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Studies on the mechanisms of adaptation of Multi Drug Resistant *Pseudomonas aeruginosa* to 2-Phenoxyethanol

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List of abbreviations

2-PE: 2-Phenoxyethanol.

ABC: ATP-Binding Cassette.

AML: Amoxicillin.

AMP: Ampicillin.

ATM: Aztreonam.

AZM: Azithromycin.

BSA: Bovine Serum Albumin.

BZC: Benzalkonium Chloride .

C: Chloramphenicol.

CAZ: Ceftazidime.

CHD: Chlorhexidine Diacetate.

CIP: Ciprofloxacin.

CL: Cepfalexin.

CN: Gentamicin.

DMF: Dimethyl Formamide.

DO: Doxycyclin.

E: Erythromycin.

EthBr: Ethidium Bromide.

EU: European Union

FOX: Cefoxitin.

HAI: Hospital-Acquired Infection.

HBV: Hepatitis B Virus.

HCV: Hepatitis C Virus.

HIV: Human Immunodeficiency Virus.

IM: Inner Membrane.

IPM: Imipenem.

KCl: Potassium Chloride.

kD: kilo Dalton

KHCC: King Hussein Cancer Center.

LEV: Levofloxacin.

LPS: Lipopolysaccharide.

MATE: Multidrug and Toxic Compound Extrusion.

MBC: Minimal Bactericidal Concentration.

MDR: Multi Drug Resistant.

MFS: Major Facilitator Superfamily.

MH: Minocyclin.

MHA: Mueller Hinton Agar.

MHB: Mueller Hinton Broth.

MIC: Minimal Inhibitory Concentration.

MRSA: Methicillin-Resistant *Staph aureus* .

MSSA: Methicillin Susceptible *Staph aureus.*

Na_2HPO_4: Disodium Hydrogen Phosphate.

NF: Nafcillin.

NOR: Norfloxacin.

O. D: Optical Density.

OFX: Ofloxacin.

OM: Outer Membrane.

P. aeruginosa: Pseudomonas aeruginosa.

P. stutzeri: Pseudomonas stutzeri.

PBS: Phosphate Buffered Saline.

PG: Peptidoglycan

PPK: Polyphosphate Kinase.

PRL: Piperacillin.

QACs: Quaternary Ammonium Compounds.

RD: Rifambicin.

RND: Resistance Nodule Division.

RPM: Round Per Minute.

S. enterica serovar Typhimurium: Salmonella enterica serovar Typhimurium.

SDS: Sodium Dodecyl Sulfate.

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

SMR: Small Multidrug Resistance .

S. aureus: Staph aureus.

SXT: Sulphamethoxazole.

TE: Tetracycline.

TEMED: Tetramethylethylenediamine.

TIM: Ticarcillin/Clavulanic acid.

TOB: Tobramycin.

VA: Vancomycin.

ZOX: Ceftizoxime.

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Abstract

Studies on the Mechanisms of Adaptation of Multi Drug Resistant *Pseudomonas aeruginosa* to 2-Phenoxyethanol

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Introduction

Biocide use and abuse and its relation to antibiotic resistance are debatable. However, a considerable amount of evidence has shown that abuse and misuse of biocides could furnish to antibiotic resistance. Recently a multidrug resistant strain of *Pseudomonas aeruginosa* was shown to retrieve its susceptibility to different antibiotics after its exposure and adaptation to 2-phenoxyethanol (2-PE).

Aim

This study aims at investigating the mechanisms of retrieving antibiotic susceptibility in Multi-Drug Resistant strains after 2-PE adaptation as well as investigating the possibility of MDR strains adaptation to other hospital used biocides.

Methodology

Alterations in the outer membrane proteins of the 2-PE adapted and non- adapted MDR strains were examined using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Efflux inhibition by 2-PE was investigated using ethidium bromide accumulation assay. Polyphosphate kinase (PPK) activity was examined through the use of a *ppk* null mutant.

Results

Pyocyanin production seemed to disappear as the strain became adapted to 2-PE and more sensitive to antibiotics. SDS-PAGE of adapted and non-adapted strains showed appearance and absence of different proteins with a molecular weight ranging between (52.8- 127.2) kD. 2-PE adapted MDR strains showed higher level of EthBr accumulation. Strains used in this study failed to adapt to the presence of the biocides Sterillium® and Septoderm®. Though, upon exposure of the MDR strains to these biocides their susceptibility to the previously effective antibiotics increased as seen by the increase in the diameter of the zone of inhibition. Furthermore two new *P. aeruginosa* MDR strains upon adaptation to 2-PE did not improve their antibiotic susceptibility as the other strains.

Conclusion

Adaptation to 2-PE in MDR *P. aeruginosa* is possible but improvement of antibiotic susceptibility is strain specific. This improvement could be attributed to several reasons of which inhibition of virulence factors such as pigment production and alteration of outer membrane protein structure which could result in permeability changes that allow penetration of antibiotics. This protein alteration could also result in disappearance of existing efflux pump proteins which explains increased accumulation of ethidium bromide.

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Thank you

Chapter One

Introduction

Introduction

1. Biocides:

Biocides are antimicrobial agents that include disinfectants, antiseptics and preservatives (Hugo and Russell, 2011).

Biocides have been commonly used in the control of bacteria for decades, and in spite of their increasing use, bacteria generally stay susceptible to them. Biocides have a range of clinical applications including disinfection of hospital surfaces and pre-operative patient skin, sterilization of medical equipment and general infection prevention via incorporation into hospital bed linens and curtains. Examples of biocides commonly used in the hospital environment include cationic biocides such as QACs (e.g. BZC), chlorhexidine, cetrimide and triclosan (Bailey *et al*., 2009, Smith and Hunter, 2008). Biocides used to control the growth of pathogenic organisms in a clinical environment can be categorized based on the level of bacterial inactivation reached. For example, low-level disinfectants (e.g. isopropyl alcohol) may only eliminate vegetative bacteria whereas high level disinfectants (e.g. hydrogen peroxide) inactivate many microorganisms including vegetative bacteria, mycobacteria, viruses and most fungi (Rutala and Weber, 2007). Low level disinfectants are commonly used to disinfect 'non-critical' hospital devices that come into contact with intact skin (e.g. stethoscopes, electrocardiogram cables) which are unlikely to transmit infectious agents to patients and therefore do not require high level disinfection (Dettenkofer *et al*., 2004). 'Critical' devices that penetrate sterile tissues (e.g. catheters, needles) are sterilized using high-level disinfectants. Hospital-acquired infections (HAIs) cause significant morbidity and mortality (Klevens *et al*.,

2007), which that emphasizes the requirement for effective disinfection procedures and the correct use of the appropriate biocidal products in order to prevent the spread of infection. On the other hand, increased use of biocides will expose bacteria in the clinical environment to biocidal products more frequently.

The indiscriminate use of biocides in human medicine, cosmetics, agriculture, livestock farming, food production, personal care products and household products has raised concerns about the development of bacterial biocide resistance and potential cross resistance to antibiotics (Gilbert and McBain, 2003). Despite the establishment of the European Union (EU) biocidal product regulation to regulate the authorization and use of biocidal products throughout the EU, the total amount of biocide use in the EU remains unknown(Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2010)). Concerns have also been raised over the release of biocides into waste water and the surrounding environment as a result of their increased use, and the potential effect of this on microbial populations in soil and aquatic habitats.

1.1 Adaptation to biocides:

It is proposed that concentrations present in the environment may be sufficient to select for bacterial strains with reduced antimicrobial susceptibility and furthermore could result in increased exposure of the human and animal population to biocides which could lead to alterations in biocide susceptibility of microflora in humans and animals (SCENIHR, 2010). Sub minimal inhibitory concentration of biocides due to misuse in domestic settings and hospital environments have resulted in biocidal adaptation of microorganisms.

In fact exposure of microbes to biocide stress could lead to genetic, biochemical, functional or physiological changes in the bacterial cell that select for bacteria with

greater tolerance to these conditions (Scenhir, 2010). Laboratory strains and clinical isolates of *P. aeruginosa* were successfully adapted to Benzalkonium chloride (BZC) by sub-culturing the bacteria in subminimal inhibitory concentrations of BZC (Joynson *et al*., 2002).

Studies showed successful adaptation of *P. aeruginosa* to sub minimum inhibitory concentrations (MIC) of 2-Phenoxyethanol (2-PE) that resulted in 2 folds increase in the MIC while adaptation of *E. coli* was able to grow only in for two passages of 2-PE with a slight increase in the MIC of 2-PE (Abdelmalek and Badran, 2013) which means that adaptation to certain biocide might be species specific.

1.2 Generating resistance to biocides

The generation of biocide-resistant mutants is useful because it allows the exploration of mechanisms behind resistance to a particular biocide, investigation of the effect of the developed resistance on virulence or growth and the identification of any cross-resistance to other biocides or antibiotics.

Biocides abuse though exposing microbes to low concentrations could predispose to biocide resistance. Thus a common in vitro method that has successfully generated biocide resistant bacteria includes the stepwise passaging of bacteria through gradually increasing concentrations of biocide, either in broth or on agar. For example *E. coli* mutants adapted to Benzalkonium Chloride (BZC) via 24 h subculture of isolates in nutrient broth supplemented with gradually increasing concentrations of BZC were produced by Pagedar *et al*., (2012). They reported that 29 resistant isolates showed a major increase in efflux pump activity and were better than non-resistant isolates at biofilm formation.

Nevertheless it must be noted that the bacterial species and mechanism of action of the biocide used may have an effect on the successful generation of resistant mutants. This means that a single, universal method might not be possible because ideally the method would be based on conditions under which the biocidal product is used (Maillard and Denyer, 2009). Parameters that could be kept consistent might include the preparation of the test inoculum, the number of repeats performed, suitable neutralization of the test product and investigation in to resistance to the in-use concentration of the product (Maillard and Denyer, 2009).

1.3 Biocides resistance

Microbial resistance towards biocides is emerging (McDonnell and Russell, 1999). Biocide have multiple target sites in bacterial cells and is therefore less likely for bacteria to become resistant to a particular biocide via the alteration of a target site although there are some exceptions (Bailey *et al*., 2009). Biocide resistance therefore generally occurs as a result of mechanisms that decrease the concentration of a particular biocide to a level that is not lethal to the bacterial cell or prevent the entry of the biocide in to the cell.

1.3.1 Mechanisms of bacterial resistance to biocides

1.3.1.1 Alteration of cell permeability

Bacterial intrinsic insusceptibility to biocides may be associated with changes to the outer layer that alter cell permeability and restrict the uptake of the biocide into the bacterial cell. Gram-negative bacteria such as *Salmonella* and *Burkholderia* spp. tend to be less susceptible to biocides than Gram positive bacteria due to the presence of a lipid-rich outer membrane. This membrane is composed of phospholipids, fatty acids, lipopolysaccharide (LPS) and porins that aid in limiting the uptake of the biocide into

the cell it. The negative charge may result in the repulsion of biocide molecules away from the bacterial cell. Figure 1 illustrates both the Gram-positive and Gram-negative bacterial cell walls. *Mycobacterium spp*. possesses a lipid-rich cell envelope composed of mycolic acids which have been associated with antimicrobial resistance. This layer prevents the Gram staining of this species, and maintains the structural integrity of the membrane and has been associated with resistance to oxidizing agents such as hydrogen peroxide. (Dubois-Brissonnet *et al*., 2011, Ferreira *et al*., 2011, Mensa *et al*., 2011)

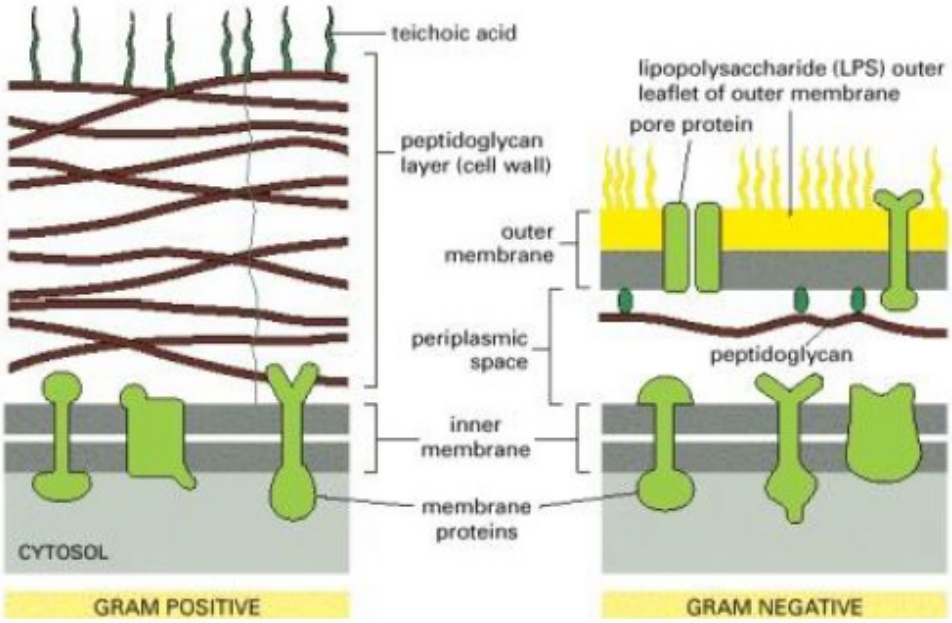


Figure 1: Gram-positive and Gram-negative bacterial cell walls (Alberts *et al*., 2002).

Alteration and modification of the bacterial cell membrane in response to biocide exposure has been described on more than one occasion. Dubois-Brissonnet *et al*., (2011) described the modification of membrane fatty acid composition resulting in increased tolerance to peracetic acid and dodecyl ammonium bromide in *Salmonella enterica serovar Typhimurium*. They found that exposure to sub minimum inhibitory concentrations of natural plant derived terpenes used as chemical preservatives in the food industry resulted in the increased production of saturated fatty acids throughout

all bacterial growth phases. This change was thought to stabilize the membrane in the presence of antimicrobials that exert their effects on the cell via the partitioning of the lipid membrane. Tattawasart *et al*., (2000) reported that chlorhexidine diacetate resistant *Pseudomonas stutzeri* had an altered outer membrane protein profile and found the expression of two additional protein bands. They also observed changes in LPS that were thought to contribute to cross- resistance to other antimicrobial agents aside from chlorhexidine diacetate, such as polymyxin B sulphate and gentamicin. Despite the increased biocide susceptibility observed in Gram-positive bacteria (compared to Gram-negatives), certain Gram- positive species of bacteria, such as *Clostridium* spp. and *Bacillus* spp. have the ability to form spores when under environmental stress such as nutrient starvation. Bacteria in spore form exist in a dormant state and can survive in this state for many years (Leggett *et al*., 2012). The presence of a spore coat, composed of highly cross-linked proteins is thought to contribute to intrinsic resistance to antimicrobials. Treatment of spores with chemical disruptors of disulphide bonds has been shown to increase spore susceptibility to hydrogen peroxide and lysozyme (Gould, 1970).

