides such as daptomycin, affects the outer membrane and disrupts the cytoplasmic membranes. The fourth mechanism inhibits the formation of nucleic acids which are crucial for cell division and growth, by acting on enzymes such as DNA gyrase and topoisomerase, and includes antibiotics such as fluoroquinolones. Finally some antimicrobials, such as trimethoprim-sulfamethoxazole, have antimetabolite activity which inhibits the production of folic acid which is necessary for cell growth.[124]

It has been established that in *P. aeruginosa* the biofilm will cause the movement of antibiotics, especially aminoglycosides, into the biofilm to be retarded.[47] This is however restricted by the limited receptors for inhibiting antimicrobial diffusion in the EPS. Another factor contributing to antibiotic resistance within biofilms is multidrug efflux pumps which are speculated to be upregulated in a biofilm.[125]

It is important to establish and understand the effect of antibiotics on biofilm formation for future treatment and prevention of biofilm associated infections. In this study we aim to describe the effect of different concentrations of antibiotics on the formation of *P. aeruginosa* biofilms. Four different antibiotics were tested on a subset of isolates and the impact on biofilm formation measured after various time periods to investigate their effect at different stages of biofilm formation.

Four different antibiotics were chosen to represent different classes and mechanisms of action. The selected antibiotics are commonly used to treat *P. aeruginosa* infections. One antibiotic each from the cephalosporin, quinolone, carbapenem and aminoglycoside groups were selected. Cefepime is a 4th generation cephalosporin which is active against both Gram positive and Gram negative bacteria, and acts by binding to the penicillin binding proteins which will disrupt cell wall synthesis. Ciprofloxacin belongs to the fluoroquinoloneclass of

antibiotics and inhibits DNA replication by binding to DNA gyrase, which is responsible for the “supercoiling” of DNA. Imipenem is a carbapenem which also targets the penicillin binding proteins to disrupt cell wall synthesis. The fourth antibiotic, gentamicin belongs to the aminoglycoside group. Gentamicin binds to 16S ribosomal RNA within the 30S ribosomal subunit, close to tRNA, which affects the process of protein synthesis by inhibiting translocation of the ribosome.[124][126] In particular, we wanted to further investigate the effect of strain lineage on the effect of antibiotics on biofilm formation, and we also aimed to determine whether strong and weak biofilm forming organisms (as in Chapter 3) were affected differently by antibiotics. A better understanding of the effect of specific antibiotics on *P. aeruginosa* biofilm formation may help to advise future treatment and reduce the burden of morbidity and mortality it causes.

4.2 Materials and methods

4.2.1 Isolate selection

A subset of isolates was selected to test the effect of antibiotics on biofilm formation. Five isolates were selected from three MLVA types according to their level/strength of biofilm formation. Two isolates (with strong or weak biofilm forming ability; section 3.3.4 were selected from each of the two most common MLVA types (types 1 and 2; section 2.3.2). The selected isolates included TBP 47 & 20 (MLVA type 1), TBP 16 & 23 (MLVA type 2) and TBP 73 (MLVA type 9) was included as a sensitive strain representative. A control strain (*P. aeruginosa* PAO1) was included in the assay as this strain is a known biofilm former.[112] The PAO1 and TBP 73 strains were included to represent sensitive strains as they are susceptible to all antibiotics used in the assay.

4.2.2 MIC determination (Minimum inhibitory concentration)

Isolates were tested for susceptibility to cefepime, imipenem, ciprofloxacin and gentamicin by determining the minimum inhibitory concentration (MIC) for each antibiotic using gradient diffusion (Etest strips, Biomerieux). Etest strips release a concentration gradient of antibiotics onto the agar and allow the MIC to be read at the point where the elliptical growth intersects the strip (Figure 4.1).

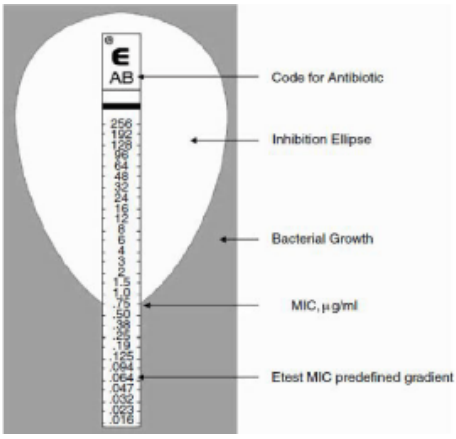

Figure 4.1: Etest strip representation of elliptical growth intersecting with the strip at 0.75µg/ml. Source: Nasir *et al.,* (2015)[127].

Each isolate was grown on TBA (tryptose blood agar, NHLS Greenpoint Media Laboratory) overnight at 37°C, where after single colonies were suspended in 2 ml of saline. The suspension was made up to a cell density of 0.5 McFarland as measured with a nephelometer (DensiCHEK plus, Biomerieux). The suspension was carefully spread on MH agar plates (Mueller Hinton, NHLS Greenpoint Media Laboratory) with a sterile cotton swab, covering the entire surface of the plate. Two Etest strips were used per plate, placed parallel to each other in reverse orientation on each half of the plate. The MH plates were incubated for 20h at 37˚C. The inhibition of growth of the organism was visualized on the plate and the MIC's were interpreted and noted in µg/ml. The raw (uncorrected) MIC data were used to determine the antibiotic concentrations used in the biofilm assays.

4.2.3 Antibiotic dilutions

The following antibiotics and solutions were used: cefepime dihydrochloride monohydrate (40mg/ml, European pharmacopoeia), ciprofloxacin hydrochloride (30mg/ml, European pharmacopoeia), imipenem monohydrate (10mg/ml, Sigma) and gentamicin sulphate (50mg/ml, Sigma). Stock solutions and dilutions were made according to each antibiotic's solubility and stored at -20˚C. Stock solutions for each antibiotic were made up as follows.

- Cefepime: 32mg/ml dissolved in H_2O
- Ciprofloxacin: 16mg/ml dissolved in HCl
- Imipenem: 5mg/ml dissolved in H_2O
- Gentamicin: 25.6mg/ml dissolved in H_2O

4.2.4 Biofilm assays.

Biofilm assays were performed as described in Chapter 3, section 3.2.2, with the addition of antibiotics. Each isolate was tested with concentrations of each of the 4 antibiotics that corresponded to 10%, 50% and 100% of the MIC that had been determined by Etest for each isolate. Biofilm formation was measured at 4, 10 and 24 hours. Replicate plates were prepared for each time point.

Antibiotic stock solutions were diluted in TSB (tryptic soy broth, Sigma) in 15ml falcon tubes, and then added to equal volumes of overnight culture of the respective organisms to achieve final concentrations that corresponded to 10%, 50% and 100% of the MIC that had been determined for each antibiotic and each isolate (Table A3, Appendix).

The total volume inoculated into each well of the 96 well micro titre plate was 200µl; containing 100µl of overnight culture diluted 1:100 and 100µl of antibiotic in TSB as

described above. Each 96 well plate allowed for assessment of biofilm formation of two isolates in the presence of all 4 antibiotics at the 3 concentrations. Figure 4.2 provides the layout of the 96 well plate, including technical triplicates for each concentration of each antibiotic, as well as control wells containing only culture in the absence of antibiotic, culture medium only (Negative control) and antibiotic in the absence of culture, to control for contamination of the culture media or antibiotic. Each assay was performed in biological triplicate. The average biofilm formation and standard deviation for each antibiotic concentration was calculated for each organism from the technical and biological replicates.

