GENOMIC CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES BY PULSED FIELD GEL ELECTROPHORESIS METHOD

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that,as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

GENOMIC CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES BY PULSED FIELD GEL ELECTROPHORESIS METHOD

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Pseudomonas aeruginosa is a common cause of nosocomial infections, particularly in intensive care units (ICUs), bronchoscopy, oncology, and urology. The aim of this study was to characterize *P. aeruginosa* clinical isolates clonally relatedness by pulsed-field gel electrophoresis (PFGE) typing and it's cut off value determination.

We did retrospective study and analyzed genotypically a collection of 58 clinical isolates recovered during the period 2006–2011 from MESA private hospital microbiology department's bacterial archive, PFGE was carried out at the Rafik Saydam National Hygiene Center.

We used isolates' drug resistance pattern as a control phenotypic character and compared it with observed "A-Z" PFGE pulsed types and drawn dendograms.

Most of the *Pseudomonas aeruginosa* isolates were resistant to chloramphenicol (60 %), gentamycine (55 %) and to a lesser extent to imipenem (11 %), meropenem (39 %), pipracilin (31 %), ciprofloxacilin (10 %), ceftazidim (23 %), cefepim (6 %), cefoxitin (16 %), tobramycin (25 %), and aztreonam (10 %), 33 % were multidrug resistant. Pulsed-field gel electrophoresis with SpeI-digested genomic DNA resulted in 53 different pulsed types "With upper 95 % dice similarity cut off", while with decreasing cut off value to upper 80 % dice similarity strains accumulated in 25

clusters each one representing different wards of hospital and nine individuals. This cut off value fully described same patients' different samples "*Pseudomonas* placement in a same cluster correctly, while former cut off value interpret them with contradiction in separate genotypes.

PFGE cut- off value with SpeI restriction enzyme is different from XbaI. Tenover criteria, scales must be adjusted to such restriction enzymes due to halfing "restriction fragment bands" numbers and scales.

A-Z pulsed types were observed; special pulsed types were merely observed in bronchoscopy patients isolates and was not observed in other wards of hospital, with correct sterilization of bronchoscope, incidence of above pulsed types was decreased. Special pulsed types were observed in intensive care units (ICU). Multi drug resistant strains showed to be genetically identical origin. PFGE demonstrated the existence of a common clone in a (ICU) critical care area and bronchoscope unites due to this, we defined it bronchoscope related infection. Despite not existing horizontal transferring from patient to patient due to high level of hygiene plans of this hospital, isolating similar strain (*Pseudomonas aeruginosa*) between year 2006-2010 maybe due to biofilm formation and non-correct sterilization of medical equipments e.g. bronchoscope, Oxygen mask, laryngoscope These means may be sources of infection in this hospital. Reinforcement of infection control measurement is needed to avoid iatrogenic and horizontal transmission and severe infections.

Morphological typing method based on anti-biotic Resistance method fully overlapped with PFGE method and confirmed our results in higher level of sensitivity and reliance.

Aztreonam, colistin appeared to be the most effective agent against multidrug-resistant *Pseudomonas aeruginosa* isolates.

KEYWORDS: Pulsed field gel Electrophoresis, DNA finger printing, *Pseudomonas aeruginosa*, Bronchoscope; Nosocomial infection BAL (Bronchial Aspirate lavage) ENT, ICU, MDR.

Öz

KLİNİK ÖRNEKLERDEN IZOLE EDİLEN PSEUDOMONAS AERUGINOSA SUŞLARIN' DA PFGE YÖNTEMİ İLE GENOMİK PROFİL ARAŞTIRMASI

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Pseudomonas aeruginosa hastane enfeksiyonlarının en yaygın sebebidir, özellikle yoğun bakım ünitelerinde, bronkoskopi, onkoloji, ve üroloji bölümlerinde görülmektedir. Bu çalışmanın amacı PFGE yöntemiyle *P. aeruginosa* klinik isolatlarının klonal ilişkisinin ve PFGE'nin cut off değerinin belirlenmesidir.

2006-2011 yılları arasında MESA özel hastanesi mikrobiyoloji bölümünün bakteri arşivinden elde edilen 58 klinik isolat nümunesi retrospektif ve genotipik olarak incelendi. PFGE, Refik Saydam Hıfzısıhha Merkezinde yapıldı.

Isolatların ilaç direnç profili kontrol "fenotipik karakteri" olarak kullanıldı ve bunu gözlemlenen "A-Z" PFGE pulse tipleriyle mukayese edilerek, dendogramları çizildi.

P. aeruginosa isolatlarının antibiyotiklere direnç oranları; chloramphenicol (% 60), gentamycine (% 55) ve imipineme (% 11), meropenem (% 39) pipracilin (% 31), ciproflaxacilin (% 10), ceftazidin (% 23), cefepim (% 6), cefoxitin (% 16), tobramycin (% 25), ve aztreonam (% 10), % 33 ise birden fazla ilaca direnç gösterdi. PFGE, SpeI restriksiyon enzimi ile yapıldığında % 95 üzeri cut off değeri 53 farklı pulse tipiyle sonuçlandı. Fakat cut off değeri % 80 üzerine indirildiğinde strainler 25 kümede toplanıldı. Her küme hastanenin farklı bir bölümünü temsil etmektedir. Dokuz sonuç ise kümelerin dışında ayrı olarak ve tek tek çıktı. Bu ikinci cut off ile

aynı hastanın farklı isolat bakterileri aynı kümeye doğru yerleşti, halbuki bir önceki "cut off"ta çelişik, farklı genotiplerde çıktı.

SpeI enziminin PFGE cut off değeri XbaI'nınkinden farklıdır.

Tenover kriterleri, yeni enzimleri de kapsaması gerekmektedir.

A-Z pulse tipleri gözlemlendi;

özel pulse tipleri sadece bronkoskopi hastalarınını isolatlarında ve başka yerde görülmedi.

Bronkoskopların doğru sterilizasyonu sonucunda yukardaki pulse tiplerinin görülme sayısı azaldı.

Özel pulse tipler yoğun bakımda gözlemlendi.

Çok sayıda ilaça dirençli strainlerin aynı genetik kaynaklı olduğu gösterildi.

PFGE yoğun bakım ve bronkoskop ünitesinde ortak bir klonun varlığını gösterildi. Bunu bronkoskop alakalı enfeksiyon olarak tanımlandı.

Adı geçen hastanenin yüksek hijyen seviyesi sayesinde hastadan hastaya bulaşma olmamasına rağmen, 2006-2011 yılları arasında aynı isolatların çıkmasının sebebinin, cihazlarda biyofilm oluşumu ve tıbbi cihazların, bronkoskop, oksijen maskesi, larinkoskopun, yanlış sterilizasyonu olabileceği tahmin edildi. Bu cihazlar bu hastanenin enfeksiyon kaynağı olabilir. Iatrogenik ve yatay geçiş ile oluşan enfeksiyonlardan sakınılabilmesi için enfeksiyon kontrol ölçümlerinin arttırılmsı gerekmektedir.

Anti-biyotik direnç yöntemine dayalı morfolojik tipleme yöntemi, PFGE yöntemi ile tam örtüştü ve bulgularımızı yüksek hassasiyet ve güvenilirlik ile doğruladı.

Aztreonam and colistin çok ilaça dirençli *P .aeruginosa* isolatlarına karşı en etkili madde olarak öne çıktı.

Anahtar Kelimeler: Pulsed field Gel Elektrophorez,DNA parmak izi,Pseudomonas aeruginosa,Bronkoskope,Hastane enfeksiyonu,KBB,YBU,ÇID

To: My parents, My siblings,

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16S rRNA: 16S Ribosomal Ribonucleic acid

AAC: Aminoglycoside acetyltransferase

ADP: Adenosine Di-phosphate

ADPRT: Adenosine Di-phosphate Ribosyl Transferase

AES1: Australian Epidemic Strain I

AES2: Australian Epidemic Strain II

AES3: Australian Epidemic Strain III

ANT: Aminoglycoside Nucleotidyltransferase

API: Analytical profile index:classification of bacteria based on Biochemicals

Apr: Alkaline Protease

APH: Aminoglycoside Phosphoryletransferase

ATCC: American Type Culture Collection

AX2: *Dictyostelium discoideum* Axenic strain 2

AX4: *Dictyostelium discoideum* Axenic strain 4

BAL: Broncho-alveolar lavage

BMI: Body Mass Index

BSAC: British Society of Antimicrobial Agents and Chemotherapy

COPD: Chronic Obstructive Pulmonary Disease

CLSI: Clinical Laboratory Standards International

CF: Cystic Fibrosis

CFTR: Cystic Fibrosis Transmembrane Regulator

CHO: Chinese Hamster Ovary cell line

DIG: Digoxigenin-dUTP

DNA: Deoxy-ribonucleic Acid

ExoA: Exotoxin A

ESBL: Extended Spectrum β-lactamase

FAFLP: Fluorescent Amplified Fragment Length Polymorphism

FEV1%: Forced Expiratory Volume in one second

FVC%: Predicted Forced Vital Capacity

G+C: Guanine and Cytosine

GTP: Guanosine Tri-phosphate

HCN: Hydrogen Cyanide

HIV: Human Immunodeficiency Virus

HSL: Homoserine Lactone

ICU: Intensive Care Unit

IDSA: Infectious Diseases Society of America

IV: Intravenous

IOM:institute of medicine

LasB: Elastase

LES: Liverpool Epidemic Strain

LPS: Lipopolysaccharide

MBC: Minimal Bactericidal Concentration

MDR: Multi-drug Resistant

MES: Manchester Epidemic Strain

MMβL: Mobile Metallo-β-lactamase

MDCK: Madin-Darby Canine Kidney cell line

MDR: Multi drug resistance

MIC: Minimal Inhibitory Concentration

NATA: National Australian Testing Authority

NCTC: National Collection of Type Cultures

NPV: Negative Predictive Value

NSU: Neurosurgery Unit

PA14: *Pseudomonas aeruginosa* laboratory control strain 14

PAI-1: N- (3-oxododecanoyl)-L-homoserine lactone

PAI-2: N-butyral-L-homoserine lactone

PAO1: Pseudomonas aeruginosa laboratory control strain 01

PA14: Pseudomonas aeruginosa laboratory control strain 14

PA99: Pseudomonas aeruginosa laboratory control strain 99

PA103: Pseudomonas aeruginosa laboratory control strain 103

PAK: Pseudomonas aeruginosa laboratory control strain K

PCN: Pyocyanin

PCR: Polymerase Chain Reaction

PDR: Pan drug resistance

PFGE: Pulsed Field Gel Electrophoresis

PPV: Positive Predictive Value

PQS: 2-heptyl-3-hydroxy-4-quinolone

QS: Quorum Sensing

RAPD: Random Amplified Polymorphic DNA

RFLP: Restriction Fragment Length Polymorphism

RHH: Royal Hobart Hospital

RND:resistance/nodulation/cell division

MFS :major facilitator subfamily

SMR: small multidrug regulator

SpeI:kind of Restriction Enzyme name

T1SS: Type I Secretion System

T2SS: Type II Secretion System

T3SS: Type III Secretion System

TFP: Type IV Pili

TLR4: Toll-like Receptor 4

TSI: Triple Suger Iron Agar

UK: United Kingdom of Great Britain and Northern Ireland

UTI: Urinary Tract Infection

XbaI: kind of Restriction Enzyme

CHAPTER 1

LITERATURE REVIEW

1.1 Definitions

1.1.1 "Plasmids: are small ***DNA*** molecules that are physically separate from, and can duplicate independently of, chromosomal DNA within a cell. Most commonly found as small circular, double-stranded DNA molecules in bacteria, plasmids are sometimes present in archaea and eukaryotic organisms. In nature, plasmids carry genes that may benefit survival of the organism (e.g. antibiotic resistance), and can frequently be transmitted from one bacterium to another (even of another species) via horizontal gene transfer (Lipps G, 2008, Lederberg J, 1957). Artificial plasmids are widely used as vectors in cloning, serving to drive the replication of recombinant DNA sequences within host organism (Lipps G, 2008, Lederberg, J, 1957). Plasmids are considered replicons, capable of replicating autonomously within a suitable host. Plasmids may carry genes that provide resistance to naturally occurring antibiotics in a competitive environmental niche, or the proteins produced may act as toxins under similar circumstances.Plasmids can also provide bacteria with the ability to fix nitrogen or to degrade recalcitrant organic compounds that provide an advantage when nutrients are scarce" (Lipps G, 2008, Lederberg J, 1957).