1.3.1.2 Biofilm formation

It has been estimated that > 90 % of bacteria in nature exist as a biofilm (Branda, *et al*., 2005) .A biofilm is a structured community of bacteria attached to a surface by exopolymeric substances (Baugh *et al*., 2013). Biofilms can consist of monocultures of several diverse species or of mixed phenotypes of a given species. An environment limited in nutrients has been shown to induce a ‘stress’ response where bacteria adopt a resting or dormant phenotype similar to that of endospores which are resistant to numerous chemical agents (Leggett *et al*., 2012). Bacteria within a biofilm exist in a slow growing, nutrient-depleted state, or non-growing state (Gilbert and McBain,

2003) and have an altered phenotype in comparison to planktonic (non-biofilm) species. In a hospital environment biofilms are generally found on moist surfaces such as catheters, disinfecting soap dispensers, instruments regularly immersed in fluids, as well as in patients which can lead to the increased spread of infection (Vickery *et al*., 2012).

Some biofilm-forming bacteria have been shown to be 10 - 100 fold more resistant to antimicrobials in comparison to their planktonic counterparts. Wong *et al*., (2010a) tested the efficacy of benzalkonium chloride (BZC), chlorhexidine diacetate (CHD), citric acid, sodium hypochlorite and ethanol against planktonic *S. enterica serovar Typhimurium* cells and 3 day old *S. enterica serovar Typhimurium* biofilms at recommended in-use concentrations, and found that all biocides were able to reduce the number of biofilm cells and still left some viable cells whereas all planktonic cells were eliminated. This demonstrates the reduced biocide susceptibility of cells present in a biofilm and the potential for further spread of infection if a biofilm is not completely eliminated. It has also been reported that biofilm age has no effect on the efficacy of the antimicrobial and that older biofilms are no more or less susceptible to biocides than new biofilms (Wong *et al*., 2010a).

The exchange of mobile genetic elements between bacteria within biofilms has been reported (Antonova and Hammer, 2011). Antonova and Hammer (2011) observed that Vibrio cholera present in a biofilm produce an autoinducer molecule that allows them to become naturally competent to take up extracellular DNA. This demonstrates the possibility of the acquisition of resistance genes amongst bacteria present in a biofilm, and may further contribute to the reduced biocide susceptibility of these bacteria. Wong, *et al*., (2010b) suggested that an increase in concentration and contact time was the only way to ensure 100 % reduction of viable cells present in a biofilm. The

need to increase biocide concentrations in order to kill biofilms could result in high environmental toxicity and increased costs, and may also select for increased biofilm development amongst bacterial species.

1.3.1.3 Metabolism of biocides

Biocides generally have multiple targets (e.g. cell wall, cytoplasmic membrane, DNA, proteins) in the bacterial cell therefore; it is unlikely that biocide resistance would occur due to biocide inactivation by bacteria. However, the break down and inactivation of quaternary ammonium compounds (QACs) has been reported. Nishihara *et al*., (2000) showed that *Pseudomonas fluorescence* TN4 isolated from sludge was able to degrade didecyldimethylammonium chloride. This isolate was also able to degrade other QACs via an N-dealkylation process. The initial parent compound was broken down after 24 h and the first metabolite was then further broken down after a period of 7 days. This strain was found to be highly resistant to the compounds it could break down demonstrating the presence of bacteria that are able to degrade QACs in the environment. Biocide use in waste water treatment, industry, building materials and fuel may result in the release of biocide residues into the environment, although the exact quantities are unclear (SCENIHR, 2010). Biocide residues in the environment may create a selective pressure for the clonal expansion of bacteria with the ability to degrade certain compounds, contributing to biocide resistance.

Although there are very few reports of bacteria that are able to directly break down biocides, there is evidence of bacteria making alterations to a specific metabolic pathway targeted by a biocide in order to prevent damage to the bacterial cell. Bailey *et al*., (2009) described a triclosan-specific alteration in metabolism in *S. enterica serovar Typhimurium* that assisted in the protection of the bacterial cell from the

biocide. They demonstrated through the use of DNA microarrays that the bacterium was able to down-regulate expression of the *fab* gene cluster associated with fatty acid biosynthesis and up-regulate pyruvate synthesis genes in order to by-pass the inhibitory effects of triclosan, which inhibits fatty acid biosynthesis at low concentrations, preventing cell membrane synthesis. Due to the specificity of this alteration in response to biocide exposure there is not yet any reported evidence of cross -resistance to antibiotics (Bailey *et al*., 2009).

1.3.1.4 Biocide efflux

Efflux is the pumping of a solute out of a cell, and efflux pumps are present in all organisms. In bacteria efflux pump genes can be chromosomally encoded or found on mobile genetic elements such as plasmids. Efflux pumps are proteins that span the bacterial cell membrane and can either transport a single, specific substrate or a range of structurally similar compounds (Nikaido and Pages, 2012). Examples of bacterial efflux pump substrates include dyes, detergents, antibiotics (e.g. quinolones, fluoroquinolones, chloramphenicol, and tetracycline) and biocides (e.g. cetrimide, triclosan). Many bacterial efflux pumps are now well characterized and have been associated with a multidrug resistant (MDR) phenotype (Guo *et al*., 2013, Buroni *et al*., 2009, Smith and Hunter, 2008). There are five well described efflux pump families in bacteria: Bacterial efflux pump multidrug and toxic compound extrusion (MATE), major facilitator superfamily (MFS) , small multidrug resistance (SMR), resistance nodule division.(RND) and ATP-binding cassette (ABC) (Piddock, 2006a). These pumps are classified based on the number of components a pump has, the energy source required for the pump to transport substrates, the number of trans-membrane spanning regions and the type of substrate (Piddock, 2006b). Different species of bacteria can express more than 1 type of efflux pumps. In Gram-negative

bacteria, the type of efflux pump most commonly associated with multidrug resistance is the resistance nodule division (RND) type pump. RND pumps are composed of a tripartite system.

Brozel and Cloete (1994) showed that adaptation of *P. aeruginosa* resulted to Kathon® resulted in a gradual decrease in susceptibility towards increasing concentrations of this biocide accompanied with the concurrent disappearance of proteins T-OMP.

1.4 Biocide resistance in the clinical environment

Russell (2004) suggested that biocides are only really required in high risk areas where the spread of HAIs is high e.g. in the sterilization of medical equipment but not perhaps in areas that are rarely heavily contaminated such as hospital walls and ceilings. For example Block and Furman (2002) found that in clinical areas of a hospital where chlorhexidine was used more intensely, micro-organisms isolated from patients showed decreased in susceptibility to this biocide. It has been argued that bacterial strains showing reduced susceptibility to biocides are still not a major health concern as generally biocides are used at high concentrations that are lethal to these strains (Thomas *et al*., 2005). However Duarte *et al*., (2009) reported an epidemic of rapidly growing *Mycobacterium massiliense* in patients that had undergone surgery in one of 63 hospitals in Rio de Janeiro, Brazil. Five isolates belonging to a specific clonal group referred to as BRA100 were resistant to 2 % gluteraldehyde solution which had been commercially used in the sterilization of surgical instruments. All isolates tested were also found to be clinically resistant to ciprofloxacin, cefoxitin and doxycycline. This finding contradicts the claim that much higher, in-use concentrations of biocides are highly effective.

1.5 Biocide–antibiotic cross resistance:

External chemical stress, such as biocide exposure may promote the maintenance of transferable resistance genes and increased transfer of these genes. Gilbert and McBain (2003) have speculated that the increased use of biocides could potentially create a selection pressure for mutant strains that hyper-express these multi substrate efflux pumps when exposed to biocides. Hyper-expression could occur via a point mutation in the efflux gene promoter or a mutation in the global repressor (Baucheron *et al*., 2004) leading to the over-expression of the gene in the absence of the substrate that induces its expression. This would result in reduced susceptibility to any other substrates that come into contact with the bacterial cell, including antibiotics, which may lead to limited therapeutic options when treating an infected patient.

The potential for biocide-selected cross-resistance to clinically important antibiotics is the main subject of some discussion in the literature (Schweizer 2001). Loughlin *et al.* (2002) showed that adaptation of *P. aeruginosa* to BZC resulted in cross resistance to polymyxin and Triclosan-resistant *P. aeruginosa* showed elevated resistance to some antibiotics as a result of mutational up-regulation of endogenous multidrug efflux systems which accommodate both antibiotics and biocides (Chuanchuen *et al*., 2001).

In contrast, adaptive resistance to some biocides has been reported to improve antibiotic susceptibility (Abdel Malek 2009; Joynson *et al*., 2002). and adaptation to it was accompanied by a decrease in minimum inhibitory concentration (MIC) to different antibiotics and accelerated the loss of resistance to imipenem BZC was also suggested to be used in hospital hygiene to help overcome antibiotic resistance in clinical environment (Joynson *et al*., 2002).

2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is an example of an emerging nosocomial pathogen that is notoriously sometimes difficult to control with antibiotics or disinfectants because of its remarkable ability to develop antibacterial drugs resistance (Kirecce and Dara Kareem, 2014).

P. aeruginosa belongs to the family Pseudomonadaceae. It is metabolically versatile opportunistic bacteria of a wide ecological distribution found in soil, aquatic environment and skin flora. It is the main cause of chronic lung infections and a main factor of mortality in patients with cystic fibrosis as well as other serious infections in the cornea, urinary tract, soft tissues, joints, bones, burned tissues and other mammalian tissues especially in the immunocompromised patients (Imperi *et al*., 2009).

Its pathogenic ability derived from the presence of several cell-associated and secreted virulence factors in addition to the organism's intrinsic high resistance to many antimicrobials (Morales*, et al*., 2004).

2.1 Characteristics

P. aeruginosa is a rod shape Gram negative bacteria 0.5 to 0.8 µm by 1.5 to 3.0 µm in size. The first part of the name is gained from the Greek two word (Pseudo: False, Monas: Unit) and the other part from the Latin word (aeruginosa: Cupper rust) as it secretes a green- blue pigment (Bhawsar *et al.,* 2014). It exists in versatile ecological niches: soil or water and is fast swimming using the single polar flagellum (some of them have two or three flagellae), It exists in planktonic form and is capable of forming biofilms on surfaces (Palleroni, 2010). The organism is aerobic and requires O_2 for growth or NO_3 in the absence of O_2 as a respiratory electron receptor. Simple nutrients can support its growth where the simplest medium for growth of *P.*

aeruginosa consists of acetate as a source of carbon and ammonium sulfate as a source of nitrogen. The optimum growth temperature is 37℃ but it can grow at temperature up to 42℃ (Bhawsar *et al*., 2013).

2.2 *Pseudomonas aeruginosa* cell envelope structure

P. aeruginosa is generally as resistant to hydrophobic molecules as other Gram-negative bacteria due to the presence of the LPS molecules (Vaara 1992). However, *Pseudomonas aeruginosa* also exhibits resistance to small hydrophilic antibiotics, like tetracyclines and β-lactams. Hence, the outer membrane of *P. aeruginosa* is suspected of having a lower level of permeability than that of other Gram-negative bacteria. *P. aeruginosa* has 12 to 100 fold less outer membrane permeability than that of *Escherichia coli* (Russell and Chopra 1996; Hancock 1998). The cell envelope of *P.aeruginosa* is very much like that of other Gram-negative bacteria (Figure 2). The cell envelope is made of two concentric bilayer membranes surrounding the peptidoglycan cell wall and confining the periplasmic space (Koebnik *et al* 2000). Both membranes contain proteins that assist in the passage of matter and information, but the two membranes differ markedly with respect to structure and function. The inner cytoplasmic membrane (Figure 2) renders the bacteria impermeable to large molecular weight hydrophilic compounds (Russell and Chopra 1996). It is made of inner and outer leaflets. The two leaflets are made exclusively of phospholipids which makes it highly hydrophilic and impermeable to hydrophopic molecules. The cytoplasmic membrane contains proteins that assist in the passage of materials and information in and out of the cell. The major phospholipid in *P. aeruginosa* is phosphatidylethanolamine that constitute 59% of the total phospholipid of the cell (Anderes *et al* 1971). Other phospholipids such as phosphatidylglycerol and cardiolipin are equally distributed among the inner and outer leaflets (Koebnik *et al*

2000). The outer leaflet of the cytoplasmic membrane is bounded by the cell wall (Kadurugamuwa and Beveridge 1996). The cell wall is a rigid highly structured layer that contributes to the mechanical stability of the bacteria. It is composed of peptidoglycan polymer, which is made of two different N-acetylated aminosugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAMA). A short peptide chain is connected to the carboxyl group of NAMA. Glycan strands are cross linked through direct peptide bonds between the adjacent peptide chains or peptide interbridges to create a covalent network with great mechanical strength (Russell and Chopra 1996). *P. aeruginosa* has an acyl group on the 6^{th} position of the NAMA, which renders the peptidoglycan insensitive to degradation by lysozyme. The presence of an acyl group in that position prevents effective binding of the lysozyme to the β-1, 4 glycosidic bond.