Figure 4.2: 96 well micro titre plate layout for two different organisms. Culture – organism with no antibiotic, AB – Antibiotic only (100% MIC), NEG – Sterile TSB with no antibiotic, PM – Cefepime, CI – Ciprofloxacin, IP – Imipenem, GM – Gentamicin. Antibiotic at 10%, 50% and 100% of the MIC for the antibiotic / organism combination.

	Organism 1				**Organism 2**			
	PM	**CI**	**IP**	**GM**	**PM**	**CI**	**IP**	**GM**
	A	B	C	D	E	F	G	H
1	Culture	Culture	Culture	Culture	Culture	Culture	Culture	Culture
2	10%	10%	10%	10%	10%	10%	10%	10%
3	10%	10%	10%	10%	10%	10%	10%	10%
4	10%	10%	10%	10%	10%	10%	10%	10%
5	50%	50%	50%	50%	50%	50%	50%	50%
6	50%	50%	50%	50%	50%	50%	50%	50%
7	50%	50%	50%	50%	50%	50%	50%	50%
8	100%	100%	100%	100%	100%	100%	100%	100%
9	100%	100%	100%	100%	100%	100%	100%	100%
10	100%	100%	100%	100%	100%	100%	100%	100%
11	AB	AB	AB	AB	AB	AB	AB	AB
12	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

4.3 Results

4.3.1 MIC determination

The MICs of each of the antibiotics, cefepime, ciprofloxacin, imipenem and gentamicin, were determined for each of the 6 strains. For the purposes of categorisation of isolates as susceptible, intermediate or resistant, the MIC was adjusted to the next doubling dilution if necessary, according to the performance standards for antimicrobial susceptibility testing (2015) (Table 4.1). The MICs of isolates from the same MLVA type differed slightly, with the

exception of cefepime and gentamicin for MLVA type 1 and imipenem for MLVA type 2 which had the same MIC. Cefepime, imipenem and gentamicin showed no more than a 2 fold dilution difference in MIC between isolates of the same MLVA type. A single dilution difference is generally acceptable even when testing the same organism. On the other hand, ciprofloxacin showed much larger variation within the MLVA types with up to a 4 fold difference in MIC (adjusted MICs of 2 µg/ml vs 32 µg/ml for TBP 16 and TBP 23; and MICs of 16 µg/ml and 4 µg/ml for TBP 47 and 20 respectively).

Table 4.1: Raw MIC and adjusted MIC results of six isolates including *P. aeruginosa* PAO1, determined for four antibiotics. S – Sensitive, I – Intermediate. R – Resistant. MIC values are given in µg/ml.

		Cefepime		Ciprofloxacin		Imipenem		Gentamicin	
MLVA Type	Isolate	Raw MIC	Adjusted MIC	Raw MIC	Adjusted MIC	Raw MIC	Adjusted MIC	Raw MIC	Adjusted MIC
Type 1	TBP 47	3	4 (S)	12	16 (R)	8	8 (R)	256	256 (R)
	TBP 20	4	4 (S)	4	4 (R)	4	4 (I)	256	256 (R)
Type 2	TBP 16	4	4 (S)	1,5	2 (I)	6	8 (R)	256	256 (R)
	TBP 23	6	8 (S)	32	32 (R)	8	8 (R)	96	128 (R)
Type 9	TBP 73	1	1 (S)	0.06	1 (S)	0.75	1 (S)	2	2 (S)
Control	PAO1	0.5	1 (S)	0.1	1 (S)	0.5	1 (S)	1.5	2 (S)

Focussing on the categorical agreement (S, I, R), the results for isolates from MLVA type 1 were identical except for the imipenem results for which TBP 20 and 47 were respectively intermediate and resistant, due to a single dilution difference in MIC. TBP 16 and 23, the MLVA type 2 isolates, were respectively intermediate and resistant to ciprofloxacin. All isolates selected were sensitive to cefepime, although the MICs for the isolates from MLVA types 1 and 2 were in some cases close to the breakpoint, and notably higher than the MICs of TBP 73 (MLVA type 9) and PAO1. The same trend was observed for the susceptibility results to ciprofloxacin, imipenem and gentamicin, where the isolates from MLVA groups 1 and 2 were all intermediate or resistant to these agents, while TBP 73 and PAO1 were susceptible.

4.3.2 The effect of antibiotics on biofilm formation

Biofilm formation of each of the 6 isolates was measured at 3 time points, 4, 10 and 24 hours, in the presence of different concentrations of four different antibiotics and compared to the biofilm formation in the absence of antibiotics. Different time points were selected to

enable us to evaluate differences in how isolates react to antibiotics at different incubation periods.

In the absence of antibiotic, two different patterns of biofilm formation were observed, which were independent of MLVA type (Figures 4.3 - 4.6). Isolates were either classified as early onset or late onset biofilm formers. TBP 47 (MLVA type 1) and 23 (MLVA type 2) were classified as early onset biofilm formers; with a strong biofilm present after 10 hours' incubation (absorbance >1.0), followed by a decline in biofilm formation at 24 hours. The opposite was seen for TBP 20 (MLVA type 1), 16 (MLVA type 2) and 73 (MLVA type 9) which started with a weak biofilm at 10 hours, but showed a significant increase in biofilm formation by 24 hours; and were classified as late onset biofilm formers. The early and late-onset biofilm formers corresponded to their classification as strong and weak biofilm forming strains, respectively (section 3.2.1). PAO1 did not display either of these patterns, with the amount of biofilm remaining relatively consistent across all 3 time points.

Cefepime completely inhibited biofilm formation in late onset biofilm formers at all concentrations, although biofilm formation in TBP 73 was less affected than in TBP 20 and TBP 16 when exposed to cefepime at 10% of MIC. Interestingly TBP 73 also had a lower cefepime MIC than either of the other two late onset biofilm formers. PAO1, also susceptible to cefepime, showed far less effect of cefepime at any concentration, with the exception of a slight increase in biofilm at 10 hours when exposed to cefepime at 50% MIC. The isolates with early onset biofilm formation showed that cefepime concentrations of 50% of the MIC resulted in a late rise in biofilm formation (resulting in a pattern similar to a late biofilm forming isolate) while high concentrations of cefepime (100% of MIC) inhibited biofilm formation completely.

Ciprofloxacin inhibited biofilm formation in all three late onset biofilm formers, up to 24 hours, at a concentration of 50% and 100% MIC. At 10% MIC, the antibiotic appeared to have less effect, and biofilm formation mirrored the control (more so for TBP 16 and TBP 73 than TBP 20). With regards to the early onset formers, at low concentrations (10% MIC), ciprofloxacin had little effect. However at higher concentrations (50% and 100% MIC), biofilm formation again resembled a "late onset biofilm" pattern, with relatively abundant biofilm at 24 hours in the presence of ciprofloxacin at 50% of the MIC. For PAO1, ciprofloxacin at all 3 concentrations reduced the amount of biofilm present at all 3 time points, compared to the no-antibiotic control.

Imipenem showed the least effect on biofilm formation, with the pattern of biofilm formation mainly mimicking the control growth curve at all three antibiotic concentrations. Again, PAO1

was an exception, with less biofilm present at all 3 time points, although more markedly so at both 4 and especially 10 hours. This closely mimicked the effect of ciprofloxacin on PAO1.

Gentamicin at 100% of the MIC inhibited biofilm formation of the two gentamicin-resistant late onset biofilm formers (TBP 16 and TBP 20) compared to the gentamicin-susceptible late biofilm former (TBP 73). In contrast, lower gentamicin concentrations (10% and 50% MIC) appeared to result in biofilm patterns very similar to the antibiotic free control for all 3 late-onset biofilm isolates. An increase in biofilm formation was seen at 24 hours for early onset biofilm formers exposed to gentamicin at 50% MIC. Conversely, at this time point the biofilm formation of both the antibiotic-free controls and isolates exposed to gentamicin at 10% MIC had reduced to almost baseline levels, with maximum biofilm seen at 10 hours. The only isolate where exposure to gentamicin at 50% of the MIC resulted in a reduction in biofilm at 24 hours was the control strain, PAO1.