"Plasmid host to host transfer requires direct mechanical transfer by conjugation, or changes in expression of incipient host genes expression allowing the intentional up take of the genetic elements by transformation phenomenon" (Lipps G, 2008, Lederberg J, 1957).

1.1.2 "An **integron:** is a two component gene capture and dissemination system, initially discovered in relation to antibiotic resistance, and which are found in plasmids, chromosomes and transposons. The first component consists of a gene encoding a site specific recombinase along with a specific site for recombination, while the second component comprises fragments of DNA called gene cassettes which can be incorporated or shuffled" (Lipps G, 2008, Lederberg J, 1957).

"Antibiotic resistance genes maybe encoded by a cassette, although most genes in integrons are uncharacterized. An integron contains an integrase (int1) related to a phage, followed by an attI site for integration of cassettes and recognition of the integrase, with a promoter to drive expression. An integron may appear in a plasmid or on the chromosome. An att C sequence (also called 59-be) is a repeat that flanks cassettes and enables cassettes to be integrated at the attI site, excised and undergo horizontal gene transfer".

A functional integron "platform" requires:

--An integrase: *intI* (a tyrosine recombinase enzyme responsible for insertion into the genome).

--A proximal recombination site: *attI*, which act as the locus for reincorporation to the genomes and combines with an *attC* site at the insertion site.

The term *super-integron* was first applied in 1998 (but without definition) to the integron with a long cassette array on the small chromosome of *Vibrio cholera*. The term has since been used for integrons of various cassette array lengths or for integrons on bacterial chromosomes (versus, for example, plasmids) Use of "super-integrons" are now discouraged since their meaning are unclear"(Hall, RM; Collis, CM 1995, Kovalevsky, N. P. 2002, Hall, R. M.; Stokes, HW 2004, Mazel, D.; Dychinco, B; Webb, VA; Davies, J 1998).

1.1.3 "A **transposable elements** (**TE**) are DNA sequence that can change their position within the genome, sometimes creating mutations and altering the cell's genome size. Transposition often results in duplication of the TE. Barbara McClintock's discovery of these **jumping genes** early in her career earned her a Nobel Prize in 1983".

TEs make up a large amount of the C-value of eukaryotic cells. They are often considered "junk DNA". They play a critical role in development. In *Oxytricha*, which has a unique genetic system; they are also very beneficious to researchers as a means to alter DNA inside a living organism. (Kidwell, M.G. 2005, Craig NL, Craigie R (2002), Lewin B 2000)

1.1.4. "Immune-competent: Normal person have competent immune system, So bacteria can invade body in the case of any injury in skin or catheter, aspirator etc can enter body and can cause infection".

1.1.5. "Immune-compromise: Patients that has diabetes, cancer, neoplazy, or gotten immunosupressor drugs for not rejecting transplanted organ are called immunocompermised patients, they get infection with *Pseudomonas* due to deficiency in their cellular immunity system".

"Immune-competence is the ability of the body to produce a normal immune--response following exposure to an antigen.Immune-competence is the opposite Of immunodeficiency or immune-incompetent or immune-compromised"

Examples include:

A newborn that does not yet have a fully functioning immune system may have maternally transmitted antibodies – immune-deficient;

A late stage AIDS patient with a failed or failing immune system - immune-incompetent; or

A transplant recipient taking medication so their body will not reject the donated organ - immune compromised. There may be cases of overlap but these terms are all indicators of non-fully functioning immune system. In reference to lymphocytes, immune-competence means that a **B** cell or **T** cell is mature and can recognize antigens and allow a person to mount an immune response.

1.1.6. "A **nosocomial infection**, also known as a **hospital-acquired infection** or **HAI**, is an infection whose development is raised by a hospital environment, such as one acquired by a patient during a hospital visit or one developing among hospital staff. Such infections include fungal and bacterial infections and are aggravated by the reduced resistance of individual patients(Pollack, Andrew 2010, Wolf, Hanson, 201, Peter A, Nosocomial Infection 2008) Nosocomial infections can create severe pneumonia and infections of the urinary tract, blood stream and other parts of the body. Many types eradication are difficult with antibiotics, and antibiotic resistance is spreading to Gram-negative bacteria that can infect people outside the hospital (Hanninen, O 1983) In the United States, the Centers for Disease Control and Prevention (CDC) estimate that roughly 1.7 million hospital-associated infections. Nosocomial infections are commonly transmitted when hospital officials become complacent and personnel do not practice correct hygiene regularly. Also, increased use of outpatient treatment means that people who are hospitalized are more ill and have more weakened immune systems than may have been true in the past. Moreover, some medical products break or bypass the body's natural protective barriers. Since medical staff moves from patient to patient, the staff themselves serves as a means for spreading pathogens. Essentially, the staff acts as vectors" (Finland M1979, Spanos, 1996, Pruett Jr, John R2004)

1.1.7 "Iatrogenesis, or an **iatrogenic artifact,** (originating from a physician) is an inadvertent adverse effect or complication resulting from medical treatment or advice, including that of psychologists, therapists, pharmacists, nurses, physicians and dentists. Iatrogenesis is not restricted to conventional medicine: It can also result from complementary and alternative medical treatments (Braun, B.G., 1989) Causes

of iatrogenesis include chance, error, negligence, social control, unexamined instrument design, anxiety or annoyance related to medical procedures and the adverse effects or interactions of medications. Iatrogenesis is a major phenomenon, and a severe risk to patients. A study carried out in 1981 more than one-third of illnesses of patients in a university hospital were iatrogenic, nearly one in ten was considered major, and, in 2% of the patients, the iatrogenic disorder ended in death. Complications were most strongly associated with exposure to drugs and medications. In another study, the main factors leading to problems were inadequate, patient evaluation, lack of monitoring and follow-up, and failure to perform necessary tests" (Abbey, S.E.1993, Boscarino, JA 2004, Moos, R.H. 2005, Weiss, B.; 2005).

"In the United States, figures suggest estimated deaths per year of:

(Starfield B, July 2000)"

12,000 due to unnecessary surgery (Leape L May1992)

"7,000 due to medication errors in hospitals" (Phillips DP, 1998)

"20,000 due to other errors in hospitals" (Lazarou J1998)

"80,000 due to nosocomial infections in hospitals" (Lazarou J 1998)

"106,000 due to non-error, negative effects of drugs" (Phillips DP, 1998)

Based on these figures, iatrogenesis may cause 225,000 deaths per year in the United States (excluding unrecognizable error).

"These estimates are lower than those in an earlier Institute of Medicine of the national academy washington.USA (IOM) report, which would suggest from 230,000 to 284,000 iatrogenic deaths"

"Nosocomial infections and iatrogenic infections overlap with each other in most of cases, however nosocomial infection are defined to infections acquired from hospital after passing 48 hours from patient's admission to decrease case of infections to its minimum amounts. One of the most important bacteria in nosocomial infection is P*seudomonas aeruginosa".*

1.1.8 *Pseudomonas aeruginosa* Nosocomial infections

Pseudomonas aeruginosa is a recognized nosocomial pathogen, responsible for between 10% to 20% of hospital-acquired infections(Dinesh, Grundmann *et al*.2003; Matar, Chaar (2005) Indeed, *P. aeruginosa* is the second most common cause of nosocomial pneumonia, and shows the highest mortality for this type of infection (30%) (Dinesh, Grundmann *et al*. 2003) *P. aeruginosa* is also highly associated with burn wound infections, sepsis, meningitis and infections associated with indwelling devices amongst hospitalized patients (Dinesh, Grundmann *et al*. 2003; Stryjewski and Sexton 2003) Given the large number of severely ill and immune-suppressed patients in hospitals, the widespread use of antimicrobial agents and disinfectants in these patients and their surrounds, it is unsurprising that a positive selection for *P. aeruginosa* in the hospitalized patient occurs.

These pre-disposing factors are particularly applicable in the context of the intensive care units(ICU), as 29% of infections in ICUs may be attributable to *P. aeruginosa*, ranking it as the most common cause of Gram negative infection in this setting (Deplano, Denis *et al*. 2005)Nosocomial isolates of *P. aeruginosa* display markedly higher rates of antimicrobial resistance and multi-drug resistance than those recovered from community acquired infections (Brisse,Milatovic *et al*. 2000), particularly amongst ICU patients (Guitierrez, Juan *et al*. 2007).

One study found that of 419 isolates of *P. aeruginosa* from 19 separate Canadian ICUs, 12.6% showed an **Multiple Drug Resistant** (MDR) phenotype, the closest frequency of MDR phenotype in other Gram negative rods isolated in this study was that of 0.6% in Enterobacter cloacae (Zhanel, De Corby *et al*. 2008) Infection control practices play an important role in the control of *P. aeruginosa* infections within the hospital setting (Saiman and Siegel 2004, Bradbury R, 2009).

1.2 Introduction

Pseudomonas aeruginosa is a common cause of bacterial infection in humans, animals and plants worldwide. Infection may vary in severity from a mild, self-limiting illness through to severe and debilitating systemic disease, associated with significant morbidity and mortality. *P. aeruginosa* is recognized as the causative agent of a remarkable spectrum of diseases in both healthy and immune compromised patients, and is the primary cause of morbidity and mortality in cystic fibrosis (CF) patients. It is ubiquitous within the environment, having been recovered from a wide range of aqueous and vegetative environmental sources (Sekiguchi, Asagi *et al*. 2007). *P.aeruginosa* will often merely colonize (the presence and multiplication of an organism within or upon the host, without tissue invasion or damage) a host.

In cases where a breach in the integrity of host defenses occurs, strains may cause infection (the presence and multiplication of an organism within the host, leading to tissue damage through local cellular injury, secretion of toxins or antigen-antibody reactions). The incidence of severe infections caused by *P. aeruginosa* has risen dramatically in recent decades, a change that has been proposed as being prompted by a number of factors including:

a) A significant increase in the longevity of severely ill and immune compromised patients,

b) Concurrent significant increase in the use of broad spectrum antimicrobials in patients (Which in many cases will select for *P. aeruginosa* colonization and infection),

c) A significantly increased capacity for the development or acquisition of mechanisms to resist the action of antimicrobials,

d) The recent acquisition of new virulence factors by horizontal gene transfer (Kulasekara, Kulasekara *et al*. 2006, Bradbury, Richard) .

1.3 *Pseudomonas* General characteristics

P. aeruginosa is a non-spore forming, non-fermentative, Gram negative bacillus (Kiska and Gilligan 2003) The organism is catalase positive and oxidase positive and has traditionally been considered strict aerobe; unable to grow in anaerobic conditions due to its requirement for oxygen as a terminal electron acceptor in biochemical pathways. However anaerobic growth of *P.aeruginosa* has been demonstrated to be possible in the presence of nitrate or arginine, which the organism may also utilize as terminal electron acceptors (Kiska and Gilligan 2003). *P. aeruginosa* has a wide growth temperature range, growing equally well at room temperature and 37˚C. Slower growth rates are seen at 4˚C (Baron 2001; Pier and Ramphal 2005). *P. aeruginosa* is distinguishable from other clinically significant *Pseudomonas spp*. by its capacity for growth at 42˚C (Baron 2001; Kiska and Gilligan 2003, Bradbury, Richard).