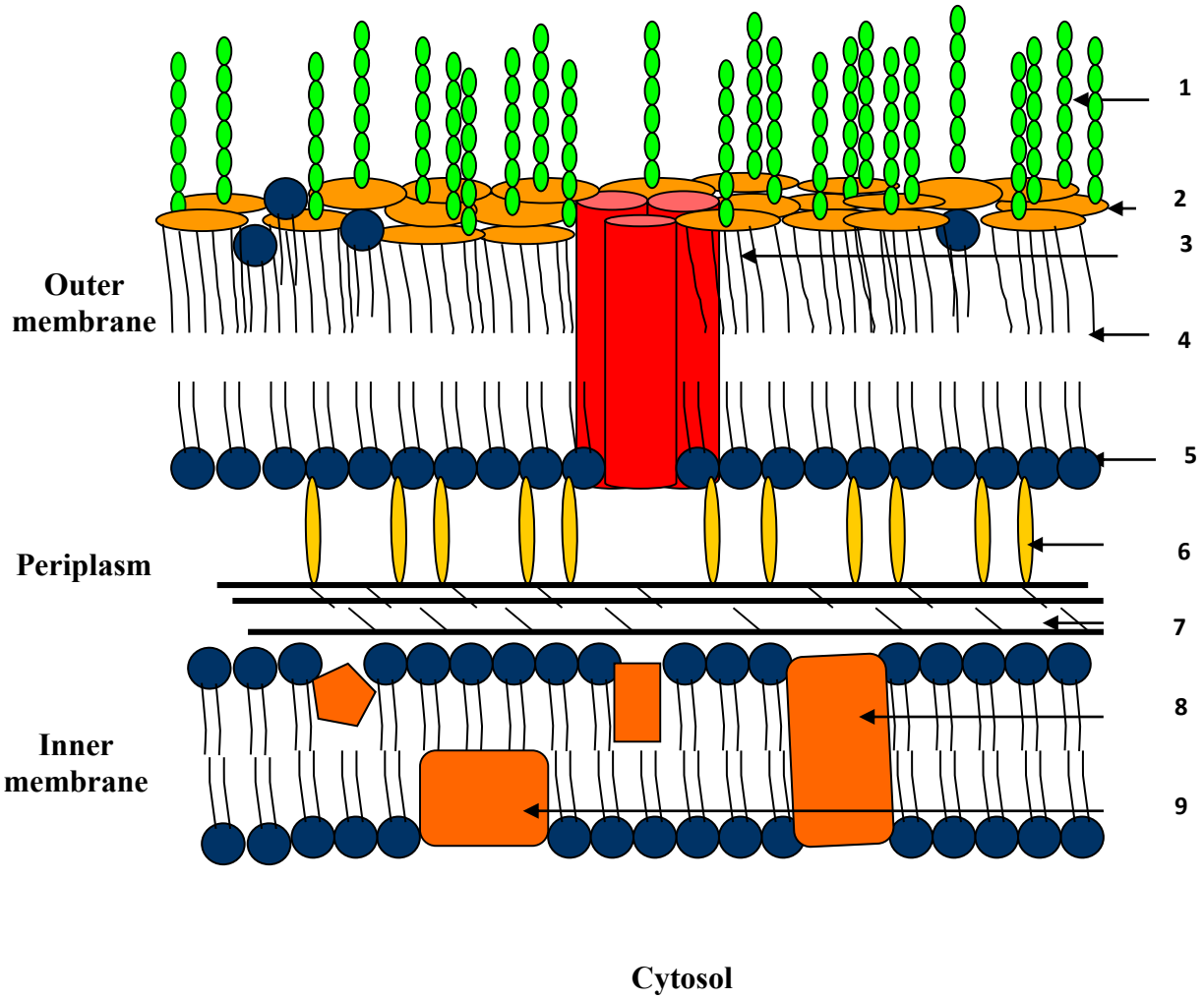
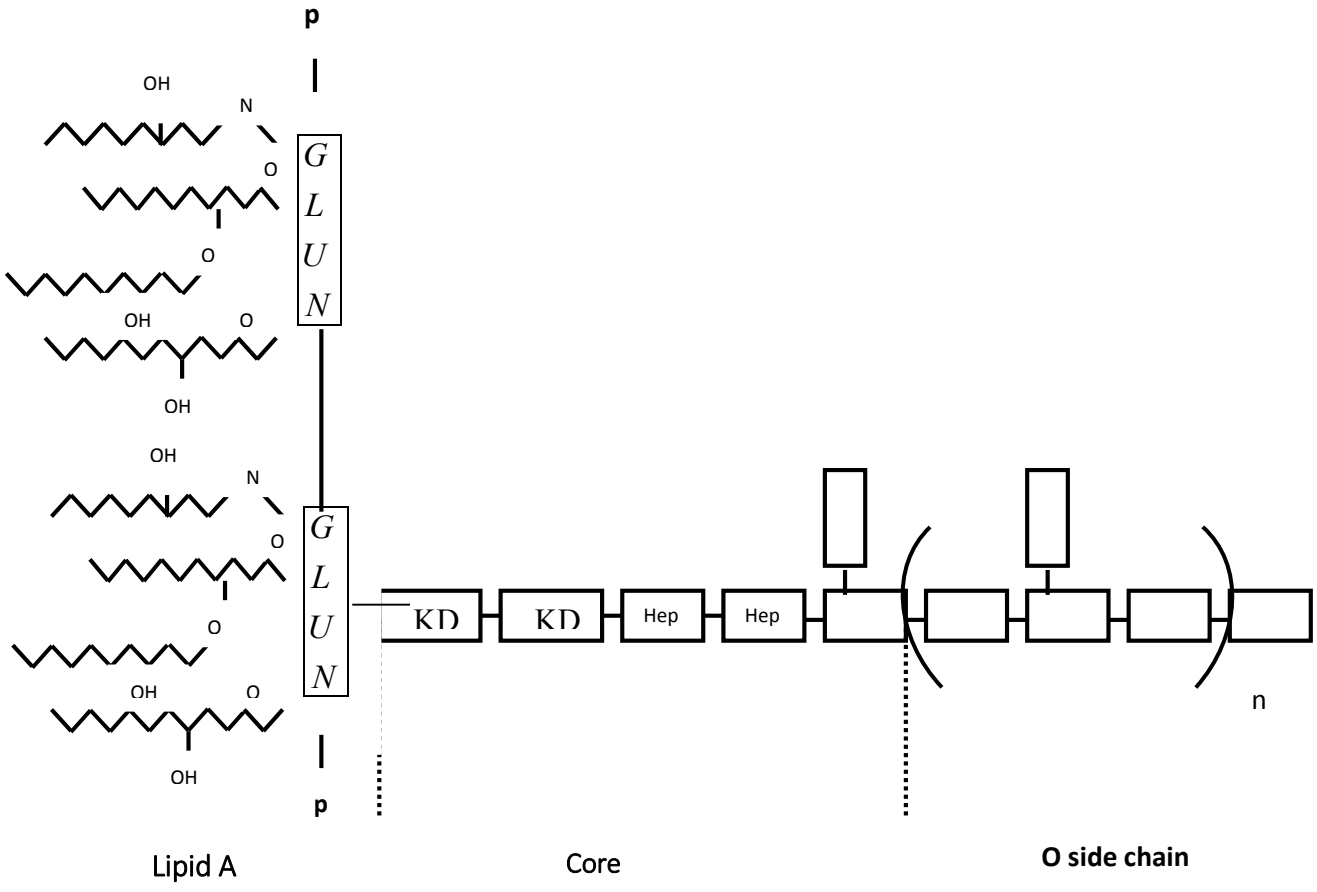


Figure 2: Cell envelope of Gram-Negative bacteria. (1) Lipopolysaccharide chains (2) Core region (3) Porin (4) Lipid A region (5) Inner leaflet of the outer membrane (6) Lipoproteins (7) Peptidoglycan (8, 9) Transport protein.

between the two aminosugars and thus will not be able to break the bond (Hammond *et al* 1984). Nearly all bacteria have an absolute requirement for peptidoglycan, which makes it a good target for antimicrobials (e.g. β-lactams). Peptidoglycan of Gram-negative bacteria is very thin (Figure 2). Peptidoglycan is surrounded by the outer membrane and is anchored in its place by covalent bonds with the Braun's lipoproteins. Braun's lipoproteins are found in the periplasmic area between the

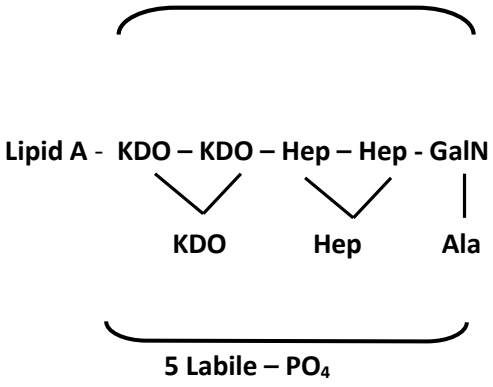
peptidoglycan and the outer membrane (Kadurugamuwa and Beveridge 1996). Lipoproteins occur in two forms, a bound form and a free form. The N- terminal of the bound lipoprotein contains a cystein moiety that is linked to the diacyl glycerol in the lipid A portion of the outer membrane by a thioester bond. The C-terminal contains L-lysine residue, which is connected to the peptidoglycan through a covalent bond between the L-lysine and the carboxyl group of the L-Diaminopimelic acid. About two thirds of the lipoprotein is freely embedded in the outer membrane (Hammond *et al* 1984). The periplasmic area is entrapped between the two membranes of the Gram-negative bacterial cell envelope (Figure 2). The periplasmic area is an essential and highly active component of these cells. It contains high concentrations of a variety of macromolecules that function as hydrolytic enzymes. These enzymes break down relatively large compounds to allow for their uptake by the cytoplasmic membrane into the cell. The periplasmic area also contains waste products and harmful substances waiting to be removed from the cell. A number of detoxifying enzymes that degrade harmful substances preventing them from injuring the cell, and a large number of binding proteins, that facilitate the transport of substrates into the cell through the cytoplasmic membrane, are also found in the periplasmic area. Constituents of the periplasmic area are ever changing in response to cellular and environmental messages (Kadurugamuwa and Beveridge 1996). The outer boundary of the periplasm is the outer membrane, which is a highly asymmetrical structure with the inner leaflet made totally of phospholipids (primarily phosphatidyl ethanolamine), while the outer leaflet consists largely of a single glycolipid compound, the lipopolysaccharide (LPS). The outer leaflet also contains a few molecules of phospholipids together with a number of functional proteins (Hancock 1997) (Figure 2). LPS is a high molecular weight, negatively charged molecule that provides the cell with a polyanionic external surface (Hancock 1997).

This anionic feature is partially neutralized by the presence of divalent cations (e.g. $Mg^{2+}$and Ca^{2+}). Divalent cations bridge adjacent LPS molecules resulting in the tight packing of these molecules (Hancock 1997). As shown in figure (1.2), LPS is made of three covalently linked regions, Lipid A, Core lipopolysaccharide and the O side chain. The lipid A region is made of six or seven fatty acid chains connected to a phosphorelated glucosamine disaccharide, unlike phospholipids that only have two fatty acid chains connected to their backbone structure. The amino moieties of the glucosamine are connected to 3-hydroxydodecanoate (C12: 0) through amide linkage. The 3-hydroxydecanoic (C10: 0), dodecanoic (C12: 0) and hexadecanoate (C16:0) of other fatty acids are ester linked to the glucosamine backbone or to the hydroxy groups of other fatty acids (Kropinski *et al* 1985). All of the fatty acids in the lipid A portion are saturated medium length hydroxy fatty acids (Nikaido and Vaara 1985). Lipid A of *P. aeruginosa* contains 3-hydroxy and 2-hydroxy fatty acids; most of these are saturated and even numbered fatty acids. The non-hydroxy fatty acids such as C_{12} myristic, C_{14} palmitic and C_{16} acids are unsaturated. *P. aeruginosa* contains variable amounts of other fatty acids such as 17,19 cyclopropane acids and tetradecanoate (Kropinski *et al* 1987). The core polysaccharide region is connected to the lipid A region by an acid labile linkage between the glucosamine disaccharide backbone and the sugar acid Ketodeoxyoctonate (KDO) (Figure 3).



(a)

3 PO_4



(b)

Figure 3: The LPS structure of Gram-negative bacteria **(a)**. Structure of the core region in the LPS of *P.aeruginosa* PAO1 **(b)**. KDO: 2-keto3-deoxyoctonic acid; Hep: Heptose sugar; GalN : galactosamine; Ala: alanin (Kropinski *et al* 1985)

The core region is made of a short chain of saccharides. The saccharide chain includes heptose, glucose, galactose and N-acetylglucosamine, rhamnose and alanine (Jarrell and Kropinski 1977). This region is highly conserved between species, variations only exist in the degree of substitution by phosphorous residues, *P. aeruginosa* strain PAO1 contain 8 phosphate residues in the core region (figure 3 b) (Kropinski *et al* 1985). The O side chain (Figure 2) is a branched or unbranched short polysaccharide chain extending outwards from the core. It has a wide range of variation in the sugars, which accounts for the large number of serotypes. The O side chain is made of a chain of six carbon sugars, e.g. glucose, galactose, rhamnose, fucosamine and mannose. Mutants of *P. aeruginosa* that lack the O-side chain are known as rough mutants (R) while mutants that expresses the side chain are known as smooth mutants (S) (Kropinski *et al* 1985). LPS is important to the Gram-negative bacterial cell, not only in the avoidance of host defense, but it also imparts a negative charge to the bacterial cell, since it contains charged sugars and phosphate. LPS surface has several divalent cations binding sites that stabilize the outer membrane (Hancock 1998). The permeability of the outer membrane can be altered when metal chelators such as EDTA are included in the growth media. EDTA chelates the divalent cations present on the surface of LPS molecules which would result in the disruption of the tight interactions between molecules and hence disruption of the outer membrane. However, the regular permeability is restored by the addition of Mg^{2+} to the growth media (Brown and Melling 1969; Ayers *et al* 1998; Ayers *et al* 1999).