All of the organisms were sensitive to cefepime while only the isolates that form an early biofilm (TBP 47, 23) showed biofilm formation at a 50 % MIC concentration. This occurrence can also be seen with the other antibiotics, where biofilm formation occurs at 50% MIC for both early biofilm forming strains.

Figure 4.3: Biofilm formation of isolates exposed to different concentrations of cefepime and incubated for 4h,10h and 24h. E – Early onset biofilm former, L – Late onset biofilm former. Control – No antibiotics. Y-axis: Absorbance (nm) X-axis: Incubation time (hours).

Figure 4.4: Biofilm formation of five isolates introduced to different concentrations of ciprofloxacin and incubated for different periods of time, 4h,10h and 24h. E – Early onset biofilm former, L – Late onset biofilm former. Control – No antibiotics. Y-axis: Absorbance (nm) X-axis: Incubation time (hours).

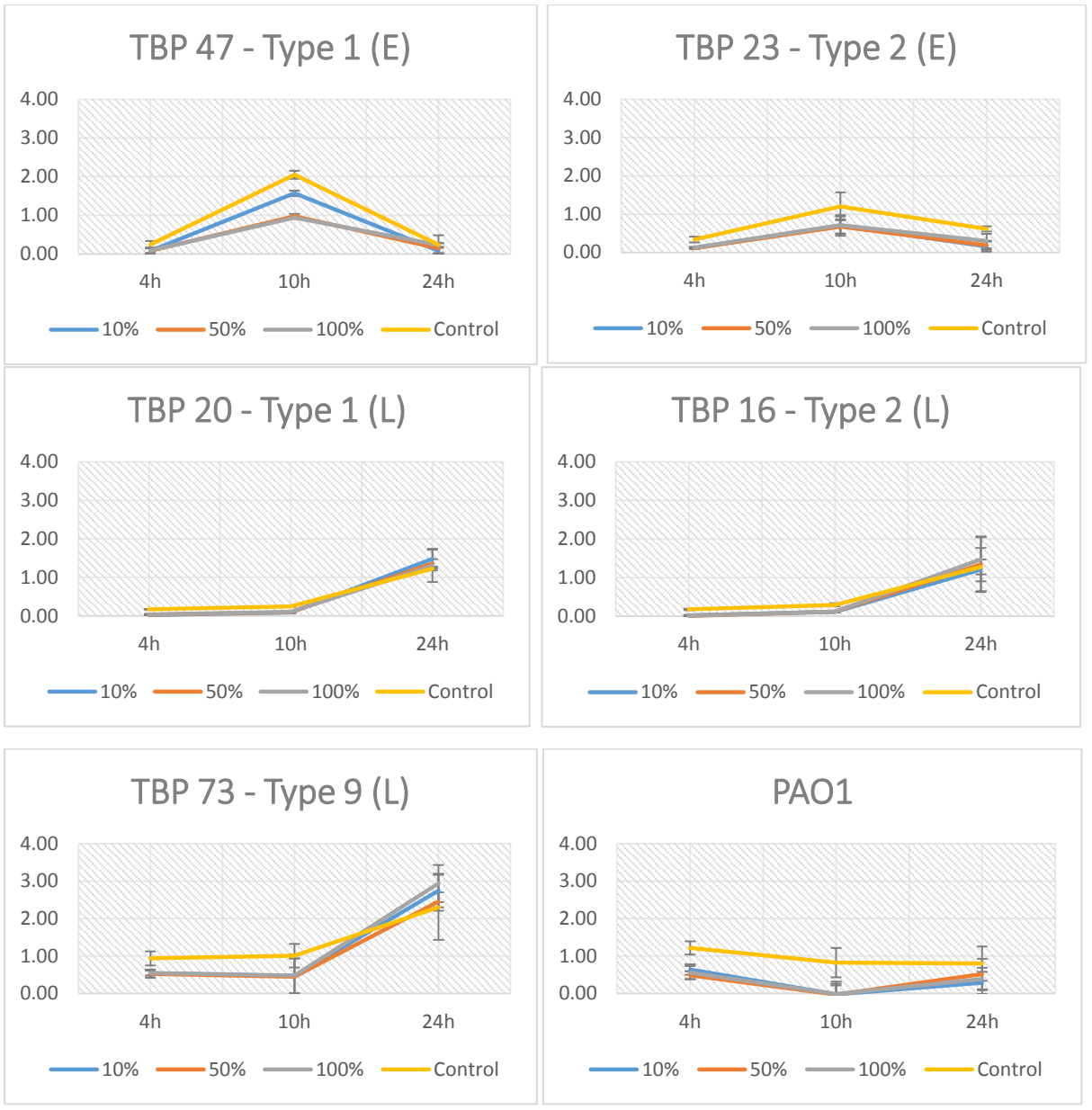

Figure 4.5: Biofilm formation of five isolates introduced to different concentrations of imipenem and incubated for different periods of time, 4h,10h and 24h. E – Early onset biofilm former, L – Late onset biofilm former. Control – No antibiotics. Y-axis: Absorbance (nm) X-axis: Incubation time (hours).