Colonies of *P. aeruginosa* are round, matt, butyrous colonies 2-3 mm in diameter after 24 hours growth on blood agar at 37ºC, with irregular edges and a floccular internal structure. Commonly described are smaller colonies, resembling coli forms, and raised, rough, umbonate or even rogues colonial morphologies have been described (Pitt 1998). *P.aeruginosa* may dissociate into multiple colonial morph types, which are regularly observed following sub-culture of an isolated colony (Zierdt and Schmidt 1964). Most distinctive of the differing morph types are the mucoid strains first described by Sonnenschein in 1927 (Çetin, Töreci *et al* 1965) often recovered from patients with CF and other chronic respiratory infections with *P. aeruginosa*. Mucoid isolates have also been recovered from contact lens associated corneal infections and urinary tract infections (particularly those UTIs associated with indwelling catheters), the common factor between all of these infections being growth in biofilm (Engel 2003). *Pseudomonas aeruginosa* was first obtained in pure culture by Gessard in 1882 from wounds that had produced blue-green discoloration (Forkner, 1960). The word '*aeruginosa*' comes from the Latin word for verdigris or copper rust. This describes the blue-green bacterial pigment seen in laboratory cultures of *P.aeruginosa.*

Pseudomonas aeruginosa is a Gram-negative, mesophilic, aerobic rod (measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm) (Bergey's manual of Systematic Bacteriology, 2001). These bacteria are commonly found in soil and water. They occur regularly on the surface of plants and occasionally on the surfaces of animals. The *pseudomonads* are better known to microbiologists as pathogens of plants rather than animals, but few *pseudomonads* species are pathogens of humans (Todar, 2004, Stover 2000). Accomplished sequencing of the complete (6.3 Mbp = 6.300.000 base pairs) genome of *P.aeruginosa*. The large genome size and genetic complexity of *P.aeruginosa* reflects evolutionary adaptations permitting it to thrive in diverse ecological niches. *P.aeruginosa* has broad capabilities to transport, metabolize and grow on organic substances, numerous iron siderophore uptake systems and enhanced ability to export compounds, e.g., antibiotics, by a large number of protein secretion and efflux systems. Pseudomonas aeruginosa is not particularly distinctive as a pseudomonad, but there are a few characteristics that are noteworthy and relate to its pathogenesis (Todar, 2004, Bradbury, Richard) (Figure34, 35, 36 AppendixB).

Pseudomonas aeruginosa possesses the metabolic versatility for which *pseudomonads* are so renowned. *Pseudomonas aeruginosa* is no fermentative and derives its energy from oxidation rather than fermentation of carbohydrates. It can utilize at least eighty organic compounds but can grow on minimal media with only acetate for carbon and ammonium sulphate for nitrogen. It does not require any organic growth factors (Bergey's Manual of Systematic Bacteriology, 2001).

Pseudomonas aeruginosa is extremely versatile biochemically and can multiply in an extraordinary assortment of environments including eye drops, soaps, sinks, anesthesia and resuscitation equipment, fuels, humidifiers and even stored distilled water, which is evidence of its minimal nutritional requirements (Todar, 2004). Although the bacterium is respiratory and never fermentative, it will grow in the absence of O_2 if NO_3 is available as a respiratory electron acceptor. Its optimum temperature for growth is 37º C, and it is able to grow at temperatures as high as 42º C. Indeed, it is this ability to grow at 42º C that distinguishes it from many other *Pseudomonas* species. No growth occurs at 5º C (Bergey's Manual of Systematic Bacteriology), its ecological capability makes it as an opportunistic pathogen. *Pseudomonas aeruginosa* does, however, show a preference for growth in moist

environments, a reflection of its origins in soil and water (Todar, 2004). *P. aeruginosa* isolates can produce three different colony types. One is large, smooth, with flat edges and an elevated center ("fried egg" appearance) and the other is small, rough and convex. Clinical materials are, in general, good sources of the large colony type, while the small is commonly obtained from natural sources (Véron and Berche, 1976). A third type (mucous) often can be obtained from respiratory and urinary tract secretions and was first observed by (Sonnenshein1927). The mucus is attributed to the production of alginate slime. The smooth mucoid colonies are presumed to play a role in colonization and virulence (Bergey's Manual of Systematic Bacteriology, 2001).

Pseudomonas aeruginosa produces many types of soluble pigments of which pyocyanin and pyoverdin are the most common. The latter is produced abundantly in media of low-iron content, and functions in iron metabolism in the bacterium. Pyocyanin refers to "blue pus" which is a characteristic of supportive infections caused by *P. aeruginosa*. (Palleroni, 1986).Other pigments produced are pyorubin (red), pyomelanin (brown) and pyoverdin (yellow/green) (Bergey's Manual of Systematic Bacteriology, 2001, Bradbury, Richard) (Figure34, 35, 36Appendix B).

P. aeruginosa is the epitome of an opportunistic pathogen of humans. It rarely causes infections in healthy individuals but is a major cause of hospital acquired nosocomial infections. Even though the bacterium almost never infects uncompromised tissues, there is hardly any tissue that it cannot infect, if the tissue defenses are compromised in some manner (Todar, 2004). It tends to infect people with immunodeficiency or burns and those with indwelling catheters or on respirators. Infection with *P.aeruginosa* can lead to urinary tract infections, sepsis (blood stream infection), pneumonia, endocarditis, pharyngitis, meningitis, and many other medical problems. It colonizes the lungs of patients with cystic fibrosis (CF) and contributes to the chronic progressive pulmonary disease and death rate in CF.Although the initial isolation of *P.aeruginosa* from sputum may be intermittent in CF and bronchiectasis, once a chronic infection is established it is almost impossible to eradicate it even with intensive antibiotic treatment (Rayner *et al*, 1994, Bradbury, Richard). *P.aeruginosa* is notorious for its resistance to antibiotics and is, therefore, a particularly dangerous and dreaded pathogen (Seol *et al*., 2002). It has a natural

tendency for the development of resistance to antibiotics. This limits future therapeutic uses of antibiotics against this bacterium and increases rates of mortality. The bacterium is naturally resistant to many antibiotics including tetracycline and benzyl penicillin due to the permeability barrier afforded by its outer membrane lipopolysaccharide (LPS) (Li *et al*., 1994 a). It can colonize surfaces in a biofilm form making the cells impervious to antibiotics. *Pseudomonas aeruginosa* has been living in the soil for millions of years in the presence of antibiotic producing bacilli, ctinomycetes and molds. Therefore, it has developed resistance to a variety of their naturally occurring antibiotics. Moreover, *P.aeruginosa* maintains antibiotic resistant plasmids, and is able to transfer these genes by means of the bacterial processes of transduction and conjugation. Only a few antibiotics are effective against *Pseudomonas*, including some β-lactams, amino glycosides and fluoro-quinolones, and even these antibiotics are not effective against all strains. The futility of treating *Pseudomonas* infections with antibiotics is most dramatically illustrated in cystic fibrosis patients, virtually all of whom eventually become infected with a strain that is so resistant that it cannot be treated (Todar, 2004). *Pseudomonas aeruginosa* produces a variety of virulence factors, which aid it in colonizing a host. These include protease enzymes, mucoid exo-polysaccharide, pili, exo-toxin A, lipopolysaccharide, pigments, lipase, haemolysin, histamine, exo-enzyme S, leukocidin and rhamnolipids (Schaber *et al*., 2004). These help the bacteria to adhere and invade to their host by damaging the host's immune responses and forming a barrier to antibiotics. No single virulence factor by itself is potent but the whole array of factors contributes to the pathogenicity of the *P.aeruginosa* (Wilsonand Dowling, 1998, Bradbury, Richard).

1.4. Taxonomy of *pseudomonas*

The genus *Pseudomonas* belongs to the bacterial family *Pseudomonadaceae* which also contains the genera "*Azomonas*, *Azotobacter, and Cell vibrio, Mesophilobacter*, *Rhizobacter* and *Rugamonas"*. These bacteria are common inhabitants of soil and water. The term *Pseudomonad* is used to describe strictly aerobic Gram-negative, non sporulating bacteria. They are oxidase positive or negative, catalase positive,

non-acid fast rods, which are generally straight, but maybe slightly curved, 0.5 – 1 μm in diameter and 1.5 – 5 μm in length. These bacteria are generally motile, with polar flagella and do not ferment carbohydrates, do not fix nitrogen and are not photosynthetic. Most species fail to grow in acidic conditions (pH 4.5 or lower) and do not require organic growth factors. The optimum growth temperature for most strains is 28ºC, but many are capable of growth between 4-45ºC (Bergey's Manual of Systematic Bacteriology, 2001) Members of the genus *Pseudomonas* are free-living organisms and occupy a dominant position in the biosphere in terms of variety of habitats and the number of species in a given habitat (Todar, 2004) One of the most striking properties of the member's of this genus is their remarkable nutritional versatility. They play an important role in decomposition, biodegradation and the carbon and nitrogen cycles. Organic compounds such as alcohols, aliphatic acids, amides, amines, amino acids, aromatic compounds, carbohydrates and hydrocarbons are all readily used by *Pseudomona* species as growth substrates. In fact the only organic compounds that cannot be attacked by the Pseudomonads are "**Teflon, Styrofoam and one-carbon organic compounds such as methane, methanol, formaldehyde etc."** (Todar, 2004 Bradbury, Richard).

The biological identity of the genus *Pseudomonas* has changed dramatically in recent years during the transition between artificial classification based on phenotypic properties and revisionist classification based on genotypic properties (Todar, 2004).

In the past, *Pseudomonas* species were subdivided on the basis of rRNA homology into five similarity groups (Palleroni, 1986). There were about forty species. More recently only members of Group I was held in the genus *Pseudomonas*. Group I is the largest group, including fluorescent strains such as *P. aeruginosa*, *P. Fluorescence* and *P.putida* and the plant pathogens *P. syringae* and *P. cichorii*. It also includes many important no fluorescent species such as *P. stutzeri* and *P. mendocina*. The members of groups II, III, IV, and V were moved into new or previously existing genera such as *Burkholderia*, *Xanthomonas* and *Comamonas* based on 16S rRNA gene analysis (Bergey's Manual of Systematic Bacteriology, 2001, Bradbury, Richard).

The use of 16S rRNA gene sequence in the classification of bacterial species has now been well established (García-Martínez *et al.*, 2001). It is the part of the DNA now most commonly used for taxonomic purposes for bacteria (Harmsen and Karch, 2004).

16S rRNA coding gene is present in all bacteria and therefore can be used to measure relationships between them. This gene can be compared not only to other bacteria but also with archaeo bacteria and the 18S rRNA gene of eukaryotes function (Clarridge III, 2004) its degree of conservation is believed to result from the importance of the 16 S rRNA as a critical component of cell. Other genes, such as those that make enzymes, can tolerate more frequent mutations because they may affect structures not as unique and essential as rRNA. Therefore, very few genes are as highly conserved as the 16S rRNA gene. Although the absolute rate of change in 16S rRNA is not known, it does mark the evolutionary distance and relatedness of organisms (ClarridgeIII, 2004).

The ribosome is an organelle in cells that assembles proteins. It is composed of both ribosomal RNA and ribosomal proteins, known as the rib nucleoprotein. Ribosoms can be found floating freely in the cytoplasm or bound to the endoplasmic reticulum or the nuclear envelope and are usually found in large number in cells. Ribosomal RNA (rRNA) is the major proportion of cellular RNA and makes up about 65% of the bacterial ribosome (Rodnina *et al*., 2007).

Ribosomal RNAs are at present the most useful and most used of the molecular chronometers (Clarridge III, 2004). Since ribosoms are an essential component of protein synthesis apparatus and the structures are strictly conserved, the DNA component of the small ribosome subunit has been proven extensively to be an important and useful molecular clock for quantitating evolutionary relationships between organisms (Ueda *et al*., 1999). They are useful because they occur in all organisms, and different positions in their sequences change at very different rates, allowing phylogenetic relationships, both close and distant, to be measured, which makes their range all encompassing (Clarridge III, 2004). They are large and they consist of many domains. There are about 50 helical stalks in the 16S rRNA structure

(Figure 1) and almost 100 in the 23S rRNA (Wimberly *et al*., 2000) The number of domains is important because non-random changes affecting one of the units will not appreciably affect the others (Bradbury, Richard).