LPS is associated with different proteins. These proteins are responsible for the protective nature of the outer membrane. They comprise nearly half the mass of the outer membrane but their number is limited in comparison to the proteins in the

cytoplasmic membrane. These proteins prevent or slow down the movement of antimicrobials or bile salts into the cell (Nikaido and Vaara 1985). Although the presence of the LPS structure in the outer membrane confers very low permeability for hydrophobic solutes and presumably hydrophilic solutes, the outer membrane is considered to be more leaky than the cytoplasmic membrane (Koebnik *et al* 2000), because the permeability of the outer membrane is also controlled by a class of proteins that are associated with the LPS molecules. These proteins produce water-filled channels that function as general or substrate-selective "*conduits*" for diffusion of certain hydrophilic molecules (Hancock 1997). The overall permeability of the outer membrane depends upon the number and properties of these pore-forming proteins that are generally called porins (Figure 2) (Trias and Nikaido 1990). Porins are proteins that form holes in the outer membrane by the clustering of three protein molecules (trimeric porins) (Nikaido 1992), by folding into β–pleated sheets to form a closed barrel. The structure of porins differs from other integral membrane proteins that are usually made of transmembrane α-helices (Koebnik *et al* 2000). Porins of the outer membrane form three types of channels (Braun 1995). Type 1 channels allow the non-specific passage of small hydrophilic substrates that are not larger than 600 D. They have some preference to some anions and cations due to the presence of some charges on the mouth of the channel; thus, the total charge on each porin gives each porin its ion selectivity. This type of porins is called general diffusion porins (also called major outer membrane proteins). They are made of homotrimers that form a barrel like structure; each monomer is made of 16 β-strands that span the outer membrane. The third loop folds back into the barrel forming a constriction zone at half the height of the channel, which keeps it either open or closed (Koebnik *et al* 2000). These porins open and close by means of a process called voltage gating. Voltage gating depends upon the critical voltage (V_C) of the membrane, if the V_C is

larger than the naturally occurring Donnan potential across the outer membrane the general porins like OmpF and PhoE of *E. coli* will close (Koebnik *et al* 2000). However, V_C is affected by several other parameters, such as pH of the media, ionic strength, and the presence of polysaccharides, membrane-derived oligosaccharides or polycations (Delcor 1997), as well as the charges present in the porin channels from specific charged residues (Koebnik *et al* 2000). General diffusion porins discriminate between solutes primarily on their size and charge; they are present in sufficient quantities that can be readily measured by polyacrylamide gel electrophoresis (Nikaido and Vaara 1985). On the other hand, Type II channels e.g OprD (minor outer membrane proteins or specific porins) are usually specific porins that are found in low concentrations. However, when induced the concentration of specific porins might reach above the concentration of normal protein constituents of the outer membrane (Nikaido *et al* 1991). Specific porins allow the diffusion of specific substrates that are larger than 600 D, but they also allow non-specific diffusion of small substrates (Braun 1994). Type II channels are homotrimers, each monomer have 18 antiparallel strands that forms a barrel with the third loop folding back inside the barrel forming a constriction. Most of the channel lining residues are conserved between different specific porins and between species but there are three positions at the constriction site that shows differences in amino acid sequence, which was recently found to determine the specificity for each channel (Nikaido and Vaara 1985; Koebnik *et al* 2000). Type III channels (e.g. TonB-dependent receptors), are proteins that are essential for uptake of large molecules (e.g. Siderophore-iron complex) (Braun 1994). The activity of these porins is dependent on the electrochemical potential of the cytoplasmic membrane and the protein complex TonB-ExbBD that is an energy transducer. Ferguson *et al* (1998) and Buchanan *et al* (1999) studied the Fhu-A receptor protein for iron-siderophore uptake in *E. coli*. They found that these

receptors have a plug-and-barrel organization. It is made of 22–stranded β-barrel and an N-terminal plug domain (cork) that is located inside the barrel. The cork domain is arranged in the barrel with the β-sheet plane inclined by ~ 45º to the membrane normal, therefore obstructing the channel interior. The presence of the cork domain suggests that the direct passage of the ferrichrome-iron complex and small molecules is not possible.The cork domain is connected to the barrell inner wall by extensive hydrogen bonding, and it delineats a pair of pockets within the Fhu A, a larger external pocket that opens to the external environment and a smaller periplasmic pocket. Transport of the ferrichrome-iron complex involves the primary binding to the Fhu-A receptor followed by translocation through the channel (Ferguson *et al* 1998). The opening of the channel is influenced by the energy transduced by the TonB system in the cytoplasmic membrane. Outer membrane proteins are of particular interest due to their involvement in transport of harmful substances and in anchoring structures that mediate adhesion and motility. Recently, *P. aeruginosa* genome was published and three large paralogous families of outer membrane proteins have been identified (Stover *et al* 2000). The OprD family of specific porins (19 genes), the TonB-family of gated porins and the OprM family of outer membrane proteins involved in efflux or secretion (18 genes) (Stover *et al* 2000).

2.3 Virulence factors:

The virulence of *P. aeruginosa* results from the interaction of many factors some of which are coupled with the cells while others are secreted extracellulary These factors play an important role in the colonization, survival and tissue invasion of the bacteria (Table 1).

Table 1. Main *P. aeruginosa* virulence factors (Anis Ben Haj Khalifa, *el al,* 2011)

Virulence factors	Mechanism of virulence	Pathogenic effect induced
lipopolysaccharide (LPS)	Stimulation of cytokine production	Shock
Pili	Adherence to respiratory epithelial cells	Pathogenicity
Flagellum	Adherence to mucins Mobility: role in the internalization	bacterial diffusion
Alginate	Alginate Causes mucous phenotype Adherence to tracheal cells Inhibition of phagocytosis, the action of antibiotics and immune response	Respiratory pathogenicity Resistance to host defenses (phagocytosis) and antibiotics Head of character mucoid strains
Exotoxin A	Inhibition of protein synthesis in target cells	Cell death: tissue necrosis Important role in virulence
Exoenzyme S	Cytotoxic Effect Proliferation of LT	Tissue necrosis Damages the glycopeptide, the vimentin and IgG and IgA
Exoenzyme U	Antiphagocytic role	Lesions of epithelial cells Bacteraemia responsible or shock Septic
Rhamnolipid Elastase (+ lasB LASA)	Detergent effect Degradation of elastin, fibrin, interferon, complement and collagen	Hydrolysis of the surfactant Destruction of elastin-containing tissues Important role in virulence
Alkaline protease	Proteolysis	Role in corneal infections
Pyocyanine + Pyoverdine	Bactericidal action on other bacteria Increase in elastase release Inhibition of cilia beat Iron uptake Induce the synthesis of free radicals	Promotes the emergence of *Pseudomonas aeruginosa* Decreased clearance of bacilli Role in the occurrence of arterial vasculitis in lung.
Soluble lectins	Inhibition ciliary beat lung cells	respiratory pathogenicity Role in chronic infection
Phospholipase C	Local cytolytic effect	Lysis of target cells (pulmonary atelectasis) Role in acute and chronic infection.

3. 2-Phenoxyethanol:

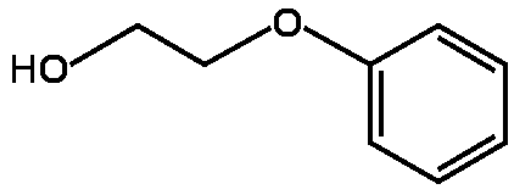


Figure 4: Structure of 2-Phenoxyethanol

2-phenoxyethanol is an oily material produced naturally by the male rabbits and synthetically by the reaction between phenol and ethylene oxide. It is used as general antiseptic against wide range of bacteria (including *P. aeruginosa)*. It is used as a bladder irrigant, in the treatment of burns, in the formulation of pharmaceuticals, insecticides and cosmetics, as anesthetic, as a fixative material in the perfumes and as a solvent for inks (Tsantilas *et al., 2006*).

4. Sterillium® and Septoderm®:

Sterillium® and Septoderm® are propanol based biocides (Sterillium® 85% w/w, Septoderm® 63% w/w) where Sterillium® is composed of (Propan-2-ol, propan-1-ol, macetromiumetilsulphate, tetradecan-1-ol and Glycerol) and Septoderm® (2-propanol, 1,3-butanediol and oxythylene), currently used in hospitals, ambulances and home care. They have bacteriocidal, yeasticidal, tuberculocidal, mycobactericidal, virucidal activity against enveloped viruses (including HBV, HIV and HCV) and adeno, polyoma and retroviruses activity. They show hygienic disinfection activity on hands within 30 seconds and surgical disinfection activity within 1.5 minutes.

Aim

To investigate:

a- Investigating the **mechanisms** of **retrieving** antibiotic sensitivity in Multi-Drug Resistant strains of *P. aeruginosa* after 2-PE adaptation.

b- Investigating the possibility of MDR strains adaptation to other hospital used biocides.

Chapter Two

Materials and methods

1. Materials:

1.1 Antibiotics

The following antibiotic discs were obtained from Oxoid (Germany): Amoxacillin (25 µg/ml), Ampicillin (10 µg/ml), Azithromycin (15 µg/ml) Aztreonam (30 µg/ml), Cefoxitin (10 µg/ml), Ceftazidime (30 µg/ml), Ceftizoxime (30 µg/ml), Cepfalexin (30 µg/ml), Chloramphenicol (30 µg/ml), Ciprofloxacin (5 µg/ml), Doxycyclin (30 µg/ml), Erythromycin (15 µg/ml), Gentamicin (10 µg/ml), Imipenem (10 µg/ml), Levofloxacin (10 µg/ml), Minocyclin (30 µg/ml), Nafcillin (1 µg/ml), Norfloxacin (10 µg/ml), Ofoxacin (5 µg/ml), Piperacillin (100 µg/ml), Rifambicin (5 µg/ml), Sulphamethoxazole 25 µg/ml), Tetracycline (10 µg/ml), Ticar/clav (85 µg/ml), Tobramycin (10 µg/ml) and Vancomycin (30 µg/ml).

1.2 Bacteriological Media

Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA) and Motility test media (Himedia, India).

1.3 Chemicals

2-Phenoxyethanol, dimethyl formamide (DMF), Ethidium Bromide, Acrylamide/Bis-acrylamide, 30% solution (37.5:1), ammonium persulfate, TEMED, sarcosine, Bovine Serum Albumin BSA Folin reagent and molecular weight marker number S8445 (Wide range 6,500-20,000 Da) were obtained from Sigma® Eldrich. 2-PE was prepared fresh by dissolving it in DMF to make a stock solution of 2.560 mg/ml and kept in a cool dark place.

1.4 Phosphate buffer saline (PBS)

PBS was prepared by dissolving: 8g of sodium chloride (NaCl), 0.2g potassium chloride (KCl), 1.4g disodium phosphate (Na_2HPO_4) and 0.24g of potassium dihydrogen phosphate (H_2KO_4P) in 1 L of distilled water.

1.5 Bacterial strains:

Four MDR strains of *Pseudomonas aeruginosa* designated as MDR1, 2, 3 and 4 all four strains were obtained from King Hussein Cancer Center (KHCC) and wild type *P. aeruginosa* PAO1 strain and *ppk null* mutants of *P. aeruginosa* PAO1 strains were the kind gift from Prof. Mike Brown (UK). All strains were identified at source. Additional identification using cultural characteristics (tear like colonies, pigmentation on nutrient agar) and biochemical reactions (growth on TSI media, Oxidase test) and Gram staining (Gram negative short rods) were also performed. All strains were maintained by sub-culturing on Nutrient agar (Himedia, India) and backup cultures were prepared. Strains were also preserved in 10% glycerol at -70°C.

2. Methods

2.1 Adjusting inoculums size

The inoculum size of all fresh overnight culture strains of *P. aeruginosa* were adjusted at an optical density of 0.7 at 470 nm (10^6 CFU/ml) (Brozel & Cloete 1994.)

2.2 Growth characteristics of strains:

Changes in cultural characteristics (pigmentation, growth density and mucoidy) .of different strains during the passage experiments were observed and recorded.

2.3 Biocides Minimal Inhibitory Concentration (MIC) Determination

Macrodilution method for MIC determination was used (Bloomfield, 1991). Briefly, aliquots of the three biocides (**2-PE, Sterillium® and Septoderm®)** were added to 9 ml of sterile MHB to yield descending concentrations. 0.1 ml bacterial culture at an inoculum size adjusted as previously mentioned was added to all tubes. Tubes were incubated for 24 hours at 37℃. MIC was determined as the lowest concentration of the three biocides that inhibits bacterial growth as seen byloss of visual turbidity.

2.4 Adaptation of *P. aeruginosa* to 2-phenoxyethanol

An aliquot of (250 µL) of 24 hrs. culture was inoculated into 25 ml of fresh MHB containing MIC/4 of 2-PE determined for each strain and incubated in a shaking incubator (Brunswick / USA) at 37°C for 24 hrs at 180 rpm (Passage 1) (Brözel and Cloete 1993). At the end of the incubation period MIC of Passage 1 was determined. These steps were repeated for several passages until 2 folds increase in MIC was obtained.

2.5 Adaptation of P. aeruginosa to Sterillium® and Septoderm®

An aliquot of (250 µL) of bacterial culture obtained from fresh cultures were inoculated into 25 ml of fresh MHB media containing MIC/4 of Sterillium® and Septoderm® determined for each strain and incubated in a shaking incubator (Brunswick / USA) at 37°C for 24 hrs at 180 rpm (Passage 1) (Brözel and Cloete 1993). When growth became, swabs from tubes where cultured to check if there is growth in the test tubes.

2.6 Antibiotic susceptibility testing

2-Phenoxyethanol

The effect of 2-PE adaptation on antibiotic susceptibility was detected using the disc diffusion. Briefly the inoculum size of a 24hrs fresh culture of each *P. aeruginosa* (MDR 2-PE adapted and MDR non 2-PE adapted, PPK mutant, PAO1) was adjusted at O.D. of 0.7 at 470 nm wavelength. Bacteria were then seeded on a MHA plate surface using a sterile swab and antibiotic discs were applied. Plates were incubated at 37°C for 24 hrs. Diameter of zones of inhibition around each disc were measured in mm. Each experiment was repeated three times.

Sterillium® and Septoderm® exposed cells were plated from MIC tubes and antibiotic susceptibility was determined for the unpassaged cells using disc diffusion method.

2.7 Motility testing:

The flagellum-mediated motility of 2-PE adapted and non-adapted strains of MDR *P. aeruginosa* was assessed by inoculation of bacterial colonies as spots on a motility test media plates for 12 hrs. at 30°C.

Migration of the cells from the point of inoculation (observed as a turbid zone) was an indicator for motility (loss of motility is indicated for by absence of turbidity zone).

Results indicate change in the zone of turbidity around the inoculation points.

2.8 Outer membrane preparation and SDS-PAGE

2.8.1 Outer membrane Preparation

Outer membranes of 2-PE adapted and non-adapted strains were prepared according to **(**Pugsely *et al*., 1986**),** ***briefly*** 24 hr cultures were washed three times in PBS (each 20 minutes at 5000 rpm (HERMLE/ Germany). Supernatants were thrown and final pellet was resuspended in 2 ml of fresh PBS and homogenized using a hand homogenizer (Ultra Turrax / Gremany). Homogenization was carried for 2.5 minutes on crushed ice at separate intervals of 30 sec with cooling intervals for 10 sec in ice and the probe was sterilized with methanol between samples. Homogenized samples were centrifuged for 1 hour at 13000 rpm at 4°C and supernatants collected and labeled as "C" which refers to the Cytosol of the bacterial cells. Pellets were mixed with 1mL of (0.5 ml Tris buffer + 0.5 ml of sarcosine) and left for 20 minutes in ice.

The previous mixture was centrifuged for 1 hour at 13000 rpm at 4°C.

Supernatants of the inner membrane were collected and pellets were re-suspended in 1ml of distilled water and centrifuged for 1 hour at 13000 rpm at 4°C. Supernatant was discarded and the remaining part of the cells labeled as "O" which refers to the outer membrane of the bacterial cells.

Outer membranes stored at -20°C were used for analysis by SDS-PAGE.

2.8.2 Outer membrane Protein estimation

- This experiment was performed to measure the protein content in the prepared outer membranes using Lowry's method (Lowry *et al.,* 1951) briefly:

Reagents used

1. BSA stock solution (1mg/ml) in distilled water.

2. Analytical reagents: (a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water.) (b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution. Analytical reagents were prepared by mixing 2 ml of (b) with 100 ml of (a).

3. Folin - Ciocalteau reagent solution (1N) commercial reagent (2N) was diluted with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml distilled water).