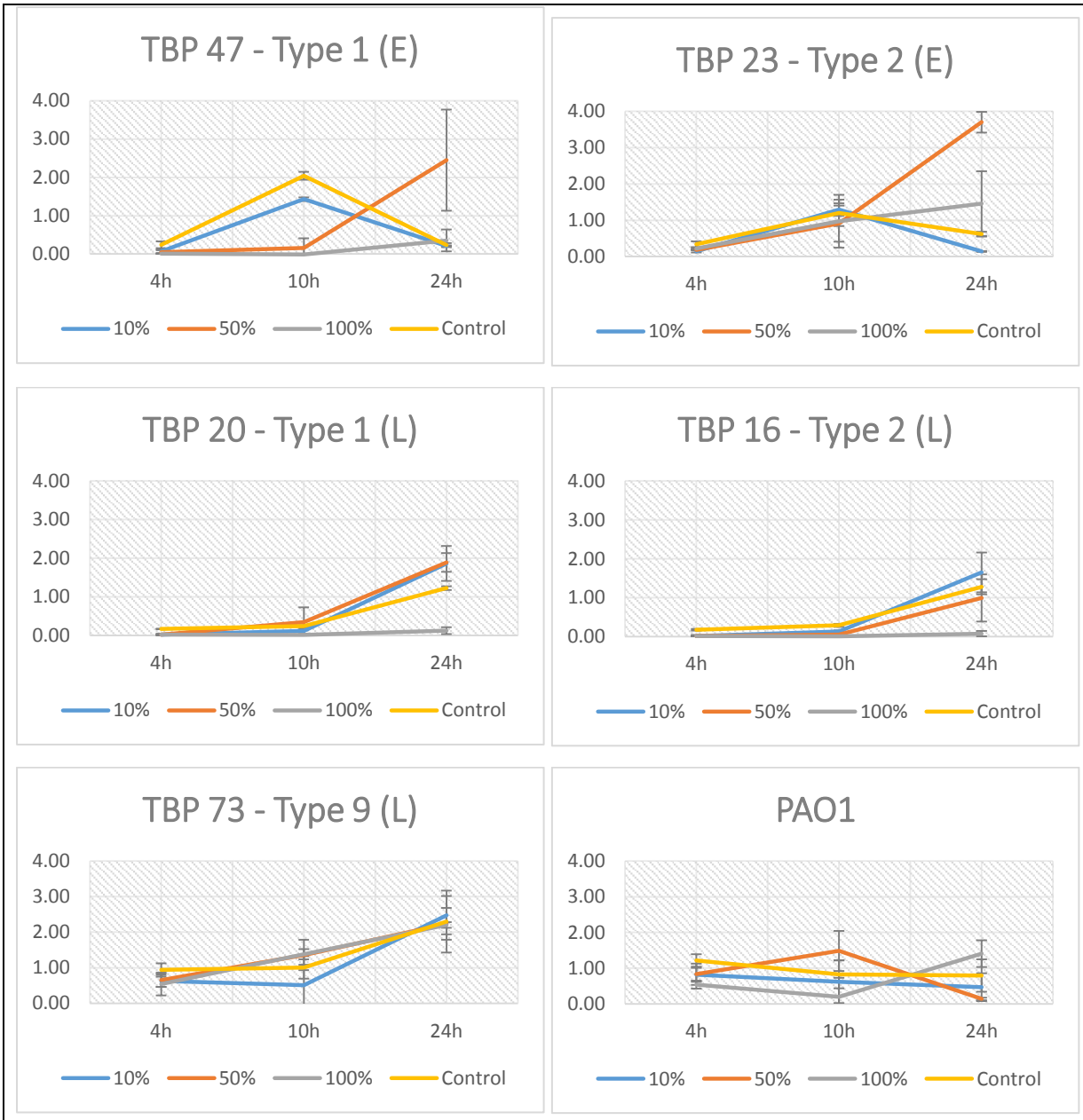

Figure 4.6: Biofilm formation of five isolates introduced to different concentrations of gentamicin and incubated for different periods of time, 4h,10h and 24h. E – Early onset biofilm former, L – Late onset biofilm former. Control – No antibiotics. Y-axis: Absorbance (nm) X-axis: Incubation time (hours).

4.4 Discussion

The aim of this study was to compare the effect of different antibiotics on different isolates with different biofilm formation abilities and belonging to different MLVA types. Two isolates each from MLVA type 1 and MLVA type 2 were chosen according to their biofilm forming abilities, as determined in chapter 3. These two MLVA types were the most common among the isolates studied. One isolate from type 9 was included as a strain sensitive to all antibiotics, as there were no such susceptible isolates among those in MLVA type 1 or 2.

Two biofilm formation patterns were observed amongst these isolates. Isolates previously chosen as weak biofilm formers were categorised in this chapter as late onset biofilm formers, while strong biofilm formers were categorised as early onset biofilm formers. In the first pattern, the biofilm formation initially seemed to be stationary followed by an increase in biofilm formation between 10 and 24 hours of incubation. The second pattern was characterised by early biofilm formation within the first 10 hours of incubation, followed by a decline in biofilm by 24 hours. These patterns were not unique to any MLVA type. Previously, in chapter 3, biofilm formation was only measured up to 12 hours, and it is possible that all the weak biofilm formers from the initial biofilm assays (chapter 3) are actually late onset biofilm formers; although this is small sample size from which to make such a generalisation.

The response to antibiotics was similar amongst the early and late onset biofilm formers, respectively, even though they had different MLVA types. This might suggest that it is not the strain type that influences the effect of antibiotics on biofilm formation but rather the initial amount of biofilm, and therefore the rate at which a strain can form a biofilm.

Imipenem had the least observable effect on biofilm formation with the growth curves in the presence of antibiotics mimicking the control growth curve. Cefepime and ciprofloxacin showed the greatest inhibition of biofilm formation at concentrations of 50% and 100% of the MIC. High concentrations of cefepime, ciprofloxacin and gentamicin appear to have very little effect on early onset biofilm formers while late onset formers are influenced more. A study from Brooun *et al.,*(2000)[128] focussed on the MexAB-OprM pump and showed cells did not show any difference in resistance to ciprofloxacin in the presence or absence of the MexAB-OprM pump. The effect of gentamicin seems to show the most consistent pattern across all isolates and concentrations. Overall all, ciprofloxacin, imipenem and gentamicin at concentrations of 10% of the MIC had no effect on biofilm formation and mirrored the control growth curve (no antibiotic) which was probably due to the concentration being too low and not having any effect on the formation of the biofilm. Cefepime on the other hand inhibited

biofilm formation in late onset biofilm formers at an antibiotic concentration of 10% of the MIC.

Some antibiotic sensitive strains were able to form biofilms when exposed to intermediate antibiotic concentrations such as TBP 47, 23 and 73 for cefepime, although concentrations equivalent to 100% of the MIC were able to inhibit biofilm formation, as was seen for resistant strains at 100% MIC. One might speculate that isolates that are resistant may be less affected by the antibiotic even at high concentrations since there is already a mechanism to counter the effect of the antibiotic activity such as MLVA types 1 and 2 for ciprofloxacin, imipenem and gentamicin, but this did not seem to be the case in this study. Comparing the isolates that are resistant to gentamicin with MICs of 256 µg/ml to those that are sensitive with MICs of 2 µg/ml, the antibiotic appeared to inhibit biofilm formation at 100% MIC in the resistant strains, but not in the sensitive isolate (TBP 73) with a low MIC, despite the fact that a concentration equivalent to the MIC should by definition inhibit growth of the particular strain. This may be attributed to the limited ability of the biofilm to retard penetration of the antibiotic; at a low concentration of antibiotic there are sufficient antimicrobial binding proteins to bind all of the antibiotic, while for high antibiotic concentrations these proteins are unable to bind enough antibiotic to influence its activity.

MLVA type 1 isolates which are resistant to ciprofloxacin and all isolates resistant to gentamicin still showed some biofilm formation (more than susceptible strains) at an antibiotic concentration of 50% but much less than expected from a resistant strain. Exposure to concentrations of 100% of the MIC did indeed have an impact on most isolates and stopped biofilm formation of the resistant strains completely, except for TBP 23 at gentamicin and TBP 47 at ciprofloxacin. One would expect a biofilm to be more resistant, since the biofilm will reduce the penetration of antibiotics to the cells which are already somewhat resistant, resulting in an even higher concentration needed to eradicate the organism completely; however this was not observed in this study. The effect of the antibiotic may have been different if the organism had been given a chance to form a biofilm before the antibiotic was added, as then the biofilm may have been able to restrict penetration of the antibiotic, or alter its activity in some way.[129]

The mechanism of action of the antibiotic also appears to play little to no role in its effect on biofilm formation. Cefepime and ciprofloxacin, which have different mechanisms of action, showed similar effects on biofilm formation patterns, producing almost identical patterns. On the other hand, when looking at the results of exposure to cefepime and imipenem, which have the same mode of action, one would have expected similar effects on biofilm patterns; this was however not the case. This leaves us to question whether the mechanism of action

really does play a significant role. Not much is known about the impact of the mechanisms of antimicrobial action on the formation of biofilms. Since both ciprofloxacin and gentamicin display concentration dependant pharmacokinetics, one might expect greater inhibition of biofilm formation at higher antibiotic concentrations. However, this only holds true for late onset biofilm formers exposed to these antibiotics. On the other hand, β-lactams such as cefepime and imipenem show time dependant killing, and the longer the organisms are exposed to the drug the more inhibition will occur. This phenomenon was also more noticeable in late onset biofilm formers exposed to cefepime.