This is a major advantage of using the larger rRNAs (16S and 23S) over the smaller 5S rRNA (Woese, 1987) Figure 1.

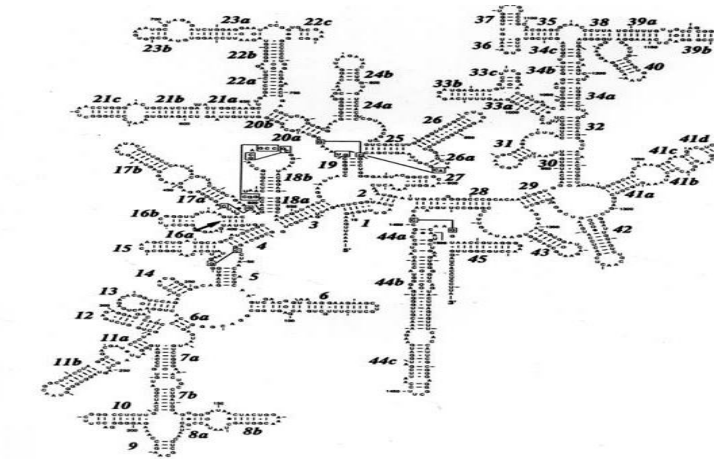


Figure 1. The secondary structure of 16S ribosomal RNA (Gut *et al.,* 1994). The small numbers indicates nucleotide numbers and the large numbers indicate the loop number

Since the development of molecular techniques such as the polymerase chain reaction and DNA sequencing in the 1980s, the phylogenetic structure of bacteria has been studied by comparing sequences of 16S ribosomal RNA. Fox *et al*. (1980) described the process, which changed the way microorganisms were identified and classified. It was identified that the 16S rRNA gene is highly conserved within a species and among a species of the same genus. Nucleotide substitutions occur within ribosomal nucleic acids at steady rate through out evolutionary history (Woese, 1987) some regions of rRNA genes evolve at different rates resulting in regions of nucleotide conservation and variability. The conserved regions allow for the selection of universal primers for PCR amplification of almost all prokaryotes (Bradbury, Richard).

Bacteria can be identified by amplifying the 16S rRNA gene, sequencing it and comparing it to other bacterial sequences in a database, such as Gen Bank, the largest database of nucleotide sequences. The reliability of DNA sequences generated in laboratories has been greatly improved by the introduction of automated sequencing systems and DNA alignment software. However, other factors, such as the purity of the DNA template and number of overlapping nucleotide fragments in the alignment, contribute to the reliability of the final sequence (Sacchi *et al.*, 2002, Bradbury, Richard).

A phylogenetic tree can be constructed which shows the bacterium's position in the evolutionary order based on base differences between species. This process is fast and very accurate and is aided by the large number of available programs and databases. Databases are available that have thousands of 16S rRNA sequences from almost all known genera of bacteria (Zhang *et al*., 2002). Advances in sequencing technology have also increased the speed with which sequence information can be obtained. 16S rRNA gene sequencing is now the gold standard of bacterial identification. It enables the identification of non-cultivable microorganisms and elucidates the relationship between unknown species and known ones (Woo *et al*., 2000, Bradbury, Richard).

Ribosomal RNA sequences do not always coincide with characterizations based on classic taxonomic methods. Whereas genotypic classifications are based on relatively stable and uniform molecular targets, phenotypic classification is subject to variations in morphology, metabolic status and interpretation. When sequence data are included with other methods (e.g. API, Biologic identification kits) in a polyphasic approach, a comprehensive taxonomic and phylogenetic assessment can be obtained (Kolbert and Persing, 1999, Bradbury, Richard).

1.5. Environmental range

P. aeruginosa is ubiquitous in the environment, being found in soil, water, animals and on plant matter (Schmidt, Tummler *et al*. 1996). It is a well-recognized phyto-

pathogen (Kiska and Gilligan,2003), and it has a predilection for moist environments, and thus is often cultured in very high numbers from drains and similar semi-aquatic environments (Grundmann, Schneider *et al*. 1995;Talon, Callous *et al*. 1996; Brisse, Milatovic *et al*. 2000). It has displayed an ability to use varied substrates as a basis for growth. The organism is particularly common in hospital environment; it has been isolated from soap, disinfectants, respiratory equipment, mattresses, endoscopes, distilled water and suction apparatus (Pier and Ramphal 2005). Thus, avoidance of exposure to *P.aeruginosa* in the general environment is impossible. Isolates from hospital environments generally display greater resistance to antimicrobials than environmental samples and are common causes of opportunistic infection in patients (Talon, Cailleux *et al*. 1996; Brisse, Milatovic *et al*. 2000, Bradbury, Richard).

1.6. Antibiotic classes and their targets

Antibiotics (meaning "against life") are molecules that stop microbes from growing or kill them outright.It effect on both bacteria and fungi. Antibiotic that stops bacteria from growing are called bacteriostatics, exemplified by the drug chloramphenicol. Antibiotic that cause bacterial cell-death is called bactericidal. Penicillin and aminoglycosides are examples of these bactericidal agents (Russell and Chopra, 1990) Antibacterial agents can also destroy spores. These are called sporicidal agents (Yildiz, F). Some antibiotics can display bactericidal activity in some circumstances and bacteriostatic activity in others, where sufficient damage to one or more cell pathways or structures occurs that a net bactericidal response is triggered. Some bactericidal agents are also sporicidal and vice versa, but bacteriostatic agents are ineffective against resting spores (Walsh, 2003).

The history of antimicrobial agents started at the beginning of the last century in 1907 with Paul Erlichs research and development of the "magic bullet", Salvarsan, an arsenic derived drug Alexander Fleming in 1928 discovered the effect of penicillin, but it was not until the 1940s that penicillin could be produced as an effective drug (Bentley R, 2005). New antibiotics came at a very quick pace until the late 1960s: sulfonamides, ß-lactams, aminoglycosides, chloramphenicol, tetracycline

macrolides, glycopeptides, lincosamides, streptogramins, trimethoprim, and quinolones between 1968 and 2000 no new class of antimicrobial drug was introduced. The oxazolidin owns lipopeptides and glycyl cyclines (Greer ND 2006, Paterson DL 2006) were introduced in the early 2000. The first two mentioned are designed for Gram-positive bacteria (Bradbury, Richard).

Although a variety of agents targeting Gram negative bacteria are being investigated, none has entered the clinical development phase and it may take 10 to 15 years before any of them may be available for clinical use (Payne DJ 2007, Bradbury, Richard).

Antibiotic agents can either be synthetic chemicals or natural products, designed to block some crucial process in microbial cells' selectivity. They specifically interfere with the biochemical processes of bacteria and hence they can be safely used in mammalians (Todar, 2002). Many of the antibiotics in human clinical use today are natural products. Both bacteria and fungi produce natural antibiotic products, with the major group of antibiotic-producing bacteria being the actinomycetes. Antimicrobial compounds can be antibacterial or antifungal but there are almost no therapeutically useful agents that are effective as both antibacterial and antifungal agents because of different molecular and cellular targets and microbial cell penetration issues (Walsh, 2003) The establishment of infections in humans and animals by a pathogenic bacterium usually involves the following steps: (a) attachment to the epithelial surfaces of the respiratory, alimentary or urogenital tracts; (b) penetration of the epithelial surfaces by the pathogen; (c) interference with, or evasion of, host defense mechanisms; (d) multiplication in the environment of the host's tissues; (e) damage of the host tissues. Antibiotics usually interfere at step (d) either by killing the pathogen or by slowing their growth to the point where host defense mechanisms can clear the infection (Russell and Chopra, 1990).

The worldwide genome sequencing efforts have completed approximately 400 bacterial genomes to date (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

Specifically *pseudomonas* genome data has been indicated in (www.pseudomonas.com). The number of genes in most of these organisms varies from between 1000 and 5000 genes. It is estimated that only between 20 and 200

genes are essential for the survival of most bacteria (Fang *et al*., 2005) Therefore the proteins encoded by these genes are potential targets for antibiotics. Other types of antibiotics interfere with assemblies of these gene products or with structural components that result from their actions.

The cell wall, bacterial envelope or ribosome Known antibiotics interfere with a handful of biochemical processes.These are interference in metabolic pathways, disruption of the integrity of the cytoplasmic membrane, inhibition of protein biosynthesis, inhibition of DNA and RNA biosynthesis and disruption of the biosynthesis of cell wall (Golemi-Kotra, 2002) (Figure 2). A list of antibiotic classes and their targets are shown in Table1.

Table1. Antibiotics and their targets (Adapted from Todar, 2002)

Antibiotic	**Target**
β-lactams	Cell wall synthesis
Glycopeptides	Cell wall synthesis
Quinolones	DNA replication and repair
Aminoglycosides	Protein synthesis
Tetracycline's	Protein synthesis
Macrolides	Protein synthesis
Chloramphenicol	Protein synthesis
Sulphonamides	Folic acid pathway
Trimethoprim	Folic acid pathway
Lipopeptides	Cell membrane

The targets for the antimicrobial substances are despite the large number of drugs surprisingly few (Payne DJ 2007). The main targets are in gross: the cell wall

synthesis and cell membrane, DNA construction and repair, RNA translation and transcription, Protein synthesis and folic-acid metabolism. The basic mechanisms for antimicrobial action on the bacterial cell are shown in Figure 2 Mechanisms of action for the most important groups of antibiotics

Four major targets of antibiotics are cell wall synthesis, DNA replication and repair, protein synthesis and metabolic pathways. These targets and others are illustrated in Figure.2.

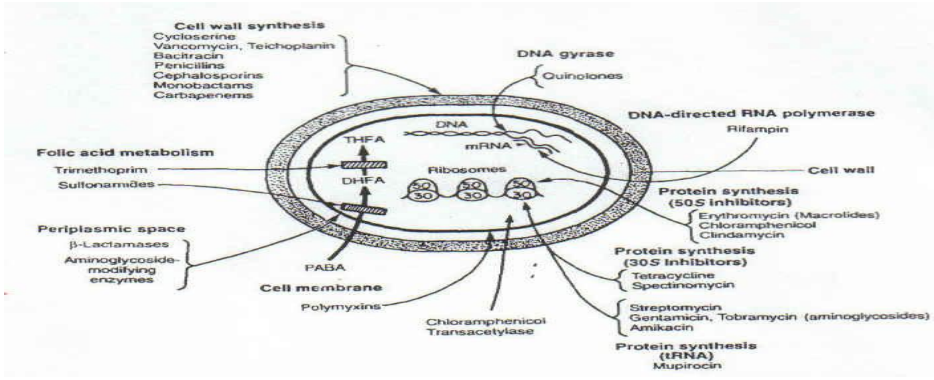


Figure 2. Antibiotics targets in bacteria (Todar 2002)

1.6.1 Antibiotics that act on cell wall biosynthesis

The Gram stain is probably the most widely used staining procedure in microbiology (Forster, 2002) it is a differential stain that differentiates between Gram-positive and Gram-negative bacteria. Gram-positive stain purple and Gram-negative stains pink.

Bacteria such as *Pseudomonas aeruginosa* and *E. coli* are Gram-negative whereas *streptococci* and *staphylococci* are Gram-positive. Gram positive and Gram negative stain differentially, because of fundamental differences in the structure of their cell walls (Figure 3) (Bergey's Manual for Systematic Bacteriology, 2001, Bradbury, Richard).

The bacterial cell wall serves to give the organism its size and shape and also to prevent osmotic lysis. Peptidoglycan (also called murein) is the component of the cell that confers its rigidity. Both Gram-positive bacteria and Gram-negative bacteria have a peptidoglycan layer as part of their cell wall structure. The peptidoglycan layer is substantially thicker and multilayered in Gram-positive bacteria. It is a vast polymer consisting of interlocking chains of identical peptidoglycan monomers.