Procedure

- Different dilutions of BSA solutions were prepared by mixing aliquots from the BSA stock solution (1 mg/ ml) and different volumes of water in the test tube to achieve BSA concentration range between 0.05 to 1 mg/ ml in a total volume of 5 ml.
- 2 ml of alkaline copper sulphate reagent was added (analytical reagent) to 0.2 ml of protein solutions and mixed well and incubated at room temperature for 10 mins.
- 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) was added to each tube and incubated for 30 min at room temperature. The absorbance was measured using a spectrophotometer that was calibrated with blank (D. W.) and at optical density of 660 nm.
- Optical density at 600nm was plotted against BSA concentration.
- The absorbance of bacterial outer membranes were measured and the concentrations of the proteins in the samples were determined using the standard curve.

2.8.3 SDS-PAGE

Preparation of gels

- Stacking gel is prepared by mixing 750 µL of Acrylamide/Bis-acrylamide30% with 3ml of D.W., 1.5 ml of stacking buffer (1g of SDS with 15.1 g of Tris base and the pH adjusted to 6.8 using HCL), 15 µL of ammonium persulfate (10%) then 5 µL of TEMED just before pouring the gel.
- Separating gel is prepared by mixing 10 µL of Acrylamide/Bis-acrylamide with 12 ml of D. W., 7.5 ml of stacking buffer (1g of SDS with 45.5 g of Tris base and the pH adjusted to 8 using NaOH), 45 µL ammonium persulfate and then 15 µL of TEMED just before pouring the gel.

Pouring of the gel:

***Separating gel*:**

- The separating gel ingredients were mixed in a beaker and it was ensured that there were no air bubbles, and then the solution poured between the two glass plates of the electrophoresis apparatus that are held in clippers with spacers between the two plates.
- Immediately after this step a layer of Butanol in water is placed over the gel layer in order to get a smooth horizontal level of the gel.
- Then the gel is left to solidify in an incubator where the solidification is faster with increased temperature.
- The Butanol in water layer is removed using a syringe and the gel surface washed with water for 3-5 times.

***Stacking gel*:**

-The ingredient were mixed and poured over the surface of the separation gel

-A comb with suitable thickness is placed between the two glass plates for a depth of 1cm from the upper edges of the plates to make holes in the stacking gel where the samples were placed.

-The gel is left to solidify.

Preparation of samples for loading on gels

1. 50 µl of samples mixed with 100 µl of cracking buffer (prepared by mixing 3.75 mL of Tris of 0.5 HCL, 1.5 mL mercaptoethanol, 0.6 SDS, 3g glycerol and 1 mL bromophenol blue at pH of 6.8 without adjustment.
2. The mixture is boiled for 5 minutes in a water bath and left for cooling.
3. The Glass plates with gels between them placed an a tank of reservoir buffer (prepared by mixing 3 g of Tris Base and 2 g SDS and the pH supposed to be 8.3 without adjustment).
4. Samples added in wells in stacking gel using special tips.
5. Gels were connected to the power pack (Biorad/USA) at 70 mA for 1.5 hour or until the blue front reach the bottom of the gel.

Coomassie blue staining:

Gels were stained using coomassie blue solution (0.25 g dissolved in 125 ml methanol and 125 ml glacial acetic acid and 100 ml D.W.) and left for 1-8 hours on shaking platform (Stuart/UK) and de-stained (100 ml methanol, 100 ml glacial acetic acid and 800 ml of D.W.) and left until the bands appeared clearly.

2.9 Ethidium bromide accumulation assay:

The role of 2-PE as an efflux inhibitor was investigated using ethidium bromide accumulation assay which is modified to suit the conditions of the experiment briefly (Li *et al.*, 2003):

1. Bacterial cultures were grown overnight in MHB at 37°C.
2. Inoculum size was adjusted to 0.8 - 1 at 600nm.
3. 10 µL of (1mg/1ml) of ethidium bromide added.
4. Bacterial cells were incubated for 30 minutes at 37°C.
5. Cells were centrifuged at 5000 g for 10 minutes at room temperature.
6. Cells washed with 50 mM of Sodium Phosphate Buffer (PH= 7.2) containing (100 mM NaCl and 0.1% (vol/vol) glycerol) and re-suspended in the same buffer at O.D = 600 of 0.5-1.
7. The fluorescence of EthBr that interchelated with bacterial DNA was measured at zero time and after 30 minutes without intervals at an excitation wavelength of 520 and emission wavelength of 590 nm with slit width of 5 nm for excitation and 10 nm for emission in a spectroflorometer (Glomax®/ USA).

2.10 Data analysis

Differences in zones of inhibition between 2-PE MDR adapted and non-adapted strains as well as PAO1 and *ppk null* mutants were subjected to t test to check for significance ($p \leq 0.05$) t test used to check the significance of results using Microsoft Excel software to compare between the adapted and non-adapted strains as well as between *ppk null* mutant strains and wild type *P. aeruginosa* PAO1. P value less than 0.05 considered as significant.

Chapter Three

Results

1. Results

1.1 Observational Changes in cultural characteristics upon 2-PE adaptation

Four MDR *P. aeruginosa* strains were used in this study, MDR 1 and 2 secreted green pigment (pyocyanin). As 2-PE MIC increased during the passage experiments this pigment disappeared. Strains 3 and 4 secreted yellow green pigment (Pyoveredine) (Prasad *et al*., 2014) that did not disappear during adaptation experiments. As strains proceeded in the passage experiments mucoid type of growth was observed. There was also decrease in the growth density of bacteria and this was observed by the difficulty in achieving the desired inoculum size.

1.2 Adaptation to biocides

All strains adapted to 2-PE with an increase in MIC. MIC of 2-PE for PAO1 was 16 µg/ml, while the MIC of that biocide for MDR strains was higher (24 µg/ml for both MDR1 and MDR2and 26 µg/ml for both (MDR3 and MDR4) (Figure 5), all stains went through four passages to reach the mentioned MICs (Figure 6). On the other hand none of the strains was able to adapt to the commercially used biocides sterillium® nor to septoderm®.

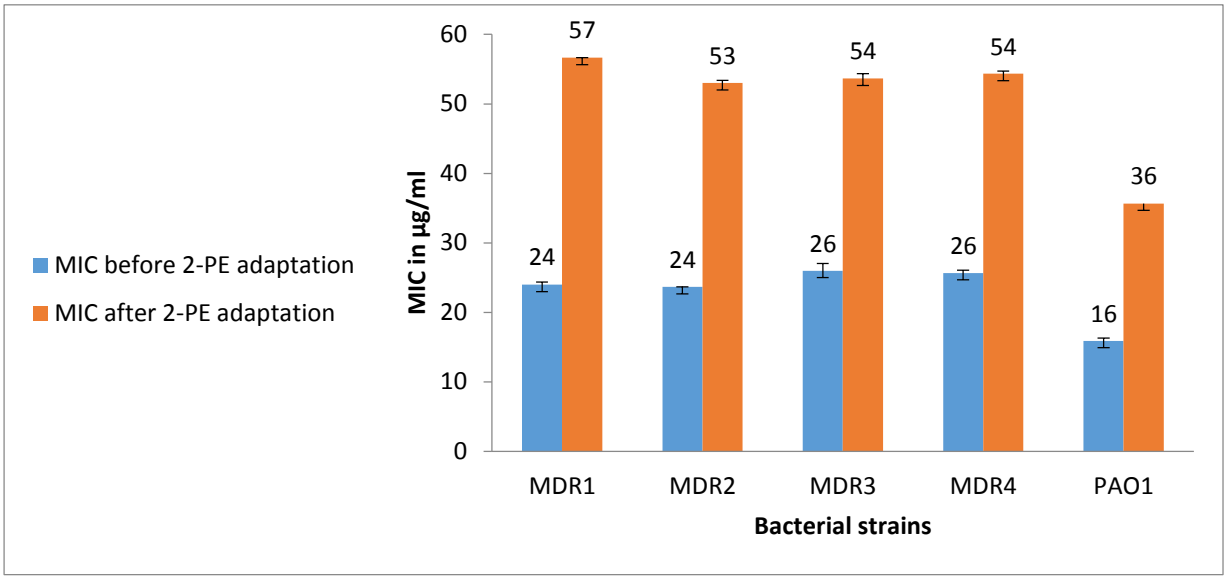
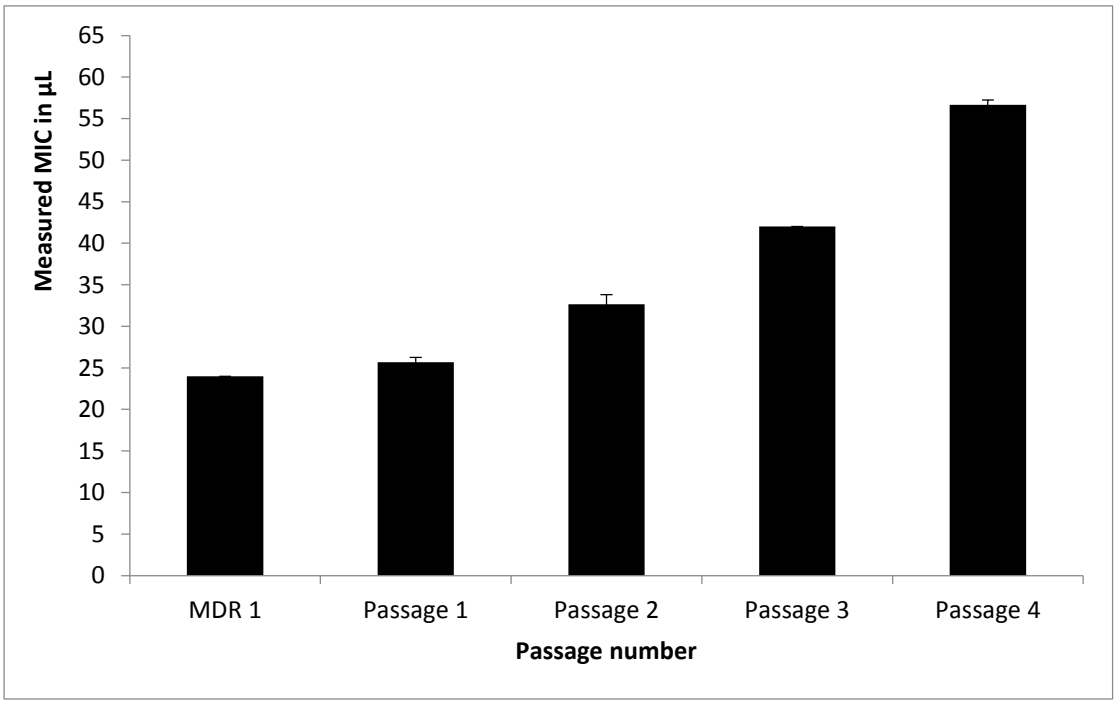
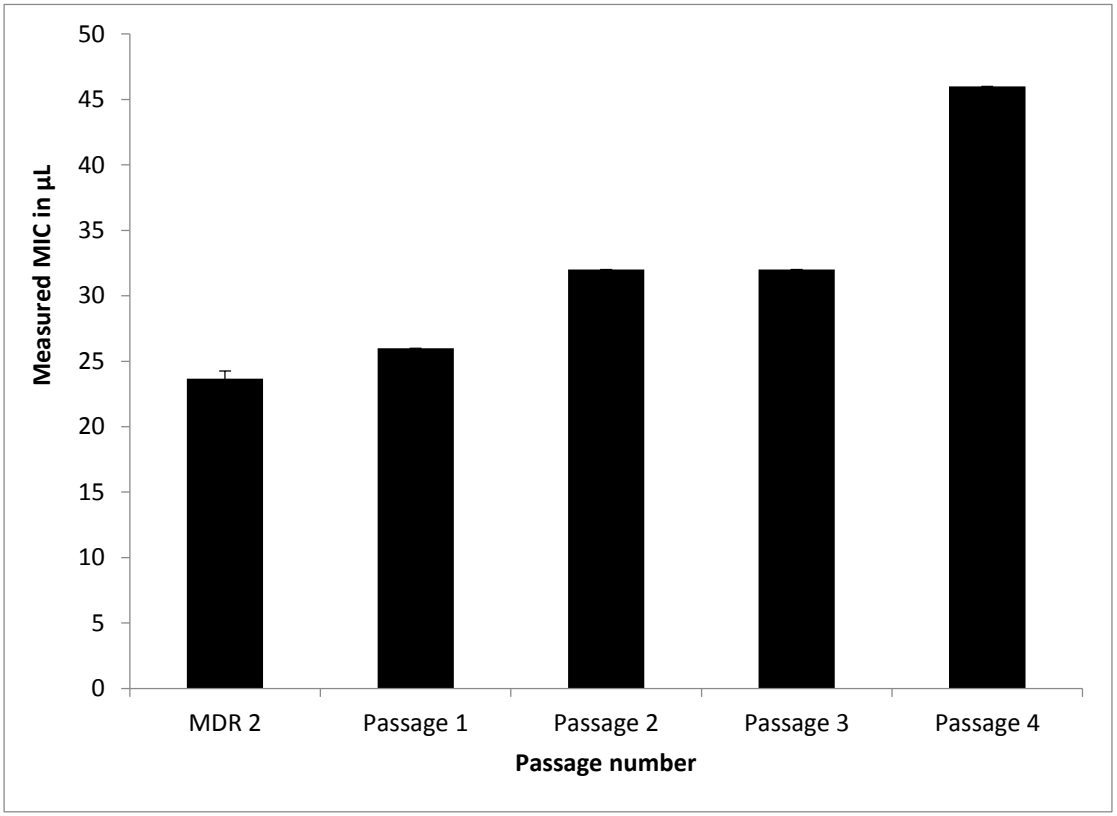


Figure **5: Minimal inhibitory concentration of 2-PE on MDR strains before and after adaptation**. Different concentrations of 2-PE were added to MHB tubes and a fixed inoculums size of *P. aeruginosa* Strains (PAO1 and MDR) of OD=0.7 at 470 nm were then added and incubated for 24 hrs. at 37°C. MIC is the lowest concentration of 2-PE that inhibited growth as seen by loss of visual turbidity.