Studies have shown that sub inhibitory concentrations of some antibiotics may promote biofilm formation and that organisms respond in a defensive manner[130]. A study done by Hoffman *et al.,*(2005) showed that sub inhibitory concentrations of aminoglycosides can induce biofilm formation in *E. coli* as well as *P. aeruginosa* and that the *arr* response regulator is responsible for this. This effect of sub inhibitory concentration was also observed in our study, for example we observed increased biofilm formation in early onset biofilm formers exposed to gentamicin and cefepime at an antibiotic concentration of 50% MIC. This may have clinical implications since patients receiving antibiotic treatment at sub inhibitory concentrations may be more likely to develop biofilm-related infections. Sub inhibitory concentrations of β-lactams such as imipenem have also been shown to enhance the production of EPS found in biofilms.[131] However, this did not seem to be true in our study since imipenem showed little to no effect on biofilm formation, and mimicked the control growth curve most of the time.

A study similar to ours, but focusing on *E. coli,* showed that increasing concentrations of antibiotics, mainly imipenem and ciprofloxacin, caused high levels of cell death within a biofilm after the first few hours of incubation, where after a sub population emerged that was less susceptible and had the potential to expand.[128] They obtained similar results to those seen in our study for isolates treated with cefepime, ciprofloxacin and gentamicin; where initial biofilm formation was weak and slow when treated with 50% MIC concentration of antibiotics, followed by a sudden increase in formation after 10 hours of incubation. Our results show that antibiotics at concentrations of 100% MIC completely inhibited biofilm formation the majority of the time, suggesting that the initial concentration was high enough to inhibit or even kill the organisms (as per the definition of MIC), while the 50% concentration only kills the susceptible organisms leaving a sub population of less sensitive organisms that has the potential to reform a biofilm at a lower antibiotic concentration, as described in Figure 4.8. This was also described by Spoering *at al.,*(2001)[132] where low concentrations of fluoroquinolones were shown to have significant biofilm eradication effect, but what remained was a sub population of resistant cells with the potential of reforming a

biofilm. Again, the same was seen for ciprofloxacin in our study, although more noticeably for early onset biofilm formers than late biofilm formers.

There are three ways that a biofilm can counter antibiotic activity and cause a persisting biofilm (Figure 4.7). Firstly, physically delaying of antibiotic penetration through the EPS, secondly, the persisting variant cells within a biofilm can be resistant to the antibiotics and thirdly, a sub population of slow growing cells can serve as a last resort for biofilm regrowth when all the sensitive and peripheral cells are eradicated, since these slow growing cells are not actively targeted by antibiotics.[95]

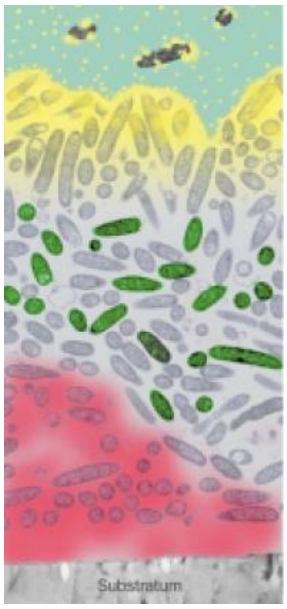

Figure 4.7: Three ways in which biofilms can develop resistance. Yellow – EPS delaying antibiotic penetration, Green – Persisting resistant cells, Red – Slow growing, metabolically slow/inactive organisms. Source: Stewart *at al.*, (2001).[95]

In our study we found that isolates from the same MLVA type did not show the same biofilm formation patterns and also did not have the same response to antibiotics. Studies have found that a population can contain phenotypic variants within a biofilm community in vivo and in vitro. These variants can be resistant to 10^6 fold higher levels of antibiotics and may cause a change in the response of a biofilm when treated with antibiotics.[133] Changes such as high surface hydrophobicity which will aid in cells attaching more easily to a surface result in increased biofilm formation. Phenotypic variants are common amongst Gram negative organisms and can be caused by environmental factors.[133] This indicates that there is a connection between phenotypic variation and the ability of a biofilm to become resistant to antibiotic treatment.[133] These phenotypic changes have also been shown to occur in *Staphylococcus aureus* when exposed to gentamicin.[12]

Two types of variants can be present within a biofilm; one is a phenotypic variant and the other a persister variant.[133] A phenotypic variant has the ability to grow in the presence of high concentrations of antimicrobials, but can be eradicated with prolonged treatment. Persister variants have the ability to withstand antibiotic eradication completely. A study has

shown that antibiotics are able to kill phenotypic variants effectively within a biofilm but when the dosage of antibiotics is increased there are still some cells that can survive and reform the biofilm, known as persister cells.[128][111] The same occurrence was found in our study at higher antibiotic concentrations. We saw that an increase in biofilm formation occurs at high concentrations of antibiotics particularly for gentamicin for both early (MLVA type 1 and 2) and late (MLVA type 9) onset biofilm formers which means there are still some cells that are able to grow after being exposed to an antibiotic. The process is explained in Figure 4.8. An initial biofilm will form and be eradicated by antibiotic treatment. A group of persister cells will remain unharmed and be able to form a mature biofilm resistant to antibiotics. These persister cells can emerge within 24 hours of biofilm formation triggered by malnutrition, a possible factor in our assay.[134][135] A study revealed that cells may have the same sensitivity to antibiotics when in a planktonic, stationary or biofilm state but high levels of resistance may remain due to the persister variants present within the community.[132] Cells deep within the biofilm (such as the persister cells) also have a limited supply of nutrients and oxygen which makes them metabolically slow or inactive, and antibiotics will target more metabolically active cells. It has been speculated that this phenomenon can decrease the susceptibility of a biofilm to antibiotics.[129] Slow growing *P. aeruginosa* cells have also been shown to be less affected by β-lactams, while growth rate does not show any significant impact on the effectiveness of fluoroquinolones.[136] Further studies will have to be done to establish whether incubation time may influence the results.

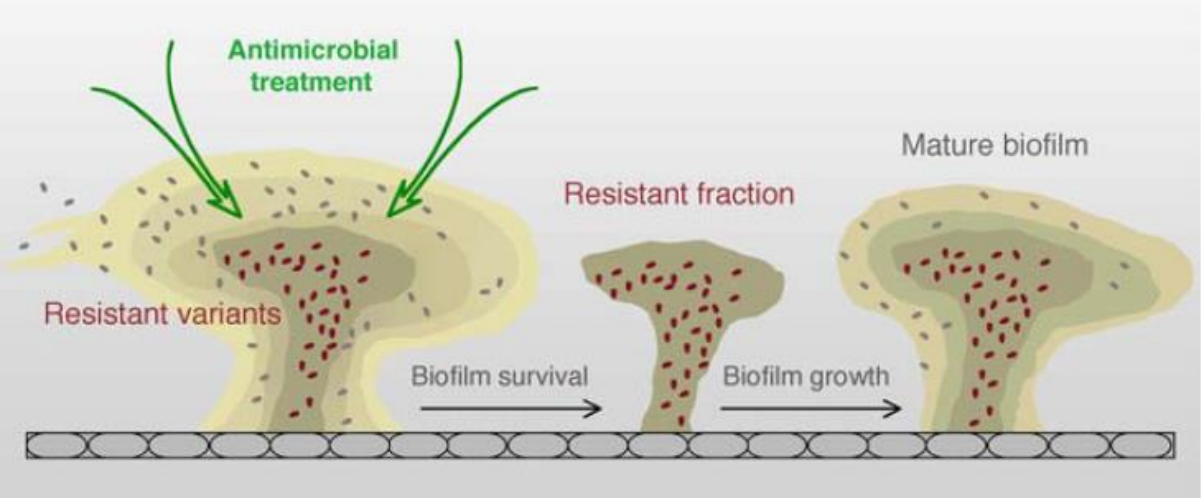

Figure 4.8: Mechanism of antimicrobial resistance in a bacterial biofilm. Antimicrobial treatment is effective against the peripheral areas of the biofilm while a sub population in the middle remains resistant against antibiotic treatment. Source: Drenkard (2003).[12]