The monomer consists of two joined sugars, N-acetyl glucosamine (NAG) and N-acetylmuramic acid (NAM), with a pentapeptide coming off the NAM (Figure3). Peptidoglycan monomers are synthesized in the cytoplasmic region of the bacterium where they attach to a membrane carrier molecule called “bactoprenol”.Bactoprenols transport the peptidoglycan monomers across the cytoplasmic membrane and work with enzymes to insert the monomers into existing peptidoglycan enabling bacterial growth following binary fission (Russell and Chopra, 1990).

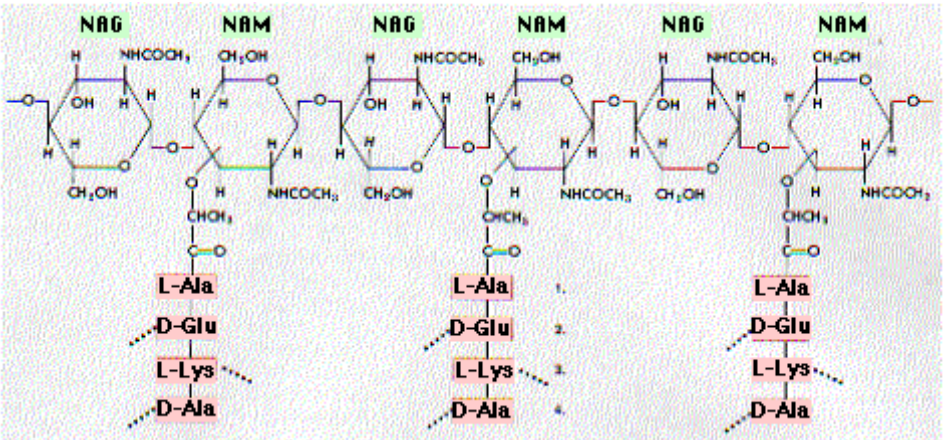


Figure 3. Structure of peptidoglycan (Walsh, 2003)

Firstly, the new peptidoglycan monomers are inserted, glycosidic bonds then link these monomers into the growing chains of peptidoglycan. These long sugar-chains are then joined to one another by means of peptide cross-links between the peptides coming off the NAMs. The peptide cross-links introduce covalent connectivity to the

meshwork, impart mechanical strength and provide the major structural barrier to osmotic pressure forces that could kill the bacterium (Walsh, 2003, Bradbury, Richard).

In order for bacteria to increase in size following binary fission, links in the peptidoglycan monomers must be inserted and the peptide cross-links must be resealed Williams *et al*. (1996) described the synthesis of peptidoglycan (Bradbury, Richard).

Peptidoglycan is made in several stages. The initial reactions occur in the cytoplasm. Further transformations are then effected in the cell membrane and the final incorporation of peptidoglycan into the bacterial cell wall occurs at the point of extension of the cell wall (Bradbury, Richard).

1.6.1.1. Cytosolic phase

The basic unit of peptidoglycan made in the cytoplasm consists of NAM attached to the inactivating nucleotide uridine diphosphate (UDP) and bearing a pentapeptide.N-acetylglucosamine-1-phosphate is first converted into UDP-NAG. The lactic acid residue that converts UDP-NAG into UDP-NAM is incorporated as pyruvate from the glycolytic intermediate phosphoenol pyruvate (PEP), followed by the reduction of this pyruvate to lactate. The lactate is joined by an ether link between its hydroxyl and the 3-hydroxyl of the NAG. The peptide is attached to the carbonyl group of the lactic acid residue. The stepwise addition of three amino acids by peptide bonds forms the tri-peptide derivative of UDP-NAM-tripeptide. The final two amino acids of the pentapeptide are added as a dipeptide of D-alanine, which is synthesized separately by D-Ala-D-Ala synthase. **Fosfomycin** is an antibiotic that inhibits the cytosolic phase of phase of peptidoglycan synthesis (Bradbury, Richard).

1.6.1.2. Membrane phase

The membrane carrier used in the membrane phase of peptidoglycan synthesis is the 55-carbon lipid undecaprenyl phosphate (Und-P), comprising 11 isoprene units. The lipid accepts phospho-NAM-pentapeptide from UDP-NAM-pentapeptide in a

reaction involving translocase I, releasing UMP into the cytoplasm. These lipid complexes in the membrane then accept NAG from cytoplasmic UDP-NAG in the transfer using translocase II, so that the growing peptidoglycan subunit now contains the NAM-β1:4-NAG disaccharide subunit and pentapeptide. **Bacitracin** is an antibiotic that inhibits the membrane phase of peptidoglycan synthesis. It binds to Und-P-P and inhibits the membrane pyro phosphatase that releases undecaprenyl phosphate from the pyrophosphate, causing an accumulation of the lipid carrier in the pyrophosphate form.

1.6.1.3. Cell wall phase

Growing bacteria have lytic enzymes to hydrolyze the mucopeptide locally in order to allow new components to be added at growing points. The peptidoglycan subunit in the membrane is detached from the undecaprenyl pyrophosphate (Und-P-P) carrier and transferred to a growing point in the peptidoglycan by a bond to the NAM-NAG disaccharide. The released undecaprenyl lipid bears pyrophosphate, which must be hydrolyzed by a specific pyro phosphatase to form monophosphate that may again accept UDP-NAM-pentapeptide from the cytoplasm. In the cross-linkage of peptidoglycan by transpeptidation, the side-chain amino group of the pentaglycine of one glycan chain reacts enzymatically with the peptide bond between two D-alanine residues of a pentapeptide from another glycan chain. The reaction involves the migration of the peptide bond and the transfer of a proton from the pentaglycine amino group; free D-alanine is released. There is no requirement for energy input because these enzymes work outside the cell on the periplasmic face of the membrane where ATP and other energy sources are not routinely available (Walsh, 2003, Bradbury, Richard).

1.6.1.4.β -lactams

The β -lactams were the first antibiotics to be discovered and used. Without doubt, the β -lactams are the most important group of drugs that inhibit the final stage of peptidoglycan synthesis (Russell and Chopra, 1990). They are favored because of

their efficacy, broad spectra and low toxicity. All β-lactams are bactericidal agents (Walsh, 2003). The penicillins are derived from the fungus *Penicillium* and modifications made upon the parent compound can alter the drug's spectrum of action. The β-lactam antibiotics include the penicillin (oxacillin, ampicillin, carbenicillin, piperacillin etc. (Table 1).Where the chemical war head, the four member β-lactam ring, is fused to a five-member sulphur ring system (Merck, 2007). Penicillins are primarily active against non- β –lactamase producing, aerobic Gram-negative, some fastidious, aerobic Gram-negative bacteria and some anaerobic bacteria. Amino-penicillins (ampicillin and amoxicillin) are active against additional Gram-negative species, including some members of the *Enterobacteriaceae*. Carboxypenicillins (carbenicillin and ticarcillin) and reidopenicillins (mezlocillin and piperacillin) are active against an expanded list of Gram-negative bacteria including many *Pseudomonas* and *Burkholderia* spp. Penicillinase-stable penicillins (cloxacillin, dicloxacillin, methicillin, nafcillin and oxacillin) are active against predominantly Gram-positive bacteria including penicillinases-producing *staphylococci* (Clinical and Laboratory Standards Institute CLSI (M100-S16, 2006).

The cephalosporins (cephalothin, ceftazidime etc.) are β -lactams in which the β-lactam is fused to a sulphur-containing ring expanded system (Walsh, 2003). Different cephalosporins exhibit somewhat different spectrums of activity against aerobic and aerobic Gram-positive and Gram-negative bacteria. The cephalosporins antimicrobial class includes the classical cephalosporins. Cephalosporins are often referred to as "first-", "second-", "third-" or "fourth-generation" cephalosporins based on the extent of their activity against the more antimicrobial agent-resistant, Gram negative aerobic bacteria. All representatives of a specific group or generation do not necessarily have the same spectrum of activity (Clinical and Laboratory Standard Institute CLSI, M100-S16, 2006, Bradbury, Richard).

The ß-lactam antibiotics are bactericidal cell-wall synthesis inhibitors. Beta lactam drugs are the most widely used group of antibiotics, owing to their high effectiveness, low cost, ease of delivery and minimal side effects (Wilke MS 2005). They include penicillin (e.g. Ampicillin), cephalosporin, mono-bactams, and penems.

The characteristic of the ß-lactam antibiotic structure is the four member lactam ring (Bryskier A 2005) various chemical side chains have been synthetically linked to the ring structures producing antibiotics with different properties. The cell wall of bacteria is a complex structure composed of a tightly cross-linked peptidoglycan net that protects the cell from the osmotic pressure. The ß-lactam antibiotics bind and inhibit enzymes involved in the cross-linking of peptidoglycan: the penicillin-binding proteins (PBP) once cell wall synthesis is inhibited, enzymatic autolysis of the cell wall can occur (Bradbury, Richard).

Other variants of the β-lactam natural products are the penems and monobactams (Todar, 2002). The penems (imipenem and meropenem) structure differs slightly from that of the penicillins. They have a broader spectrum of activity against both Gram-negative and Gram-positive bacteria because they are a lot more resistant to β-lactamase hydrolysis. Monobactam antimicrobial agents are monocyclic β-lactams. Aztreonam is the only approved monobactam antimicrobial agent. It only has activity against Gram-negative aerobic bacteria (Clinical and Laboratory Standard Institute, M100-S16, 2006).

Carbapenems are ß-lactam antibiotics. They have the widest spectrum of antimicrobial activity, including Gram-positive and negative bacteria as well as anaerobic bacteria. They have a side chain with a hydroxyl-ethyl side chain in trans configuration at position 6, which confers stability toward most ß-lactams, including the extended spectrum ß-lactamases(ESBL)(Bonfiglio,G 200).

Clinically available carbapenems are Imipenem, Meropenem and Ertapenem. Ertapenem has no effect on *Pseudomonas aeruginosa*. Carbapenems exert their action in *P.aeruginosa* by binding to protein-binding protein 2 (PBP2) (Yang Y,1995).Carbapenems are indicated for severe infections in the lung, abdomen, central nervous system, septic arthritis and for initial treatment of fever of unknown origin in neutropenic patients. Carbapenems are among the most used antibiotics in Swedish ICUs (Erlandsson, M2000).

There are also antimicrobial agents which are combinations that include a β-lactam and a second agent that has minimal antibacterial activity but functions as an

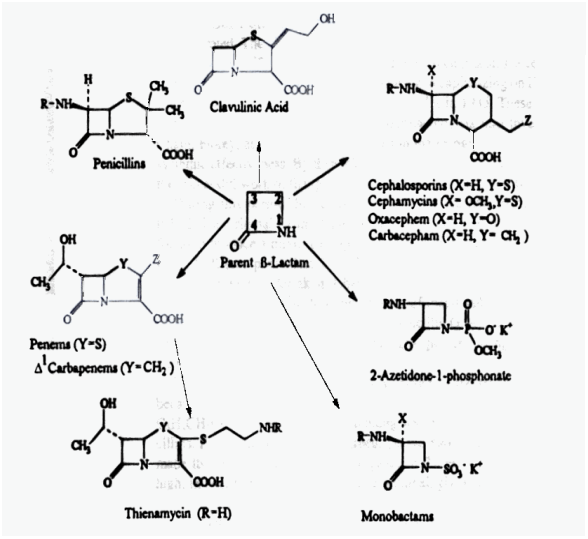
inhibitor of some β -lactamases (Walsh, 2003). Currently, three β-lactamase inhibitors are in use: clavulanic acid, sulbactam and tazobactam. The results of tests of only the penicillin portion of the combination against β-lactamase-producing organisms are often not predictive of susceptibility to the two-drug combination (Clinical and Laboratory Standard Institute, M100-S16, 2006, Bradbury, Richard).

β-lactams stop bacterial cells reproducing by inhibiting the synthesis of a new cell wall, which is essential for the survival of the bacteria. Penicillin, as well as other β-lactams, inhibits the enzyme that places essential cross-links between the individual polymer strings of the cell wall. It does this specifically by using the β -lactam ring to irreversibly block the active site of the enzyme, which catalyzes the reaction, trans-peptidase. This inhibition allows the bacteria to newly synthesize a cell wall and to elongate, but not divide. This is due to the lack of cross-linking. The result is disruption of cell wall integrity, making the cell osmotically unstable and susceptible to lysis (Walsh, 2003).