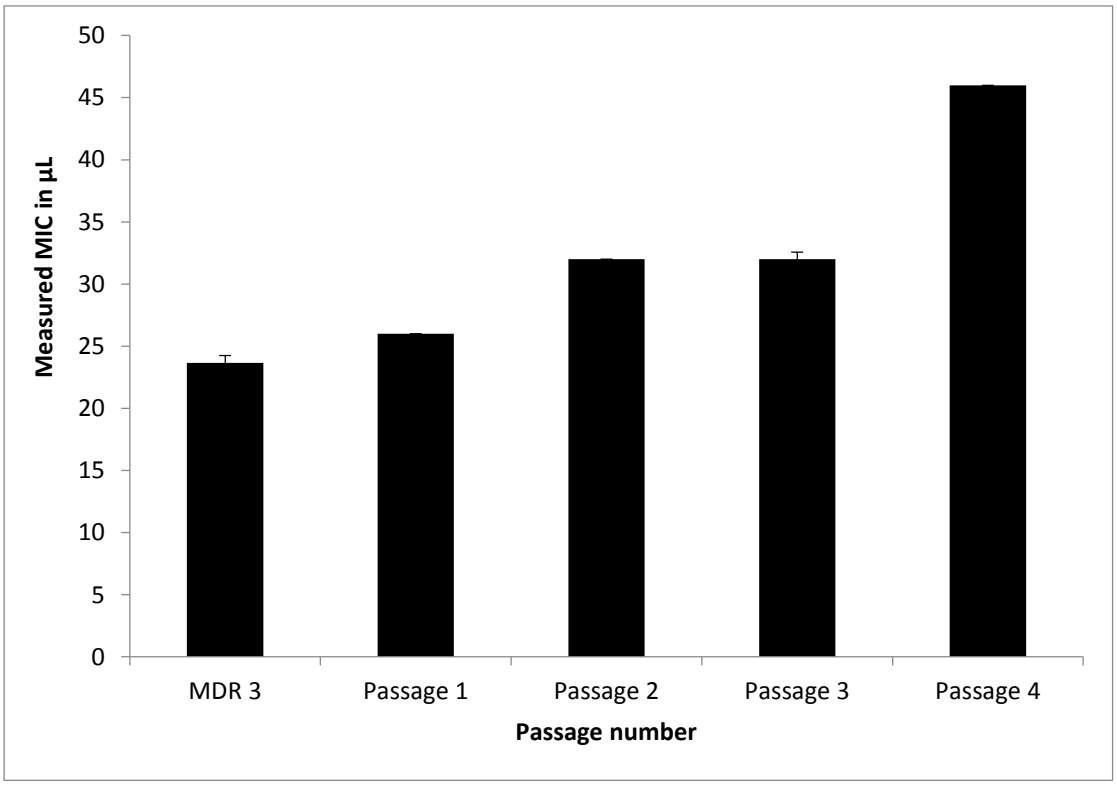
A)



B)


50
45
40
35
30
25
20
15
10
5
0
Measured MIC in µL
MDR 2
Passage 1
Passage 2
Passage 3
Passage 4
Passage number

C)


50
45
40
35
30
25
20
15
10
5
0
Measured MIC in µL
MDR 3
Passage 1
Passage 2
Passage 3
Passage 4
Passage number

D)

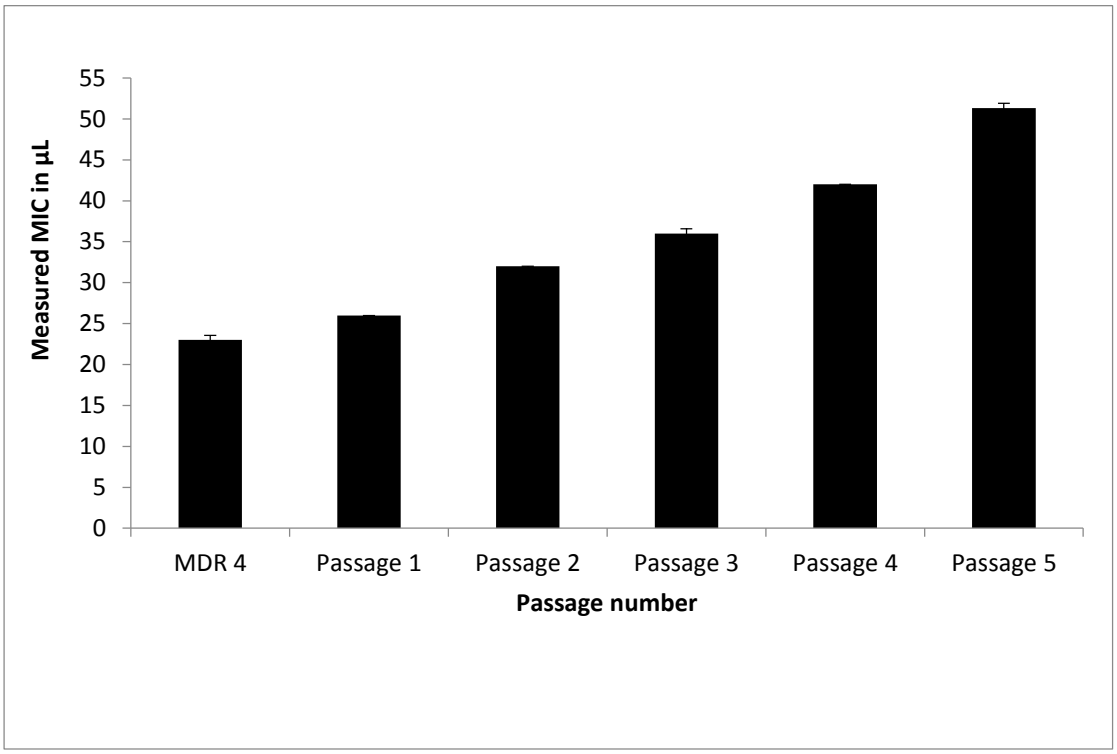


Figure 6: Minimum inhibitory concentrations changes during passage experiment for A: MDR1 B: MDR2 C: MDR3 D: MRD4.

1.3 Antibiotic susceptibility

When 2-PE adapted strains were tested for their antibiotic susceptibility MDR1 showed significant changes in the antibiotic susceptibility at MIC of 2-PE of 57 µg/ml. ($P< 0.05$) (Table 2a, 2b). MDR 2 showed significant changes in the susceptibility to antibiotics at MIC of 2-PE 53 µg/ml. ($P< 0.05$). On the other hand adaptation of strain MDR3 and MDR4 to 2-PE did not result in improvement in antibiotic susceptibility (Table 2c and 2d).

antibiotic susceptibility tests have revealed retrieval of antibiotic sensitivity in strains 1,2 towards Amoxacillin (25 µg/ml), Ampicillin (10 µg/ml), Chloramphenicol (30 µg/ml), Cepfalexin (30 µg/ml), Erythromycin (15 µg/ml), Rifambicin (5 µg/ml), Sulphamethoxazole (25 µg/ml), Vancomycin (30 µg/ml) and increase in the zone of

inhibition of Azithromycin (15 µg/ml), Gentamicin (10 µg/ml), Doxycyclin (30 µg/ml), Imipenem (10 µg/ml), Minocyclin (30 µg/ml), Norfloxacin (10 µg/ml), Ofloxacin (5 µg/ml), Piperacillin (100 µg/ml), while the adapted bacteria showed no improvement in susceptibility tawards Cefoxitin (10 µg/ml) ,Nafcillin (1 µg/ml) and Tobramycin (10 µg/ml). There was a decrease in the susceptibility toward Aztreonam (30 µg/ml), Ceftazidime (30 µg/ml), Ciprofloxacin (5 µg/ml), Levofloxacin (10 µg/ml), Tetracycline (10 µg/ml), Ticarcillin/clavulinic acid (85 µg/ml) and Ceftizoxime (30 µg/ml).

Table 2: Antibiotic susceptibility of MDR strain before and after adaptation with 2-PE.

A)

Antibiotic (Concentration in µg/ml)	Zone of inhibition in (mm) (± Standard error)	
	MDR 1	MDR 1 Adapted
AML	R	2.5 (±0.1)
AMP	R	3 (±0.1)
ATM	2.5 (±0)	R
AZM	1.2 (±0.1)	1.8 (±0.1)
C	R	3.5 (±0.1)
CAZ	2.3 (±0.1)	2 (±0.1)
CIP	3 (±0.1)	2.5 (±0.1)
CL	R	2.5 (±0)
CN	2 (±0.1)	3.2 (±0.1)
DO	1 (±0.1)	4 (±0.1)
E	0.5 (±0.1)	0.5 (±0.1)
FOX	R	R
IPM	2 (±0.1)	3.5 (±0.1)
LEV	2.5 (±0.1)	2 (±0.1)
MH	1.2 (±0.1)	2.3 (±0.1)
NF	R	R
NOR	2.5 (±0.1)	2.7 (±0.1)
OFX	2.3 (±0.1)	4 (±0.1)
PRL	1.5 (±0.1)	3 (±0.1)
RD	R	3 (±0.1)
SXT	R	3.5 (±0.1)
TE	1.2 (±0.1)	0.5 (±0.1)
TIM	1.8 (±0.1)	1 (±0.1)
TOB	1.5 (±0.1)	1.5 (±0.1)
VA	R	2.5 (±0.1)
ZOX	2.7 (±0.1)	2 (±0.1)

B)

Antibiotic (Concentration in µg/ml)	Zone of inhibition in (mm) (± Standard error)	
	MDR 2	MDR 2 Adapted
AML	R	2 (±0.1)
AMP	R	1.3 (±0.1)
ATM	0.8 (±0.1)	R
AZM	R	1 (±0.1)
C	R	2 (±0.1)
CAZ	R	1.5 (±0.1)
CIP	0.5 (±0.1)	1.5 (±0.1)
CL	R	2 (±0.1)
CN	R	2 (±0.1)
DO	R	1.5 (±0.1)
E	R	R
FOX	R	R
IPM	R	1 (±0.1)
LEV	R	1 (±0.1)
MH	R	0.7 (±0.1)
NF	R	R
NOR	R	R
OFX	R	2.5 (±0.1)
PRL	0.5 (±0.1)	1 (±0.1)
RD	0.5 (±0.1)	1 (±0.1)
SXT	2.2 (±0.1)	2.5 (±0.1)
TE	R	0.7 (±0)
TIM	R	1 (±0)
TOB	0.5 (±0.1)	0.7 (±0.1)
VA	R	1 (±0.1)
ZOX	2 (±0.1)	2 (±0.1)

C)

Antibiotic (Concentration in µg/ml)	Zone of inhibition in (mm) (± Standard error)	
	MDR 3	MDR 3 Adapted
AML	R	R
AMP	R	R
ATM	R	R
AZM	2 (±0.1)	2.2 (±0.1)
C	0.5 (±0.1)	0.5 (±0.1)
CAZ	R	R
CIP	R	R
CL	R	R
CN	R	R
DO	R	R
E	1	R
FOX	R	R
IPM	0.5 (±0.1)	0.5 (±0.1)
LEV	R	R
MH	R	R
NF	R	R
NOR	R	R
OFX	R	R
PRL	0.7 (±0.1)	0.7 (±0.1)
RD	R	R
SXT	R	R
TE	R	R
TIM	R	0.5 (±0.1)
TOB	R	R
VA	R	R
ZOX	R	R

D)

Antibiotic (Concentration in µg/ml)	Zone of inhibition in (mm) (± Standard error)	
	MDR 4	MDR 4 Adapted
AML	R	R
AMP	R	R
ATM	R	R
AZM	R	R
C	R	R
CAZ	R	R
CIP	R	R
CL	R	R
CN	R	R
DO	R	R
E	1.5 (±0.1)	1.5 (±0.1)
FOX	R	R
IPM	R	R
LEV	R	R
MH	R	R
NF	R	R
NOR	R	R
OFX	R	R
PRL	R	R
RD	R	R
SXT	R	R
TE	R	R
TIM	0.7 (±0.1)	0.7 (±0.1)
TOB	R	R
VA	R	R
ZOX	R	R

Table 2: **Antibiotic susceptibility of MDR strain before and after the adaptation with 2-PE.** A: MDR1 B: MDR2 C: MRD3 D: MDR4 of an OD 0.7 at 470 nm were grown in MHB and incubated for 24 hrs at 37°C then sterile swabs used for plating MHA and discs of antibiotics were applied to each plate and incubated for 24 hrs at 37°c. Next day, zones of inhibition were measured. Results represent the average of three experiments. R means no zone of inhibition.

After exposure of these MDR strains to the two biocides in the MIC experiment, disc diffusion test was performed and improved sensitivity (increased size of zone of inhibition) towards some antibiotics in comparison to the non-exposed MDR strains (Table 3).

Table 3: Zone of inhibition of antibiotics for MDR, Sterillium® exposed MDR and Septoderm® exposed MDR.

Antibiotics	Zone of inhibition(mm) (± Standard error)		
	MDR	**MDR exposed to Sterillium®**	**MDR exposed Septoderm®**
AML	**R***	**R**	**R**
AMP	**R**	**R**	**R**
ATM	**24 (±0.1)**	**39 (±0.1)**	**26 (±0.1)**
AZM	**12 (±0)**	**21 (±0.1)**	**21 (±0.1)**
C	**R**	**R**	**R**
CAZ	**24 (±0.1)**	**35 (±0.1)**	**26 (±0.1)**
CIP	**3 (±0.1)**	**4 (±0)**	**3 (±0.1)**
CL	**R**	**R**	**R**
CN	**21 (±0.1)**	**24 (±0.1)**	**2 (±0)**
DO	**12 (±0.1)**	**24 (±0.1)**	**1.4 (±0.1)**
E	**5 (±0)**	**6 (±0.1)**	**5 (±0.1)**
FOX	**R**	**R**	**R**
IPM	**2**	**31**	**25**
LEV	**26 (±0.1)**	**4 (±0.1)**	**3 (±0.1)**
MH	**12 (±0)**	**21 (±0.1)**	**15 (±0)**
NF	**R**	**R**	**R**
NOR	**24 (±0.1)**	**39 (±0.1)**	**32 (±0.1)**
OFX	**23 (±0.1)**	**35 (±0)**	**31 (±0.1)**
PRL	**15 (±0.1)**	**26 (±0.1)**	**16 (±0.1)**
RD	**R**	**R**	**R**
SXT	**R**	**R**	**R**
TE	**12 (±0.1)**	**22 (±0.1)**	**11 (±0.1)**
TIM	**18 (±0)**	**25 (±0.1)**	**21 (±0.1)**
TOB	**15 (±0.1)**	**25 (±0)**	**21 (±0.1)**
VA	**R**	**R**	**R**
ZOX	**28 (±0)**	**43 (±0.3)**	**31 (±0.1)**

Table 3: Zone of inhibition of antibiotics for MDR, Sterillium® exposed MDR and Septoderm® exposed MDR. Strains grown in MHB for MIC experiment and incubated for 24 hrs at 37°C were

seeded on MHA and antibiotic discs were applied to each plate and incubated for 24 hrs at 37°c. Zones of inhibition were measured in mm. R: resistant.