An interesting observation revealed that cells within a biofilm are not mutants of the parental strain and that these cells, when isolated from a resistant biofilm and exposed to antibiotics

have the same MIC results as to the original inoculated strain.[128] This indicates that the physical protection of a biofilm does in fact play a significant role in cells being resistant. On the other hand, a study by Brooun *et al.,*(2000)[128] compared planktonic cells to cells isolated from a biofilm, and showed that the cells isolated from the biofilm are more resistant, implying that cells become intrinsically more resistant. Furthermore, a study from Liao and Sauer revealed that a transcriptional regulator *brlR* plays a major role in the in the antibiotic susceptibility of *P. aeruginosa* biofilms to multiple antibiotics and also regulates the expression of the MexAB-OprM efflux pump.[137]

Studies have shown that the penetration rate of antibiotics such as aminoglycosides (gentamicin) is reduced in *P. aeruginosa* biofilms, while fluoroquinolones such as ciprofloxacin can penetrate a biofilm without any delay.[138][139] Delayed penetration of an antibiotic can be due to embedded transport molecules binding the antibiotic molecules.[12] This is not consistent with our findings, with respect to gentamicin. Gentamicin seemed to inhibit the formation of the biofilm within the first 10 hours, regardless of the concentration, where after a sudden increase in biofilm formation occurred in isolates exposed to concentrations of 50% MIC while a concentration of 100% MIC inhibits biofilm formation. This was particularly marked for the early biofilm forming isolates. Based on an assumption of delayed penetration of gentamicin, one would have expected ongoing biofilm formation within the first few hours followed by a steady decline in biofilm formation. Our results are however consistent with the finding that ciprofloxacin can penetrate the biofilm without delay, since minimal biofilm formation occurred during the initial 12 hour incubation period, implying that ciprofloxacin takes immediate action. This suggests that resistance in biofilms is not only related to embedded transport molecules, but that other factors might also play a role. These changes can cause stress and result in antimicrobial resistance. Stress factors include nutrition depletion, damaged DNA, and basic environmental factors such as temperature, osmotic and oxidative changes.[12] It has been shown that some antimicrobials penetrate a biofilm easily and reach the cells but still fail to kill the cells within the biofilm, indicating that the cells within the biofilm are still more resistant to the antimicrobial than planktonic cells and again suggests that resistance is not only dependant on the limited transport within a biofilm.[140] Biofilms delaying the diffusion of antibiotics will not save the bacteria from being killed but rather postpone the action of the antibiotic.

Limitations in this study include that only the formation of a biofilm was measured and not the planktonic growth. Free floating cells within the wells could be a good indication of whether the antibiotics are killing the planktonic cells and thereby preventing formation of a biofilm, inhibiting biofilm formation or breaking down the biofilm; as well as whether there is a difference in resistance between planktonic and biofilm cells. However, Spoering *et*

al.,(2001) showed that biofilms do not show differences in resistance to growth inhibition compared to planktonic cells but rather to antibiotic killing.[132] Growth and formation of a biofilm seems to be as sensitive to antibiotics as planktonic cells. Additional experiments can be performed to determine the effects of antibiotics on already formed biofilms. Furthermore, the number of time points selected for the study could be increased to determine more accurate changes occurring during the development of a biofilm. The sample set used in this study was relatively small and failed to include a large range of isolates representing different antibiotic susceptibilities. The stability of the antibiotics is also unknown and can be further studied to determine optimal and sustainable concentrations for use in biofilm studies.

4.5 Conclusion

Previously in chapter 3 we found that isolates within the same MLVA type had different biofilm forming abilities at a single time point. In this chapter, we observed that isolates within the same MLVA type also have different biofilm growth rates. This might suggest that it is not the MLVA type that influences the effect an antibiotic has on the formation of a biofilm but rather the initial rate at which a biofilm is formed. All of the antibiotics had a noticeable effect on biofilm formation after about 10-12h incubation. Varying concentrations of antibiotics appeared to influence the biofilm formation of the different isolates in different ways. The most noticeable inhibition of biofilm formation was by cefepime and ciprofloxacin. It was observed that organisms with an early onset biofilm formation pattern were impacted less than late onset biofilm formers by the addition of antibiotics. The mechanism of action of the antibiotic did not seem to play a major role since antibiotics that have different mechanisms of action showed similar effects on inhibition patterns. Establishing the nature of an isolate's biofilm forming ability may help with the early and rapid eradication of a biofilm associated infection.

Antibiotic resistance in *P. aeruginosa* is an increasing concern which affects many people daily. It is important to understand the effects of antibiotics on biofilm formation and breakdown. It remains to be determined whether the physical delaying of penetration and binding of antibiotics are the primary mechanisms of resistance of the biofilm, or whether persisting cells within the biofilm play a bigger role. Studies on persisting cells within the biofilm will assist in determining how to eradicate these cells. Further research in developing new antibiotics, and techniques such as combining antibiotics for more efficient treatment of biofilm related infections, can assist in fighting recurring chronic infections.

Chapter 5

Chapter 5

5.1. Conclusion

Pseudomonas aeruginosa is an opportunistic pathogen which commonly infects patients with a compromised immune system and is often associated with biofilm formation.[4] The ability to form a biofilm provides an advantage to the organism, by enabling it to withstand the effects of antibiotics through the physical protection of the surrounding extrapolysaccharide matrix. *P. aeruginosa* infections are a common and growing occurrence in the clinical setting.[5] Nosocomial transmission is an expanding problem, particularly in the burns unit. Biofilm formation within this patient group has not been described, but *P. aeruginosa* is commonly associated with biofilm formation.[111] It is important to understand the epidemiology of the organism, its ability to form biofilms and the effect of antibiotics on the formation of biofilms for rapid detection, treatment and eradication.

In this study we have described the genetic relatedness of *P. aeruginosa* isolates found in the burns unit and burns ICU (burns unit/ICU) compared to other wards within Tygerberg hospital by means of MLVA (Multiple locus variable number tandem repeat analysis). We have identified a number of MLVA types within as well as outside the burns unit/ICU. Forty two different MLVA types were identified, of which 10 MLVA types were shared by two or more isolates. The remaining 32 MLVA types had unique MLVA patterns. MLVA type 1 had the most isolates, all of which were isolated from the burns unit/ICU, with the exception of one isolate which came from a different ward. MLVA type 1 isolates accounted for 60% of the burns isolates, suggesting that spread within the burns unit/ICU occurs regularly. Four other MLVA types containing two or more isolates were identified in the burns unit/ICU; of which MLVA type 2 was the most abundant, with four isolates. Outside of the burns unit, seven MLVA types were identified, with a maximum of 3 isolates per MLVA type, indicating more strain diversity, which may be due to less transmission occurring in other parts of the hospital. From our study it is clear that there is a need for better infection prevention control within the burns unit/ICU at Tygerberg Hospital, and these results have been communicated to the IPC (Infection prevention control unit).

We further investigated the biofilm formation ability of the *P. aeruginosa* isolates from the burns unit/ICU as well as outside the burns unit/ICU. Biofilm assays were performed to identify the optimal incubation period to assess biofilm formation. A clear change in biofilm

formation was seen at 10-12 hour incubation. Three different biofilm formation patterns were described in our study, namely noticeable increase in biofilm formation, no noticeable increase, and an increase in biofilm followed by dispersion. There were similar numbers of strong biofilm forming (40%) and weak biofilm forming (55%) isolates amongst the burns isolates, however the majority of non-burn isolates were strong biofilm formers (72%). The biofilm formation abilities of isolates from different specimen types were also compared, and the majority of isolates from wound/pus swabs, tissue samples and sputum samples were shown to be strong biofilm formers. Further investigations need to be performed since the small sample set made comparisons difficult. Furthermore, the biofilm formation of the different MLVA types was compared. No similarity in biofilm formation ability was identified between isolates within the same MLVA type, indicating that multiple factors play a role in the ability of an isolate to form a biofilm.