The β-lactams resemble the sequence of the terminal dipeptide of uncross linked mucopeptide, D-alanine-D-alanine, the natural substrate for the cross-linking enzyme transpeptidase. The –CO-N- bond of the β-lactam ring is the analog of the peptide bond between the two alanine residues of the natural substrate. Penicillin reacts with the transpeptidase to form a stable acyl intermediate.The β-lactam ring acylates the hydroxyl group of one specific serine residue in the transpeptidase, producing an inactive penicilloyl-enzyme complex (Williams *et al*., 1996, Bradbury, Richard)

The transpeptidases "commit suicide" when they start a catalytic cycle with β–lactam antibiotics as substrates, mistaking them for immature peptidoglycans waiting to be cross-linked. The active-site serine adds into the strained four-ring carbonyl and generates an acyl enzyme intermediate in which the β-lactam ring has opened. The enzyme is then stuck in mid-catalytic cycle. The transpeptidases are designed to exclude water from intercepting the normal acyl enzyme intermediates and, therefore the penicilloyl enzyme forms are very slow to hydrolyze. These covalent penicilloyl enzymes build up and are effectively inactive until slow hydrolysis allows it to recover. It may take between hours and days for hydrolysis to occur (Walsh, 2003).

A list of common β-lactam antibiotics and their structure has been shown in Figure 4, 5.



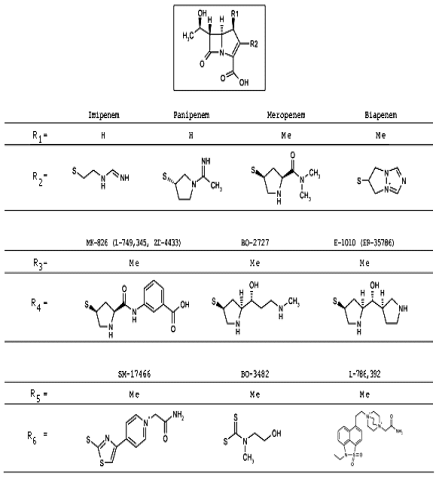


Figure 4. Structure of Beta lactams (Walsh) **Figure5.**Chemical structure of Carbapenems (Walsh)

1.6.2. Antibiotics that block DNA replication and repair

Biosynthesis of DNA and its repair has been targeted by the quinolone class of antibiotics (Oliphant and Green, 2002). Quinolones are a novel group of synthetic antibiotics that were developed in response to the increasing problem of antibiotic resistance. They are derivatives of nalidixic acid, a synthetic quinolone compound.

Quinolones are low molecular weight hydrophilic molecules. Fluoroquinolones such as ciprofloxacin, norfloxacin, sparfloxacin and gatifloxacin have a broad spectrum of activity and are widely used in the treatment of both Gram-negative and Gram-positive infections. The quinolones inhibit the replication of DNA without immediately affecting RNA or protein syntheses in sensitive bacteria. These antibiotics inhibit DNA topo-isomerases, which are necessary for DNA synthesis.

Topoisomerases are essential for cell viability. The DNA topoisomerases change the linking number in super coiled DNA by making transient cuts in the DNA substrate and then passing the DNA to be relaxed topologically through the transient break, either one strand at a time (Type I) or both strands at a time (Type II) Topoisomerase IV is essential for the separation of interlinked daughter DNA molecules. These antibiotics bind to the complex formed between DNA and DNA gyrase or topoisomerase IV, during the replication process. When the replication fork collides with the quinolones-enzyme-DNA complex, its progress is halted and the reformation of the phosphate diester is prevented (Walsh, 2003).

Nalidixic acid causes disintegration of DNA and filamentation of bacterial cells but its toxicity in animals is limited to inhibition of mitochondrial DNA replication. These antibiotics display concentration-dependent bactericidal activity. Nalidixic acid is bactericidal to most of the Gram-negative bacteria but is only useful for treatment of urinary tract infections because it does not achieve bactericidal concentrations in any bodily fluid except urine. The second-generation quinolones, norfloxacin and ciprofloxacin are more effective against a wider range of bacteria. New quinolones such as fleroxacin are active against a wide range of Gram-negative aerobes and moderately effective against Gram-positive aerobes (Williams *et al*., 1996) Figure 6.

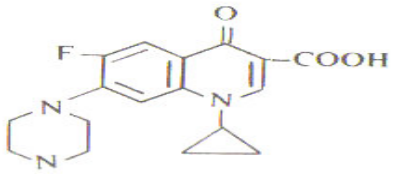


Figure 6. Structure of ciprofloxacin (Walsh, 2003)

1.6.3. Antibiotics that block bacterial protein biosynthesis

Aminoglycosides were originally isolated from soil bacteria including various species of *Streptomyces* and *Micromonospora* (Greenwood, 1995). Schatz and

Waksman (1944) reported the first aminoglycoside antibiotic. It was called streptomycin and proved to be the first chemotherapeutic agent that was effective against *Mycobacterium tuberculosis*. Streptomycin is considered to be the parent molecule for the aminoglycosides but there are now two distinct sub families of aminoglycosides based upon the structure of the aminocyclitol ring. The basic chemical structure required for both potency and the spectrum of antimicrobial activity of aminoglycosides is that of one or several aminated sugars joined in glycosidic linkages to a dibasic cyclitol. In most clinically used aminoglycosides (the larger kanamycin/neomycin group) the cyclitol is 2-deoxystreptamine and it is streptidine in the streptomycin group of aminoglycosides. The kanamycin/neomycin group consists of a central aminocyclitol ring (B ring) with two or three substituted amino glycan rings linked either at the 4 and 5 hydroxyls of the B ring (neomycin, paromomycin, butirosin and lividomycin) or at the 4 and 6 hydroxyls (kanamycin, amikacin,tobramycin and gentamicin) (Smith and Baker, 2002). There have been many more aminoglycoside antibiotics discovered since streptomycin including kanamycin, tobramycin, netilmicin and gentamicin, which have established the aminoglycosides as being very effective against aerobic Gram-negative infections (Gonzalez and Spencer, 1998, Bradbury, Richard).

Chemists have developed semi-synthetic variants that have broader spectra of activity and that are not susceptible to aminoglycoside resistance enzymes. Aminoglycosides exhibit activity against a variety of clinically important Gram-negative bacteria such as *Klebsiella* spp., *Serratia* spp. *Citrobacter* spp, *Enterobacte*r spp., *Proteus* spp. and *Pseudomonas* spp. as well as *Staphylococcus aureus* and streptococci, but they have extremely reduced activity against microorganisms growing in an anaerobic environment (Vakulenko and Mobashery, 2003). Aminoglycosides have varying spectra of antimicrobial activity. For example, gentamicin is more active than tobramycin against *Serratia* spp., whereas tobramycin has greater activity against *Pseudomonas aeruginosa* than gentamicin. The widest spectrum of activity of the aminoglycosides belongs to **arbekacin**, an aminoglycoside that is most commonly used in Japan. It has remarkable activity

against MRSA strains that show no susceptibility to other aminoglycosides (Aoki, 1994).

Aminoglycosides are very useful antibiotics as they have relatively predictable pharmokinetics, a post antibiotic effect, synergism with other antibiotics and have concentration-dependent bactericidal activity (Vakulenko and Mobashery, 2003).

They are commonly used in combination with antibiotics, which inhibit cell wall synthesis i.e., β-lactams and vancomycin, particularly in the treatment of *enterococci, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Gonzalez and Spencer, 1998).

The increased permeability afforded by these antibiotics results in an increase in intracellular uptake of aminoglycosides (Eliopoulos and Moellering, 1996).

Aminoglycosides exhibit a post antibiotic effect (Craig and Gudmundsson, 1996). That means they continue to kill bacteria after the aminoglycoside has been removed following a short incubation with the microorganism. Aminoglycosides show concentration-dependence (Gonzalez and Spencer, 1998). This means that their bactericidal activity depends more on their concentration than on the duration of bacterial exposure to inhibitory concentrations of antibiotic and is also significantly less dependent on the bacterial inoculums size. The killing potential of aminoglycosides therefore increases with increasing concentration of the antibiotic (Vakulenko and Mobashery, 2003).

Aminoglycosides are one of the commonest causes of drug-induced nephrotoxicity (Walker and Duggin, 1988). Nephrotoxicity induced by aminoglycosides manifests clinically as non oliguric renal failure (Mingeot-Leclercq and Tulkens, 1999).

Therefore, aminoglycosides are usually not the first antibiotic of choice. Aminoglycosides are usually administered parentally, although to increase the concentration of the antibiotic at the site of infection or to reduce toxicity, aerosolized tobramycin and gentamicin have been used in cystic fibrosis therapy (Heinz *et al*., 2002).

The RNA and protein machinery of the prokaryotic ribosome is sufficiently distinct from the analogous eukaryotic machinery that there are many inhibitors of protein synthesis, targeting different steps in ribosome action, with selective antibacterial action (Walsh, 2003). Aminoglycoside antibiotics are protein synthesis inhibitors. The ribosome has a complex structure made up of more than 50 proteins and three RNA molecules (Vakulenko and Mobashery, 2003).This complex, along with several GTP hydrolyzing proteinal factors, catalysis protein synthesis. The bacterial ribosome is made up of two subunits, 30S and 50S. The 50S comprises two further subunits, 5S and 23S rRNAs and 33 proteins, while the 30S is made up of a single 16S rRNA and 20 to 21 proteins (Walsh, 2003). Aminoglycoside antibiotics bind to the 30S ribosomal subunit, which plays a crucial role in providing high-fidelity translation of genetic material (Vakulenko and Mobashery, 2003, Bradbury, Richard). The ribosome has three tRNA binding sites: A (amino acyl), P (peptidyl) and E (exit) (Green and Noller, 1997).During protein synthesis; the ribosome decodes information stored in the mRNA and catalysis sequential incorporation of amino acids into a growing polypeptide chain. High fidelity translation is achieved by the ability to discriminate between conformational changes in the ribosome-induced binding of cognate and non cognate tRNA s at the A site (Bradbury, Richard) Aminoglycosides that contain the 2-deoxystreptamine ring increase the error rate of the ribosome by allowing incorporation of the non-cognate tRNA s. The structure of the 30S subunit indicates that two universally conserved adenine residues (A1492 and A1493) are directly involved in the decoding process during normal translation. In the native structure of the ribosome, these adenine residues are stacked in the interior of helix 44. Binding of the tRNA to the A site flips A1493 and A1492 out from their stacked position. It also flips G530 out from the syn to the anti-conformation. The N1of adenines interacts with the 2’-OH groups of the tRNA residues that are in the first and second positions of the codon-anticodon triplet (Vakulenko and Mobashery, 2003).

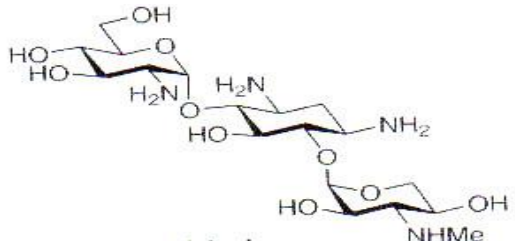


Figure 7.Structure of gentamicin (Walsh, 2003)

Aminoglycosides that contain the 2-deoxystreptamine ring bind to the major groove of helix H44 of 16S rRNA (Figure7). These results in the flipping out of the same conserved A1492 and A1493 residues that are normally displaced upon binding of the cognate tRNA. The conformational changes induced in the 30S subunit by binding of the cognate tRNA are energetically favorable because they allow the ribosome to participate in a greater number of compensating interactions between the codon and anticodon double helixes (Vakulenko and Mobashery, 2003). Because the flipping out of the adenine residues might require energy expenditure, aminoglycoside-induced flipping-out can reduce energetic cost, allowing binding of near-cognate t-RNAs and subsequent mistranslation of mRNA (Ogle *et al*, 2001, Bradbury, Richard). Streptomycin has a different structure to that of other amino-glycosides but it binds at the functional center of the ribosome in close proximity to the binding site of other aminoglycosides. Like other aminoglycosides, it induces mis-reading of the genetic code, but the mechanism is different. During translation, the 30S subunit switches between two distinct conformations. It has been shown that mutational stabilization of one of the conformations over the other results in two different fidelity phenotypes. One increases fidelity and the other decreases fidelity, i.e., ram (ribosomal ambiguity) or error-prone. The interaction of streptomycin with the ribosome is thought to preferentially stabilize the ram state. This stabilization lowers the affinity for t RNAs and allows binding of near-cognate tRNA s, which renders the A-site more promiscuous. It could also affect the proof reading by making transition to the restrictive site more difficult (Vakulenko and Mobashery, 2003, Bradbury, Richard).