1.4 Polyphosphate Kinase activity:

The loss of PPK activity in *P.aeruginosa* results in loss of motility and virulence (Rashid *et al*., 2000a). To check if improved antibiotic susceptibility was due to loss of PPK activity two approaches were made:

1.4.1 Motility testing :

The first approach was the motility test where MDR adapted and non-adapted strains were stab cultured in motility test tubes. The diameter of growth that represents motility from the stab line was measured and compared to those of non 2-PE adapted strains (Rashid *et al*., 2000b).

No comparable changes in the diameter of 2-PE adapted and non-adapted strains were observed.

1.4.2 Antibiotic susceptibility of *ppk null* mutant

It is known that poly phosphate kinase deficiency renders bacteria vulnerable and results in loss of virulence. It was suspected that the increased antibiotic susceptibility as a consequence to 2-phenoxyethanol adaptation could be a result of inhibition of PPK activity by 2-PE. To test this possibility a *ppk* deficient mutant was used to mimic the 2-PE adapted strain. Antibiotic susceptibility testing for the *ppk null* mutant showed no change in sensitivity to antibiotics in comparison to the wild type PAO1 strain Table 4.

Table 4: Antibiotic susceptibility of PAO1 and *ppk null* mutant.

Antibiotic	Wild type PAO1	*ppk null* mutant
AML	R	R
AMP	R	R
ATM	22 (±0.1)	25 (±0.1)
C	11 (±0.1)	12 (±0.1)
CFM	R	R
CIP	24 (±0.1)	20 (±0.1)
CL	R	R
CN	12 (±0.1)	20 (±0.1)
DO	R	R
NOR	20 (±0.1)	22 (±0.1)
OFX	12 (±0.1)	12 (±0.1)
PRL	7 (±0.1)	10 (±0.1)
RD	R	R
SXT	R	R
VA	R	R

Table 4: Antibiotic susceptibility of PAO1 and *ppk null* mutant strain. Bacteria from both strains were grown in MHB and incubated for 24 hrs at 37°C then sterile swabs used for plating MHA and discs of antibiotics were applied to each plate and incubated for 24 hrs at 37°c. Next day, zones of inhibition were measured. Results represent the average of three experiments. R means no zone of inhibition.

1.5 Outer membrane alterations

Biocide adaptation in bacteria causes outer membrane alterations (Abdelmalek and Bardan, 2010) Changes in antibiotic susceptibility in bacteria has been attributed to outer membrane alterations. Outer membranes profiles of 2-PE adapted and non-adapted MDR *P.aeruginosa* were prepared using SDS-PAGE and compared.

1.5.1 Protein estimation:

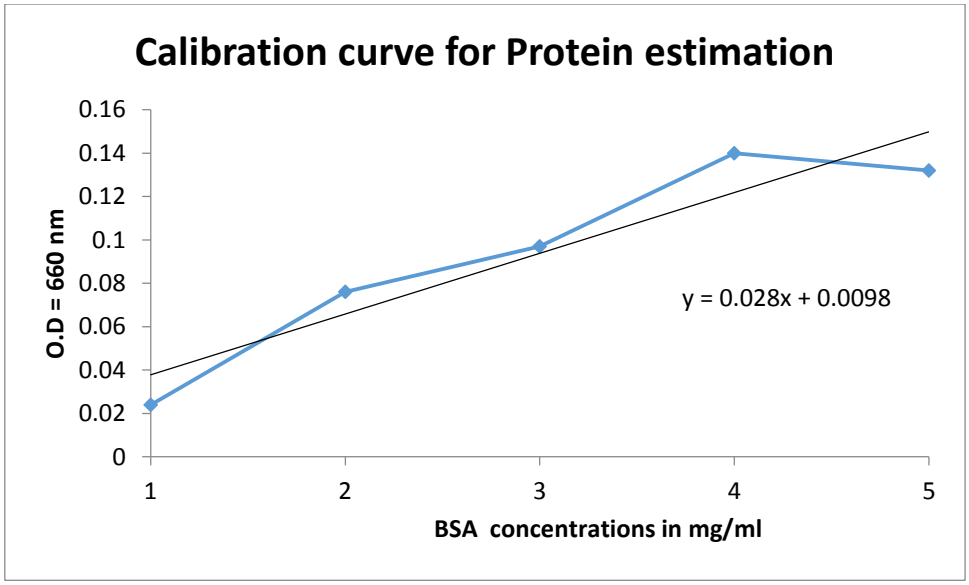


Figure 7: Protein estimation standard curve (Lowry's method).

1.5.2 SDS-PAGE

The gel electrophoresis of the outer membranes of different strains is presented in Figures 8 and 9. Analysis of the gels showed alterations in the protein bands that are summarized in table 5 and 6.

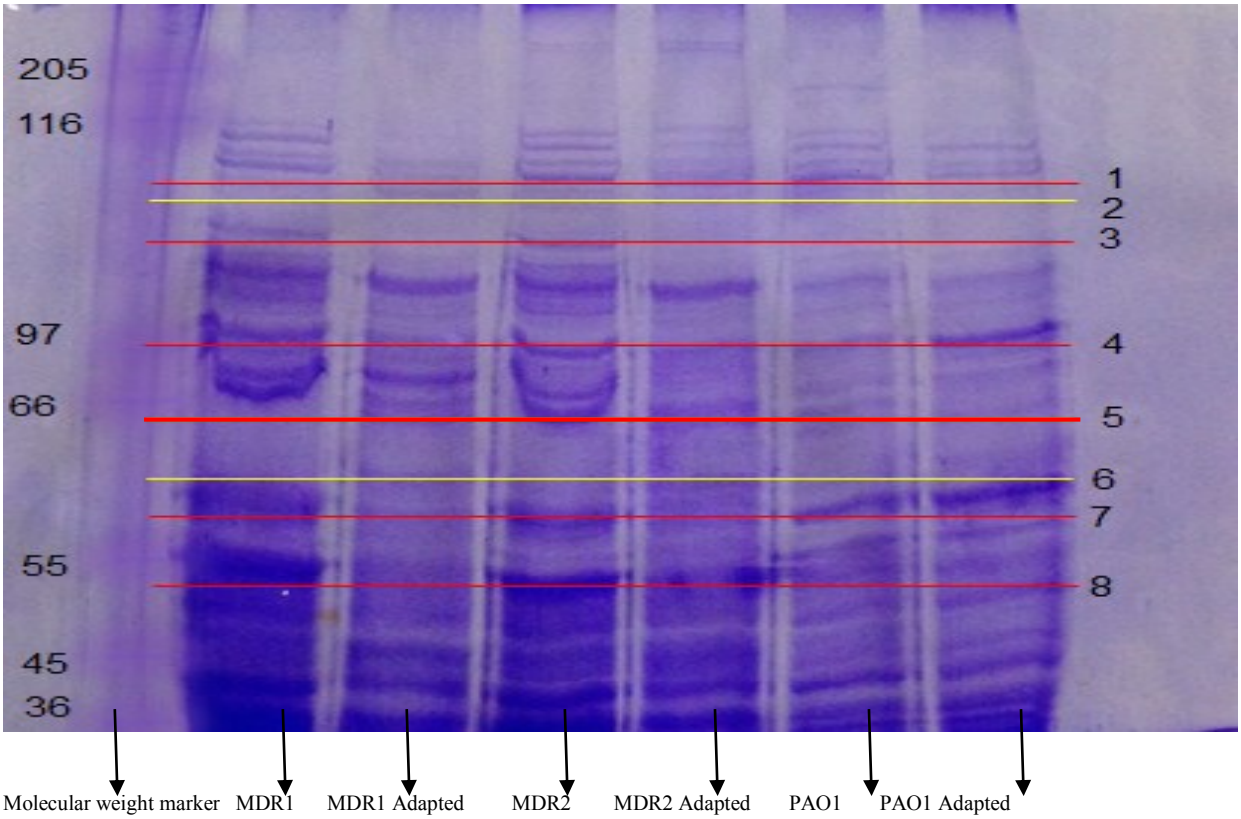


Figure 8: Outer membrane protein profile of MDR1 &1 and PAO1 and 2 PE adapted MDR1& 1 and PAO1. Protein concentration in samples **19.5, 10.1, 8.2, 11.8, 14.5, 15.8** µg/ml respectively. 1, 2, 3,4,5,6,7 and 8 represents altered bands.

Table 5: Alterations in protein bands in the outer membranes of 2-PE adapted and non-adapted MDR and PAO1 strains.

Band	MDR 1	MDR 1 Adapted	MDR 2	MDR 2 Adapted	PAO1	PAO1 Adapted	M W
1	✓	×	✓	×	✓	×	127.2
2	×	✓	×	✓	×	×	121.4
3	✓	×	✓	×	×	×	112.2
4	✓	×	✓	×	✓	✓	87.3
5	✓	×	✓	×	×	×	75.8
6	×	✓	×	✓	×	×	65.9
7	✓	×	✓	×	✓	✓	61.9
8	✓	×	✓	×	×	×	52.9

✓ Appearance or increase in concentration of a protein band. × Disappearance or reduction of a protein band.

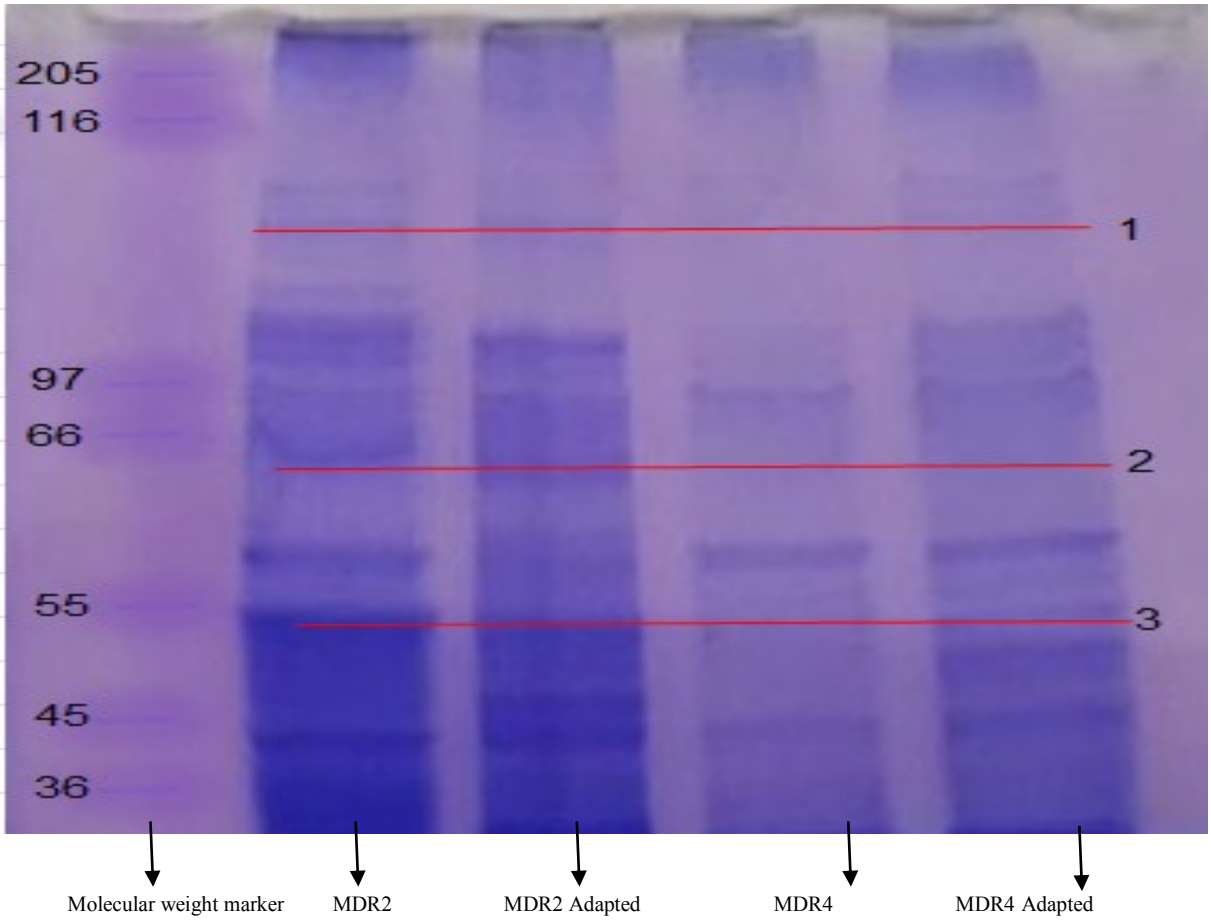


Figure 9: Outer membrane protein profile of MDR 2&4 and 2-PE adapted MDR 2&4. Protein concentration in samples **45.3, 50.5, 11.6, 54** µg/ml **respectively**. 1, 2, 3 represents altered bands.

Table 6: Alterations in protein bands in the outer membranes of 2-PE adapted and non-adapted MDR strains.

Band	MDR 2	MDR 2 Adapted	MDR 4	MDR 4 Adapted	M W
1	✓	✓	×	×	125.266
2	✓	✓	×	×	79.5
3	✓	✓	×	×	56.3

✓ Appearance or increase in concentration of a protein band. × Disappearance or reduction of a protein band.

1.6 Ethidium bromide assay:

Multidrug resistance phenotype in bacteria is accompanied with expression or overexpression of efflux pumps (Webber and Piddock, 2003). Therefore, the retrieval of susceptibility of different classes of antibiotics might indicate possible inhibition of efflux pumps. EthBr (Universal efflux pump substrate) accumulation in 2-PE adapted and non 2-PE adapted strains was measured. Figure 10 shows increased fluorescence in the 2-PE adapted strains MDR1&2 and PAO1 and no change in 2-PE adapted MDR 3&4.