Organisms within a biofilm have been shown to be more resistant to antibiotics than planktonic cells.[12] In this study we investigated the effect of common Gram negative antibiotics on the ability of an isolate to form a biofilm. Four antibiotics from different antibiotic classes and with different mechanisms of action were included in this study. A subset of isolates was selected from different MLVA types based on their ability to form a biofilm, and their biofilm formation determined over 24 hours in the presence of three different concentrations of each antibiotic. Similar to what was seen when observing biofilm formation at a single time point, no similarity in biofilm formation was observed in isolates from the same MLVA type in the presence of antibiotics. Based on the observed biofilm formation patterns, two types of biofilm formers were identified; early and late onset biofilm formers. Isolates which were defined as having the ability to form either a strong or weak biofilm (chapter 3) could be classified as early or late onset biofilm formers, respectively. Isolates within the same MLVA type could be either early or late onset biofilm formers, suggesting that MLVA type does not play a significant role in defining the biofilm response of an isolate when exposed to antibiotics. Some similarity could be seen between early onset and late onset biofilm formers respectively. Early onset biofilm formers appeared to have better biofilm forming ability in the presence of antibiotics compared to late onset biofilm formers which were more affected by antibiotics. The mechanisms of action of the antibiotics did not appear to affect the biofilm formation response. Two antibiotics, cefepime and ciprofloxacin, which have different mechanisms of action showed similar biofilm inhibition patterns, while cefepime and imipenem, which have similar mechanisms of action, evoked very different responses. Sub inhibitory concentrations of antibiotics enhanced biofilm formation in some isolates, consistent with previous reports that sub inhibitory concentrations of antibiotics given to patients can enhance biofilm related infections and

should be avoided.[130] Based on our results we speculate that the ability of an organism to withstand antibiotics and show an increase in biofilm formation relies on the initial amount of biofilm the organism are able to form within the first 10 hours of incubation, in the presence of the antibiotic. A factor that could influence this is the stability of the antibiotic and therefore concentration in the assay over time.

P. aeruginosa has been shown to be a promising model organism for biofilm formation assays and studying the antibiotic susceptibility of forming biofilms. The epidemiology of this organism was determined within the burns unit/ICU to better inform infection prevention control measurements within the hospital. An enhanced understanding of the *P. aeruginosa* population structure and biofilm formation ability in this patient group and the impact antibiotic treatment on the establishment of biofilms may enable improvements in transmission prevention and clinical treatment and outcome.

5.2 Limitations

Our study used thirteen locus MLVA typing, which has been reported to provide sufficient discriminatory power; however two loci, ms207 and ms209 were excluded from the study since the product sizes of these loci are difficult to score. Although these loci are regularly excluded from studies, they have been shown to provide additional discriminatory power which could influence the typing results.

Certain isolates showed large standard deviations between biological replicates in the biofilm assays. A number of factors could have influenced this observation. Organisms may behave differently on different days based on small changes in the environment, secondly organisms tend to behave differently at different stages of biofilm formation[119], and thirdly the staining technique that we have used, namely crystal violet staining, has previously been reported to cause variation during biofilm staining.[112] Overall, crystal violet staining may not be the best choice of stain for the quantification of *P. aeruginosa* biofilms, but it remains a common, rapid, easy and cheap method.

When investigating the effect of antibiotics on biofilms in our study we did not determine the amount of planktonic cells that were present. These cells could provide an indication of whether the antibiotics are killing the cells or merely inhibiting or breaking down the biofilm. Furthermore, these planktonic cells could also indicate whether cells within the biofilm are more resistant than these free floating cells. The assay was performed on developing biofilms and not the exposure of already formed biofilms to antibiotics, which may have different results. Mature biofilms may have better resistance mechanisms in place, such as physically delaying penetration.

5.3 Future direction

Further studies can include validation of the discriminatory power of the MLVA typing scheme in our setting and investigating the possible reasons why similar strain types have different biofilm formation abilities and different reactions to antibiotics during biofilm formation. This can be done by comparing MLVA to a well-established typing method such as PFGE (pulsed field gel electrophoresis), MLST (Multiple locus sequence typing) or whole genome sequencing. A larger sample set of *P. aeruginosa* isolates from different wards within Tygerberg can also be studied to cover a wider range of infections, and to study transmission patterns in other wards of the hospital more reliably.

Further assays comparing cells within a biofilm and free floating viable cells outside the biofilm can assist in explaining the different biofilm formation patterns that have been described. This can then also be compared to mature biofilms exposed to antibiotics since the physical barrier of a mature biofilm can have an influence on antibiotic penetration. Additional experiments can be performed using an alternative biofilm and planktonic quantification methods, since *P. aeruginosa* showed large standard deviations when using crystal violet staining. Alternative methods such as the Syto9, FDA (fluorescein diacetate), resazurin, XTT and dimethyl methylene blue assays can be used as alternative for biofilm and planktonic cell quantification.

Proteomic studies would allow us to investigate the proteins involved in biofilm formation in the presence and absence of antibiotics. The different mechanisms of action that antibiotics have and the stability of antibiotics within the assay should also be further investigated to identify possible factors influencing biofilm formation.

Appendix

Table A1: MLVA typing of *P. aeruginosa* isolates from patients in the burns unit at Tygerberg hospital: * - represents unique MLVA type, NA-no amplification

Sample #	ms77	ms127	ms172	ms142	ms211	ms212	ms214	ms215	ms216	ms217	ms222	ms223	ms213	Type
Pseudo 3	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 4	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 7	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 9	2,5	8	11	4	8	11	4	1	2	3	1	2	1	3
Pseudo 11	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 12	2,5	8	11	4	8	11	4	1	2	3	1	2	1	3
Pseudo 13	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 14	3	9	13	1	7	0.5	4	0.25	1	5	1	4	6	2
Pseudo 15	3	9	13	1	3	9	NA	0.25	1	5	1	4	6	1
Pseudo 16	3	9	13	1	7	0.5	4	0.25	1	5	1	4	6	2
Pseudo 17	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 18	3	NA	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 19	2	8	10	1	2	6	4	1	1	4	2	2	3	5
Pseudo 20	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 21	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 22	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 23	3	9	13	1	7	0.5	4	0.25	1	5	1	4	6	2
Pseudo 24	3	9	13	1	7	0.5	4	0.25	1	5	1	4	6	2
Pseudo 25	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 26	3	9	13	1	3	9	NA	0.25	1	5	1	4	6	1
Pseudo 27	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 28	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 29	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 30	1	9	12	1	2	4	5	2	1	5	2	4	1	*
Pseudo 31	1	9	11	1	5	9	5	2	1	2	1	4	5	*
Pseudo 32	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 33	3	8	14	4	3	6	5	5	2	4	3	7	9	*
Pseudo 34	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 35	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 42	4	9	13	1	3	9	4	0.25	1	5	1	4	5,5	6
Pseudo 43	4	9	13	1	3	9	4	0.25	1	5	1	4	5,5	6
Pseudo 44	3	8	11	4	5	NA	5	1	2	3	1	2	0	*
Pseudo 45	4	9	13	1	3	9	4	0.25	1	5	1	4	5,5	6
Pseudo 47	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 54	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 55	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 56	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 57	2	8	8,5	7	7	11	6	4	2	1	2	4	3	*
Pseudo 58	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 59	2	9	10	3	7	5	4	4	1	1	4	3	3	*
PAO1	4	8	12	7	5	9	3	4	3	2	2	4	5	Control