The passage of aminoglycosides (highly polar molecules) across the outer membrane of Gram-negative bacteria is a self-promoted uptake process involving the drug induced disruption of Mg2+ and Ca2+ bridges between adjacent lipopolysaccharide molecules. Penetration through porin channels is unlikely because of the large size of aminoglycosides (Mingeot-Leclercq *et al*., 1999). Uptake of aminoglycoside antibiotics across the bacterial cytoplasmic membrane is essential for antibacterial activity and is similar in Gram-positive and Gram-negative bacteria. Aminoglycoside uptake is multi-phasic with three distinct phases, one of which is energy-independent (EIP) and the remainder being energy-dependent (EDPI and EDPII) (Russell and Chopra, 1990).

The EIP phase of uptake occurs very rapidly and represents the initial binding of antibiotic to bacteria. Although, in Gram-negative bacteria, this partially represents interaction with the outer membrane, uptake during EIP also represents binding of drug molecules to the cytoplasmic membrane. EDPI represents a slow, but poorly characterized, energy-dependent uptake of drug molecules across the cytoplasmic membrane. A threshold trans-membrane potential generated by a membrane-bound respiratory chain is required for the uptake of aminoglycosides during EDPI. This is why anaerobes are resistant to these antibiotics. EDPII, associated with progressive binding of aminoglycosides to ribosomes within the cell, results in an acceleration of uptake seen towards the end of EDPI. Aminoglycosides virtually irreversibly saturate all ribosomes causing cell- death. It is not known whether the third phase of uptake neither involves a transport carrier, nor is even the exact nature of the energy source driving EDPII mediated aminoglycoside uptake (Russell and Chopra, 1990, Bradbury, Richard).

1.6.4. Antibiotics that act on metabolic pathways

Folate is a coenzyme essential for cell growth. However, bacteria cannot transport folate and have to synthesis it *de novo*. Eukaryotes cannot synthesize folate and instead scavenge it from dietary sources and transport it into cells. Therefore

selective inhibition can be achieved (Greenwood and Whitley, 2003, Bradbury, Richard).

Sulphonamides inhibit the incorporation of p-amino benzoic acid (PABA) into a precursor of dihydrofolic acid (DHF) that should then be reduced by the enzyme dihydrofolate re-educates (DHFR) to tetrahydrofolic acid (THF) THF, which is a derivative of folic acid is an important coenzyme involved in the transfer of small residues containing a single carbon atom (e.g. methyl, formyl) in intermediary metabolism. THF derivatives are also required for the synthesis of the amino acid methionine and of the nucleic acid bases, including thymine. DHF is synthesized in two stages. Firstly, dihydropteroic acid synthase catalysis the combination of pteridine derivative with PABA.This reaction is inhibited by sulphonamides. This is followed by the condensation of glutamic acid with dihydropteroic acid (Todar, 2002).

Trimethoprim inhibits the conversion of dihydrofolate to tetrahydrofolate. This limits the supply of some amino acids and nucleic acids. There is therefore a lag time between the administration of sulphonamides and the cessation of bacterial growth, which corresponds to the time taken for the bacteria to use up the stocks of biosynthetic components and folic acid already present in the cell. These drugs are bacterio-static drugs since it may take some generations for the foliate pool in the bacteria to decrease (Williams *et al*., 1996). They are active against both Gram negative and Gram-positive organisms (Clinical and Laboratory Standard Institute, M100-S16, 2006). These antibiotics are usually administered as co-trimoxazole, which is a combination of the two antibiotics. A diagram showing the sites of action of sulphonamide and trimethoprim in the folic acid synthesis pathway can be seen in Figure 8.

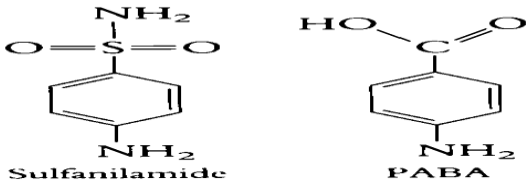


Figure 8. Structure of sulphonamide (left) and p-amino benzoic acid (PABA) (right) (Walsh, 2003)

1.7. Antibiotic Resistance

For millions of years, bacteria in the environment have been secreting specific compounds toxic to other bacterial cells. Soil bacteria, for example, are extremely prolific secretors of bactericidal chemicals such as aminoglycosides. However, these compounds are not toxic to the microorganisms that produce them. To overcome this threat, the bacteria that secrete these compounds have developed built in self-defense mechanisms, specifically, enzymes that deactivate these compounds. As a result, the bacterium can produce its toxins and, immune from their effects, gain an advantage over its neighbors. However, over time, those neighboring bacteria have incorporated some of the same enzymes into their genomes, in response, so that they are then able to deactivate the antimicrobials secreted by other bacteria. This has been helped by the fact that the genes encoding such enzymes are generally found on trans-posons and plasmids. Although this is essential for the survival of the bacteria, it is now seen as an example of acquired resistance (Smith and Baker, 2002, Bradbury, Richard).

The past half century was an extraordinarily successful period in medical history during which, most human diseases were brought under control by antibiotics.
However, the increasing number of bacteria that are becoming resistant to antibiotics is now threatening this situation (Mazel and Davies, 1999). Resistance is only just beginning to be considered as a societal issue. Resistance is not a new phenomenon. It was recognized early on as a scientific curiosity and then as a threat to effective treatment. Resistance costs money, livelihoods and lives and threatens to undermine the effectiveness of health delivery programs. Deaths from acute respiratory infections, diarrheal diseases, measles, AIDS, malaria and tuberculosis account for more than 85%of the mortality from infection worldwide. Antimicrobial use is the key driver of resistance (World Health Organization, 2001). Every time a patient takes an antibiotic for a bacterial infection; the antibiotic might kill most of the bacteria. But a few tenacious germs may survive by mutating or acquiring resistance genes from other bacteria. These surviving genes can multiply quickly, creating antibiotic resistant strains. The presence of these strains may mean that a patient's next infection will not respond to the first-choice antibiotic therapy.

The resistant bacteria may then be transmitted to others in the patient's community (Nordenberg, 1998).

The greatest risk is for patients in hospitals, nursing homes and other settings where people tend to be sick and are taking large quantities of antibiotics, increasing the chance of antibiotic resistant bacteria originating in their own bodies. Hospitalized patients are also in contact with others whose infectious diseases may spread and their immune systems may be weakened and unable to fight infections (Loeb *et al*., 2003).

Antibiotic resistance is not a new problem. Ever since the discovery and subsequent clinical use of antibiotics, resistance to these agents has been observed with a negative impact on the treatment of infectious disease. The growing problem of antimicrobial resistance has become a significant public health concern. It is no longer an isolated problem of a few bacteria; almost all important human pathogens once treatable with antibiotics have developed some resistance (Nordenberg, 1998). It involves almost all types of pathogens, including bacteria, fungi, mycobacterium, viruses and parasites.

Antibiotic resistance is now a major problem in the treatment of infections in hospitals and, with increasing and alarming frequency, in the community. For example, all strains of *Neisseria gonorrhea* were susceptible to sulphonamides when these were first employed in 1938. By 1948 resistance had built up so much that less than 20% of strains were susceptible (Walsh, 2003). Methicillin was developed to control *staphylococci*, but there are now increasing cases of methicillin-resistant *S. aureus* (MRSA) in many countries. Resistant strains have normally been controlled by vancomycin but resistance to this antibiotic is beginning to be identified. Some of these strains are still susceptible to other antibiotics but there is concern that a fully resistant strain of *S. aureus* will soon emerge (Patterson, 1999, Bradbury, Richard).

These strains are still treatable with other types of antibiotics but another serious problem is beginning to emerge, that of multi-drug resistance. There has been a recent resurgence in Mycobacterial diseases such as tuberculosis. Isoniazid, rifampin,

ethambutol and streptomycin are successful in the treatment of tuberculosis but have to be used in multidrug treatments over long periods. This has contributed to the emergence of multidrug resistant strains of *M. tuberculosis* in these patients and, more disturbingly, the spread of these resistant strains into hospitals, as reported by Russelland Chopra (1990).

1.7.1. Causes of resistance to antibacterial agents

In less than two human generations antibiotics have revolutionized medicine, but, by selecting resistance, they carry the seeds of their own obsolescence. Resistance to antibiotics is an increasing global problem and a public health threat. It has resulted in morbidity and mortality from the failure of treatments and the increased costs of treating patients with infections caused by antibiotic resistant bacteria (World Health Organization, 2001, Bradbury, Richard).

The indiscriminate usage of antibiotics in agriculture and particularly in the hospital setting can promote the survival of resistant bacteria and, in addition, impact on other normally innocuous bacteria, encouraging the development of drug-resistant strains of these species (Smith and Baker, 2002). Antibiotics are undoubtedly beneficial when used correctly, but doctors and members of the public frequently use antibiotics in appropriately. Examples of inappropriate use of antibiotics include: doctors prescribing antibiotics to treat viral infections such as colds and flus; failing to accurately identify the bacteria causing an infection; unnecessarily prescribing expensive, broad-spectrum antibiotics; not following established recommendations for using chemo prophylaxis; the availability of antibiotics over the counter in many countries and the failure of patients to complete their full course of antibiotics. All of these promote the survival of antibiotic-resistant bacteria (World Health Organization, 2001). It is essential that more stringent controls be placed on the use of antibiotics, since careful prescribing can halt the emergence of resistant strains (Smith and Baker, 2002 Bradbury, Richard).

While over use of antibiotics in human medicine is a major contributor to antibiotic resistance, agricultural use of antibiotics also contributes to the problem.Meat producers use an estimated 70 percent of all U.S. antibiotics and related drugs non-therapeutically, such as in feed additives to promote faster growth of an animal and to compensate for unsanitary and crowded conditions. The amount of antibiotics used non-therapeutically in agriculture is eight times greater than the amount used in all human medicine (Mellon *et al.*, 2001). The non-therapeutic use of antibiotics in agriculture is at a low-concentration over a long period of time. Long-term exposure to low antibiotic concentrations is the condition most likely to foster stable maintenance of resistance genes because it gives incoming elements and resistance genes a chance to adapt to their new host (Salyers and Amábile-Cuevas, 1997). Many of the antibiotics used in agriculture are also used to treat humans. The antibiotic resistant bacteria can be transferred to humans via food, contaminated soil and water and through contact with the animals (Mellon *et al*., 2001).

This inappropriate use of antibiotics exerts a selective pressure that acts as a driving force in the development of antibiotic resistance (Barbosa and Levy, 2000). As resistance to these first-line antibiotics occurs, therapy with new, broader spectrum and more expensive antibiotics increases. Increased usage of these antibiotics eventually leads to resistance to the newer drugs (Hart, 1998). However, all antibiotic use whether appropriate or inappropriate exerts selective pressure for the emergence of resistant bacteria (Seppäla *et al*., 1997). Our only means of handling the situation is through prudent use of antimicrobial agents, improved diagnostics and infection control (Fishman, 2006 Bradbury, Richard).