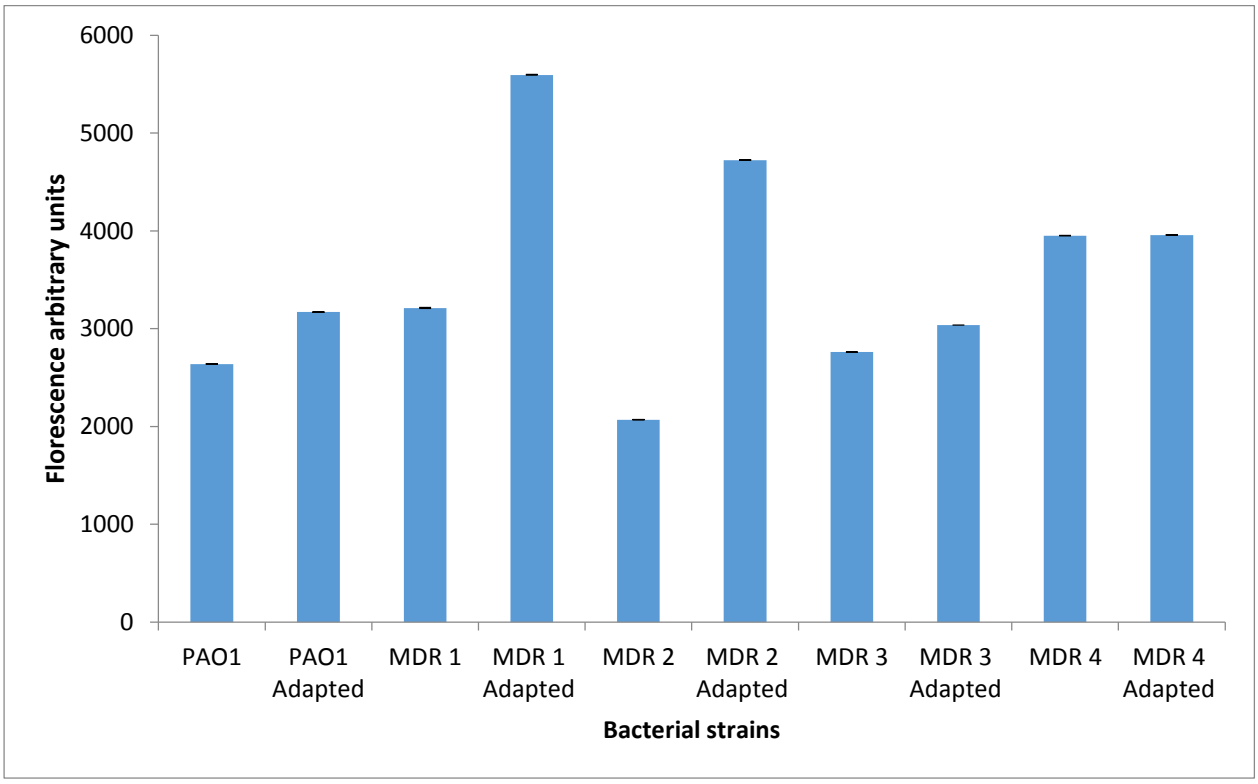


Figure 10: EthBr accumulation in 2-PE adapted and non-adapted MDR and PAO1 strains.

Chapter Four

Discussion

1. Discussion

The resulting antibiotic susceptibility of 2-PE adaptation in MDR *P.aeruginosa* is an important incident and it foretells possible retrieval of conventional antibiotics effectiveness. However exploring the mechanism of this regained susceptibility is important. This study aimed at revealing the mechanism behind the retrieval of antibiotic susceptibility following adaptation to 2-PE in MDR *P. aeruginosa* and sensitive strains. Four MDR strains were used in this study, interestingly the four MDR strains exhibited higher 2-PE MIC compared to the wild type PAO1 strain which indicates that antibiotic resistance in these strains confers decreased susceptibility towards 2-PE as well. All strains were able to adapt to 2-PE however, the effect of adaptation on susceptibility to antibiotics varied. Strains MDR 1&2 became more sensitive to antibiotics to which they were previously resistant (Table 2 a&b). While 2-PE adaptation did not affect susceptibility to antibiotics in MDR strains 3, 4 (Table 2 c&d). Strain variations were also observed in their cultural characteristics, namely pigmentation. Strains MDR 1&2 produced green pigment while strains MDR 3&4 produced yellowish green pigment. This variation in the phenotype separated the strains into two groups that appeared different in other aspects during this study.

Pyocyanin, the green pigment of *P.aeruginosa,* is a virulence factor, which loss, coincided with 2-PE adaptation as well as increased antibiotic susceptibility. This pigment was not observed in strains MDR 3&4 and interestingly, these strains did not exhibit the antibiotic sensitive phenotype after 2-PE adaptation. Pigment production in *P. aeruginosa* was shown to be associated with the multi-drug resistance phenotype (Martins *et al., 2013*). In fact pigment production has been suggested by Finlayson and Brown (2011) to be a marker for the state of virulence of an isolate. On the other hand, the yellowish green pigment (pyoveredine) of MDR 3&4 was not lost during

adaptation nor was the antibiotic resistance in these strains reversed. Interestingly though, Pyoverdine secretion was shown recently to be through an ATP-dependent efflux pump, PvdRT-OpmQ (Schalk and Guillon , 2013).

Thus it could be suggested here that 2-PE adaptation and reversal of antibiotic resistance are related to pyocyanine synthesis inhibition (Byng *et al*., 1979).

Definition of multidrug resistance in bacteria involves resistance of the isolate to three different classes or more of antibiotics (Charles *et al.,* 2003). Efflux activity has been associated with the MDR phenotype in *P. aeruginosa* (Webber and Piddock, 2003) antibiotic susceptibility tests have revealed retrieval of antibiotic sensitivity in strains 1,2 towards Amoxacillin (25 µg/ml), Ampicillin (10 µg/ml), Chloramphenicol (30 µg/ml), Cepfalexin (30 µg/ml), Rifambicin (5 µg/ml), Sulphamethoxazole (25 µg/ml), Vancomycin (30 µg/ml) and increase in the zone of inhibition of Azithromycin (15 µg/ml), Gentamicin (10 µg/ml), Doxycyclin (30 µg/ml), Imipenem (10 µg/ml), Minocyclin (30 µg/ml), Norfloxacin (10 µg/ml), Ofloxacin (5 µg/ml), Piperacillin (100 µg/ml). Thus we hypothesized that 2-PE could be interfering with efflux pumps activity by either inhibiting their function or dissipating the energy required to activate them. Ethidium bromide is an efflux pump substrate that is readily effluxed out into the media when the cell is exposed to it. When EthBr dye accumulates within the cell, it interchelates with the cellular DNA and fluoresces upon exposure to UV (Scaria and Shafer, 1991). This accumulation takes place when efflux pumps are inhibited. In the present study 2-PE adapted cells have shown a significant increase EthBr accumulation compared to the non-adapted cells ($P< 0.05$) (Figure 10). These results agree with the assumption that 2-PE is itself an efflux inhibitor thus adaptation to it by bacteria actually resulted in inhibiting efflux pumps in bacteria. Efflux pumps in *P. aeruginosa* belong to the RND group which is energized by the proton-gradient (Dinesh *et al.,* 2013). On the other hand the

previously mentioned PvdRT-OpmQ pump of pyoveredine is an ATP energized pump and has been shown not to be affected by 2-PE adaptation. Thus 2-PE inhibition is efflux pump selective. Figure 10 shows that accumulation was high regardless the strain type (wild type or MDR). Indicating that 2-PE could be deactivating constitutive pumps in PAO1 as well as other pumps in the other strains.

2-PE adaptation has been shown to induce outer membrane changes in *P. aeruginosa* (Abdelmalek *et al*., 2013). SDS-PAGE of outer membranes of Strains MDR 1&2 have shown alterations in the protein bands (Figure 8) such alterations are seen in the form of missing proteins in 2-PE adapted MDR strains MDR 1&2 of molecular weights of 127, 112, 87, 61, 52 kD. Outer membrane proteins M and J members of efflux pumps in *P. aeruginosa* falls in the molecular weight range of 50-54 kD these proteins are often overexpressed in the MDR strains (Masuda *et al.,* 1995). The disappearance of these proteins could result in loss of efflux mediated resistance. MDR 3&4 showed a slightly different outer membrane profile as seen in figure 9. Bands that are missing in 2-PE adapted MDR 1&2 are not missing in 2- PE adapted MDR 3&4 (figure 8&9).

In figure 8, band 1 & 4 & 7 appeared in MDR1 and MDR1 and PAO1 but they were absent in MRD1 and MDR2 after 2-PE adaptation while band 3,5&8 appeared in MDR1 and MDR2 and they were absent after 2-PE adaptation. Two bands appeared after 2-PE adaptation in MDR 1&2 which are bands 2&6.

Figure 9, shows a comparison between outer membrane profile of MDR2 and MDR4. Bands 1-3 were present in MDR2 strains and absent in MDR4. Which could explain variations in phenotypic characteristics of these two strains.

Change in antibiotic susceptibility post 2-PE adaptation encourages trials of similar adaptation experiments of *P. aeruginosa* to other disinfectants used in hospitals. MDR strains in this study failed to adapt to sub-minimal inhibitory concentrations of

Sterillium® and Septoderm® which are propanol based biocides. Literature review does not include previous work that shows possible adaptation to these ingredients. However an interesting result was observed, during MIC determination of these two biocides an improvement in antibiotic susceptibility of these strains was observed (Table 3).

Polyphosphate kinase is a crucial enzyme in bacteria and its presence contributes to virulent phenotype (Rashid *et al*., 2000a).The inhibition of such enzyme was found to affect virulence in bacteria in the form of loss of motility and cell become vulnerable to activity of antimicrobials (Fraley *et al*., 2006). It was hypothesized that loss of PPK activity might be responsible for the vulnerable state of the MDR 1&2 in the form of loss of antibiotic resistance. To test the hypothesis; a *ppk null mutant* was tested for its sensitivity to antibiotics and compared to the sensitivity of a wild type PAO1. Table 4 shows no significant variation ($P>0.05$) in antibiotic susceptibility between the *ppk null* mutant and the wild type except for piperacillin, gentamycin, and aztreonam which appeared to have improved effectiveness in the *ppk null* strain. General improvement in antibiotic susceptibility was not observed, nor were the antibiotics with enhanced effectiveness, indicated earlier, the same as those indicated in 2-PE adapted strains MDR 1&2. Judging from these results, it seems unlikely that PPK activity is required for antibiotic susceptibility in *P.aeruginosa*.

2. Conclusion

Exposure to biocides does not necessarily result in development of antibiotic resistance. 2-PE adaptation is strain specific. 2-PE effect on bacteria is through outer membrane protein alterations, efflux inhibition and inhibition of certain virulence factors namely pyocyanine synthesis. *P.aeruginosa* was not able to adapt to Sterillium® nor to Septoderm® although exposure of the strains to low concentrations of these two biocides improved their susceptibility to antibiotics. Such improvement in antibiotic susceptibility is promising and can provide evidence that support the role of biocide exposure in improving antibiotic susceptibility in bacteria.

3. Future work

- Further investigation of the efflux inhibition properties of 2-PE.
- Investigation of adaptation of *P. aeruginosa* to different biocides and its effect on antibiotic susceptibility.
- Does adaptation to 2-PE involve chromosomal alterations, and can it be transferred?

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الملخص

دراسة حول آليات تكيف بكتيريا الزائفة الزنجارية متعددة المقاومة للمضادات الحيوية

لمادة 2- فينوكسي إيثانول

اعداد

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المشرف المشارك الدكتور إبراهيم الأدهم

المقدمة

سوء و كثرة استخدام المطهرات و المعقمات و وعلاقته بمقاومة الاحياء الدقيقة للمضادات الحيوية مازال امراً يخضع للنقاش في المجتمع العلمي. فقد أظهر قدرا من الأدلة ان سوء إستخدام المبيدات الحيوية يمكن أن يؤدي الى زيادة مقاومة المضادات الحيوية. أظهرت دراسة اجريت مؤخرا ان سلالة الزائفة الزنجارية المتعددة المقاومة للمضادات الحيوية اصبحت قادرة على إستعادة الاستجابة لتأثير للمضادات الحيوية المختلفة بعد التعرض والتكيف على وجود المبيد الحيوي 2-فينوكسي ايثانول.

الهدف

التعرف على آلية استعادة استجابة البكتيريا المتعددة المقاومة للمضادات الحيوية وسلالات اخرى من البكتيريا بعد التكيف مع 2-فينوكسييثانول وكذلك التعرف على امكانية تكيف هذه البكتيريا على مبيدات حيوية اخرى مستخدمة في المستشفيات.

المنهجية

تمت دراسة تغيرات بروتينات الغلاف الخارجي للسلالات التي تكيفت مع 2- فينوكسييثانول والغير متكيفة عن طريق الفصل الكهربائي لهلام كبريتات دوديكل الصوديوم متعددالأكريلامايد.

تم التحقق من تثبيط مضخات الطرد بواسطة 2- فينوكسييثانول عن طريق الفحص التراكمي لمادة ايثيديوم بروماید.

تم فحص نشاط انزيم كيناز المتعدد الفوسفات باستخدام بكتيريا متحولة فاقدة للانزيم .

النتائج

اظهرت صبغة البايوسيانين تناقصاً بالتزامن مع تكيف البكتيريا مع 2- فينوكسييثانول واصبحت البكتيريا اكثر استجابة للمضادات الحيوية.

اظهر الفصل الكهربائي لهلام كبريتات دوديكل الصوديوم متعددالأكريلامايد لسلالة البكتيريا المتكيفة مع 2- فينوكسييثانول والغير متكيفة ظهور واختفاء لبعض حزم البروتينات التي يتراوح وزنها بين 52الى 127 كيلو دالتون و تجمع الايثيديوم بروماید كان اكثر في البكتيريا المتكيفة. لم تظهر البكتريا المتحولة الفاقدة لانزيم كيناز المتعدد الفوسفات فرقاً مقارنة بالبكتيريا الزائقة الزنجارية مما يدل على ان هذا الانزيم لا يلعب دوراً في زيادة حساسية هذه البكتيريا للمضادات الحيوية .

تم اختبار مبيدان حيويان اخرآن في هذه الدراسة وهم الستيريليوم والسيبتوديرم. فشلت البكتيريا في التكيف بوجود المبيدين الحيويين. ولكن ازدادت حساسية البكتيريا للمضادات الحيوية التي كانت تستجيب لهامسبقاً عند تعرضها للمبيدين وتم مشاهدة ذلك من خلال زيادة قطر منطقة التثبيط.

بالاضافة الى ذلك, فشلت سلالتان جديدتان من بكتيريا الزائفة الزنجارية في زيادة استجابتها للمضادات الحيوية اثناء تعرضها ل 2-فينوكسييثانول مقارنة بالسلالات الاخرى.

الاستنتاج

تكيف البكتريا الزائفة الزنجارية المتعددة المقاومة للمضادات الحيوية لمادة 2- فينوكسييثانول ممكن ولكن تحسن استجابتها للمضادات الحيوية مرتبط بسلالات معينة. هذا التحسن ممكن ان ينسب لاسباب متعددة من ضمنها تثبيط انتاج عوامل الخبث كإنتاح الصبغات, تغيير في هيكل بروتينات الجدار الخارجي والذي من الممكن ان يسبب تغييرات في النفاذية التي تسمح بإختراق المضادات الحيوية للبكتيريا. هذا التغيير في البروتينات ادى الى تثبيط لمضخات الطرد الذي يسبب التجمع الزائد لمادة اثيديوم بروماید.