Table A2 MLVA typing of *P. aeruginosa* isolates from patients outside the burns unit at Tygerberg hospital: * - represents unique MLVA type, NA-no amplification

Sample #	ms77	ms127	ms172	ms142	ms211	ms212	ms214	ms215	ms216	ms217	ms222	ms223	ms213	Type
Pseudo 1	2,5	8	12	2	3	8	5	6	1	4	1	2	4	4
Pseudo 2	4	9	11	1	7	4	4	2,5	2	5	1	3	4	*
Pseudo 5	2,5	8	12	4	8	9	2	4	2	4	3	3	2	*
Pseudo 6	2,5	8	8	3	8	6	2	1	2	1	1	1.125	5	*
Pseudo 8	2,5	8	12	2	3	8	5	6	1	4	1	2	4	4
Pseudo 10	2,5	8	11	6	7	3	3	2	1	3	1	2	5,5	*
Pseudo 36	3	9	13	1	3	9	4	0.25	1	5	1	4	6	1
Pseudo 37	3	8	9	5	3	9	2	4	2	3	2	4	7	*
Pseudo 38	3	8	11	12	6	3	3	2	1	3	1	2	4,5	*
Pseudo 39	3	8	12	2	3	8	5	5	1	3	1	2	4	*
Pseudo 40	1	8	11	4	5	NA	4	3	IS	3	4	7	2	*
Pseudo 41	3	8	12	4	9	9	2	4	2	4	3	3	1	*
Pseudo 46	1	8	11	5	7	NA	NA	NA	3	2	4	IS	5	*
Pseudo 48	2	8	NA	5	6	14	5	1	4	3	7	2	3.5	*
Pseudo 49	3	9	10	4	2	6	2	1	2	2	1	3	1	*
Pseudo 50	3	8	12	4	8	9	2	4	2	4	3	3	2	*
Pseudo 51	3	8	8	3	8	6	2	1	2	1	1	1.125	4	*
Pseudo 52	3	8	10	6	3	7	4	4	1	2	1	5	3	*
Pseudo 53	2	9	12	1	2	4	5	2	1	5	2	4	1	*
Pseudo 60	2	8	11	5	4	6	2	1	2	2	1,5	2	5	7
Pseudo 61	2	8	11	5	2	7	4	4	2	3	NA	2	5	*
Pseudo 62	2	8	11	5	4	6	2	1	2	2	1,5	2	5	7
Pseudo 63	2	8	11	4	8	11	4	1	2	3	1	2	1	8
Pseudo 64	3	9	NA	1	NA	3	NA	0.25	1	6	1	4	6	*
Pseudo 65	2	8	11	4	7	9	4	5	2	2	2	2	5,5	*
Pseudo 66	2	8	11	4	8	11	4	1	2	3	1	2	1	8
Pseudo 67	2	8	9	3	6	6	2	4	2	1	1	5	4,5	*
Pseudo 68	6	8	13	1	2	9	5	1	1	2	3	3	1	*
Acineto 69	NA	NA	13	4	NA	NA	NA	66bp	0	NA	2	NA	NA	*
Pseudo 70	2	8	10	1	2	6	4	1	1	4	2	2	3	5
Pseudo 71	2	8	11	4	7	6	3	2	1	1	3	4	4	*
Pseudo 72	2	8	11	6	6	3	3	2	1	3	1	2	5	*
Pseudo 73	2	8	12	4	8	9	2	4	2	4	3	3	2	9
Pseudo 74	2	8	11	4	8	11	4	1	2	3	1	2	1	8
Pseudo 75	2	8	12	4	8	9	2	4	2	4	3	3	2	9
Pseudo 76	3	9	13	1	3	3	4	0.25	1	5	1	4	6	10
Pseudo 77	4	8	8	3	2	9	3	1	2	4	2	2	5,5	*
Pseudo 78	2	8	11	3	3	9	5	2	2	4	2	2	7	*
Pseudo 79	3	8	12	1	3	9	5	2	1	3	2	2	5	*
Pseudo 80	3	9	13	1	3	3	4	0.25	1	5	1	4	6	10
PAO1	4	8	12	7	5	9	3	4	3	2	2	4	5	Control

Table A3: Antibiotic dilutions for 10%, 50% and 100% isolate MICs. Cefepime, Ciprofloxacin, Imipenem and Gentamicin. Formula: $C_1V_1=C_2V_2$

Stock in H_2O	**In TSB**	Dilutions							
Cefepime									
Stock 12,8 mg/ml	32 µg/ml	16 µg/ml	12 µg/ml	8 µg/ml	6 µg/ml	4 µg/ml	3 µg/ml		
How to prepare	25µl stock in 10ml TSB	12,5µl stock in 10ml TSB	3,75ml 32 in 6,25ml TSB	5ml 16 in 5ml TSB	1,875ml 32 in 8,125ml TSB	5ml 8 in 5ml TSB	5ml 6 in 5ml TSB		
			1,2 µg/ml	0,8 µg/ml	0,6 µg/ml				
			1ml 12 in 9ml TSB	1ml 8 in 9ml TSB	1ml 6 in 9ml TSB				
Ciprofloxacin									
Stock 16 mg/ml	64 µg/ml	32 µg/ml	24 µg/ml	12 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	3 µg/ml	1,5 µg/ml
How to prepare	40µl stock in 10ml TSB	20µl stock in 10ml TSB	3,75ml 64 in 6,25ml TSB	5ml 24 in 5ml TSB	5ml 32 in 5ml TSB	5ml 16 in 5ml TSB	5ml 8 in 5ml TSB	1,875ml 16 in 8,125ml TSB	5ml 3 in 5ml TSB
	6,4 µg/ml		2,4 µg/ml			0,8 µg/ml		0,3 µg/ml	
	1ml 64 in 9ml TSB		1ml 24 in 9ml TSB			1ml 8 in 9ml TSB		1ml 3 in 9ml TSB	
Imipenem									
Stock 5 mg/ml	16 µg/ml	12 µg/ml	8 µg/ml	6 µg/ml	4 µg/ml				
How to prepare	32µl stock in 10ml TSB	24µl stock in 10ml TSB	16µl stock in 10ml TSB	5ml 12 in 5ml TSB	5ml 8 in 5ml TSB				
	1,6 µg/ml	1,2 µg/ml	0,8 µg/ml						
	1ml 16 in 9ml TSB	1ml 12 in 9ml TSB	1ml 8 in 9ml TSB						
Gentamicin									
Stock 25,6 mg/ml	512 µg/ml	256 µg/ml	192 µg/ml	96 µg/ml					
How to prepare	200µl stock in 10ml TSB	5ml 512 in 5ml TSB	75µl stock in 10ml TSB	5ml 192 in 5ml TSB					
	51,2 µg/ml		19,2 µg/ml						
	1ml 512 in 9ml TSB		1ml 192 in 9ml TSB						

A4: Ethics approval letter.

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Approval Notice
New Application

23-Mar-2015
Van Biljon, Bernardus BD

Ethics Reference #: S15/02/019

Title: **Population structure and biofilm formation of Pseudomonas aeruginosa isolated from patients with severe burn wounds and cystic fibrosis at Tygerberg Hospital.**

Dear Mr Bernardus Van Biljon,

The **New Application** received on **10-Feb-2015**, was reviewed by members of **Health Research Ethics Committee 1** via Expedited review procedures on **23-Mar-2015** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **23-Mar-2015 -23-Mar-2016**

Please remember to use your **protocol number** (**S15/02/019**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 219389156.

Included Documents:
Protocol Synopsis
Declaration B van Biljon
Checklist

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