Resistance factors, especially those carried on mobile elements, can spread rapidly within human and animal populations. These mobile elements enable resistant bacteria to rapidly spread both locally and globally. Antibiotic resistance patterns vary locally and regionally and so surveillance data is collected from selected sources (Agustín *et al*., 2005). The data obtained from this surveillance can indicate if doctors are appropriately or inappropriately prescribing antibiotics. Although a few studies (Rice *et al*., 1990), (Seppälä *et al*., 1997) have suggested that resistant clones

can be replaced by susceptible ones, in general, resistance is slow to reverse or is irreversible (World Health Organization, 2001).

1.7.2 Mechanisms of antibiotic resistance

Bacterial resistance to antibiotics occurs in many ways, reflecting the different ways in which the various classes of antibiotics have an effect. Antibiotics fall into the following classes: cell wall synthesis inhibitors, protein synthesis inhibitors, nucleic acid synthesis inhibitors, cytoplasmic membrane function inhibitors and other agents that affect DNA and RNA (Williams *et al*., 1996, Bradbury, Richard).

"Resistance to antimicrobial drugs in bacteria can be intrinsic, or acquired. Acquired resistance is caused by genetic alterations leading to protection of the bacteria from the action of an antibiotic drug. The mechanisms of acquired resistance are multiple and varied but can be divided into four main principles (Hawkey PM, 2000):
- Inactivation of the antimicrobial drug (i.e. ß-lactamase)
- Change in target for antibiotic action: mutations in the target (i.e. PBP mutations),
Production of alternative targets or protection of the target,
-Changed access (i.e. down regulation of porins),
-Extrusion of the antibacterial agent (i.e. efflux pumps)",
Three major ways that bacterium avoid from the effect of ß-lactam drugs:

1.7.2.1. Production of ß-lactamases

"ß-lactamases are enzymes that hydrolyze the amide bond of the ß-lactam ring of the antibiotic", they there by render the drug inactive before it reaches the PBP target (Basic M 2006). They constitute the most common mechanism of resistance in Gram-negative bacteria. The ß-lactamase genes are often integrated within mobile genetic elements, such as transposons or plasmids, and can therefore easily be transferred between bacteria. Their expression is often induced by ß-lactam antibiotic (Wilke MS 2005).There is an immense and increasing number of different ß-lactamases that can hydrolyze different ß-lactam antibiotics. There are two different

classification systems for ß-lactamases: based on “amino acid sequence (Ambler classes A to C) or substrate inhibitor profile” (Bush-Jacoby-Medeiros group 1 to 4) Of particular concern, are the class C cephalosporinase (AmpC), the extended spectrum beta lactamases (ESBL) and the carbapenemases as they are able to target most beta lactams (Babic,M 2006) AmpC (Ambler class C) is a class of chromosomal ß-lactamases found in several Gram-negative bacteria (like *Pseudomonas*).

AmpC can be induced by ß-lactam antibiotics in *Enterobacter, Serratia, Citrobacter* and *Pseudomonas* and degrade cephalosporin. Ceftazidime and other cephems are stable against AmpC enzyme hydrolysis (Kazmierczak, A2005). AmpC can also be derepressed by mutations in regulator genes resulting in selection of derepressed mutants over-expressing AmpC (Juan, 2006). Examples of ampC genes being mobilized on plasmids and spreading to species normally not carrying the ampC gene has been observed (Philippon, A2002, Bradbury, Richard) .
High levels of AmpC exert resistance to penicillin, beta-lactamase inhibitors, cefoxitin and ceftazidime (Babic M 2006, Bradbury, Richard).
Most ESBL are the result of genetic mutation from other beta-lactamases (i.e.TEM-1, TEM-2and SHV-1), resulting in a “novel” beta-lactamase able to hydrolyze cefotaxime, ceftazidime and aztreonam. CTX-M ESBLs arose by plasmid acquisition of pre-existing chromosomal ESBL genes from the *Kluyvera* spp (Babic M 2006). ESBL can be plasmid mediated, and thus capable of spread. ESBL-producing isolates remain susceptible to carbapenems (Pfaller, MA2006, Bradbury, Richard).

1.7.2.2. Control of ß-lactam intracellular concentration

Some ß-lactams enter the bacteria via porins in the outer membrane. If the amount of porins is significantly decreased or the porins have a structural change, the antibiotic may no longer be able enter the bacteria and resistance develops (Bradbury).
Another way bacteria can keep the intracellular concentration of the drug low is to transport the antibiotic out of the cell by the effect of efflux pumps. Five families of efflux systems have been described. They are the ATP-binding cassette family, the

resistance-nodulation-division family (RND), the multidrug and toxic compound extrusion family and the small multi drug resistance family. RND is thought to be the most involved in ß-lactam resistance (Wilke MS2005, Bradbury).

1.7.2.3. Altered target for ß-lactam action

The target of the beta lactam antibiotic, the penicillin binding proteins (PBP), can by several mechanisms acquire induced resistance: acquisition of a“ new” less sensitive enzyme, mutation of an endogenous PBP so that it exerts less affinity for the antibiotic drug or up regulation of expression of PBP (Wilke MS, Bradbury, Richard).

1.7.2.4. *Pseudomonas* and Carbapenem Resistance

P. aeruginosa infections are difficult to treat because of high intrinsic resistant to many antibiotics and a high risk of emergence of resistance during therapy. The carbapenems, including meropenem and imipenem, are among the few therapeutic options still available for treating infections caused by *P. aeruginosa* (Bradbury, Richard).

1.7.2.5. Carrbapenem Resistance Mechanisms (molecular aspect)

The main known mechanisms of resistance to carbapenems in *P. aeruginosa* are through control of intracellular concentration of the antibiotic: alterations in or decreased production of outer membrane porin OprD (Pai H 2001), multi-drug efflux pumps (Li XZ, 2004) or hydrolysis by metallo ß-lactamases (MBL) (Li XZ, 2004, Walsh, TR2005, Livermore DM, 2001, Bradbury, Richard).

A study done by El Amin *et al* (El Amin N,2005)“indicated that also other mechanisms were involved in imipenem resistance, perhaps through mutations in genes for penicillin-binding proteins” (PBP).

As *Pseudomona*s has an “outer membrane with low permeability, many substrates necessary for growth have to utilize specialized pathways: thus the variety of gated

channels; the porins. *P.aeruginosa* has 3 large families of poring; the OprD family of specific porins, the OprM family of efflux porins, and the TonB-interacting family of gated porins" (Hancock, RE2002). OprD porin plays a major role in imipenem resistance (Livermore DM2001) Mutations in loops 2, 3 of the OprD protein (Huang H1996), lead to imipenem resistance. Changes in loop 5, 7 or 8 have been found to expand the channel, thus leading to hyper susceptibility. "The most important mechanism of resistance to imipenem in clinical strains is down-regulation of OprD (Pai H 2001). Loss of the porin OprD raises the imipenem minimal inhibitory concentration (MIC) from 1-2mg/L to 8-32mg/L (Livermore DM 2001), but does not affect meropenem susceptibility much. OprD is regulated by multiple systems; it is repressed by salicylates and catabolite repression, and activated by arginine and a variety of other amino acids" (Hancock RE 2002) MexT (PA2492) is a transcriptional repressor that down-regulates oprD and up-regulates genes for the efflux pump MexEF-OprN (so called NfxC class mutants) Mex EF-OprN efflux pump mediates resistance to several antibiotics, including quinolones. MexS (PA2491) [97] and mvaT (PA4315) (Westfall LW 2006) have similar effects.

1.7.2.6. Multi-drug efflux pumps

These efflux systems are composed of three proteins physically linked. "The systems include a pump located in the cytoplasmic membrane (e.g. MexB), an outer membrane porin (e.g. OprM) and third protein (e.g. MexA), that physically link the two other components".

Efflux pumps, all belonging to the resistance nodulation family (RND) are also involved in carbapenem resistance:

MexAB-OprM, MexCD-OprJ and MexXY (Li XZ 2004) MexAB-OprM is the one that functions primarily and effectively in the extrusion of penems (mainly meropenem) while MexCD–OprJ holds a compensatory mechanism and MexXY has a small impact (Okamoto K 2002).

1.7.2.7. Carbapenemase (ß-lactamases)

Carbapenems are stable to almost all clinically relevant ß-lactamases, but there are exceptions: the class A carbapenemases and the metallo ß-lactamases (MBL) The class A carbapenemases include chromosomal, integron or plasmid encoded enzymes. Over the past years the most notable expansion group has been the plasmid encoded carbapenemases (Bradbury, Richard).

The most important of the plasmid serine carbapenemases are the *Klebsiella pneumonia* carbapenemase and the OXA-type carbapenemase (Babic M2006). Metallo ß-lactamases (Ambler class B) are enzymes that use one of two zinc atoms for inactivating penicillins and cephalosporin. In bacteria, MBL confers resistance to carbapenems, cephalosporins and penicillin. MBL are found on a variety of genetic elements (chromosome, plasmid and integrons). Bacteria possessing MBLs are among the most resistant phenotypes encountered by clinicians. MBL are the carbapenemases found in *P. aeruginosa*, and little is known about their dissemination and spread (Bradbury, Richard).

1.7.2.8. Carbapenem (Penicilin) Binding Proteins

The bacterial peptidoglycan is a three-dimensional netlike mesh that lines the exterior of the cell membrane. It protects the bacteria from osmotic shock, determines the cellular shape, and serves as attachment sites for virulence factors and adhesions. Synthesis in an untimely manner or erroneously will lead to fragility or instability of the bacterial cell. PBP catalyze the final stages of the peptidoglycan synthesis within the peri-plasm (Macheboeuf P 2006, Bradbury, Richard).

Bacteria have multiple PBPs with different roles during cell division. There are high molecular mass (HMM) and low molecular mass (LMM) PBPs. HMM PBPs are divided into class A and B. Class A are bi-functional enzymes that catalyze both the polymerization of the GlcNAc-MurNAC chains (Glycosyltransferase, GT) and the cross linking of adjacent stem peptide (Transpeptidation,TP) reactions. Class B is mono-functional and present only TP activity (Goffin C 200, Bradbury, Richard).

LMM-PBPs catalyze a carboxypeptidation reaction that prevents further cross

linking of the peptidoglycans and are thus involved in the regulation of peptidoglycan reticulation (Morlot, C 2004, Bradbury, Richard).
There are not many studies on *P. aeruginosa* PBPs. Up to date, there seem to be 8 different PBPs, PBP-1a,-1b,-2,-3,-3a -4 -6 (or 5 depending on the nomenclature), -7 that are homologues of *E. coli* PBPs -1a,-1b, -2,-3, -4,-5 and -7 (Liao, X 1997, Liao, X 1995, Song J 1998, Handfield J1997).

1.7.2.9. Resistance to Qinolones

"Resistance to quinolones occurs through mutations in the genes encoding DNA gyrase (topoisomerase II) and topoisomerase IV subunits gyrA and gyrB, or parC and parE respectively (Hawkey PM 2003) Resistance mediated by these mutations is enhanced by porin structure changes and efflux pump activity (Lindback,E 2006) Another more recently discovered mechanism, is a plasmid mediated quinolone resistance (PMQR) gene encoding a Qnr protein capable of protecting DNA gyrase from quinolones (Robicsek,A2006). These resistance mechanisms are additive, and can be combined. In Gram-negative bacteria, DNA-gyrase tends to be the primary target for fluoroquinolones. Mutations in gyrA are found in isolates with low-level resistance, where as higher minimal inhibitory concentrations (MIC) are associated with additional mutations in parC, gyrB, parE and expression of efflux pumps (Woodford2007) The PMQR confers low level resistance to quinolones in itself, but increases resistance levels if combined with another resistance mechanism. Another aspect of plasmid borne resistance is co transmission of PMQR, amino glycoside-modifying enzymes, broad spectrum ß-lactamases and even carbapenemases" (Nazic H 2005, Bradbury, Richard) .

Resistance can be acquired (also known as active) (i.e., the result of a specific evolutionary pressure to adapt a counter attack mechanism against an antibiotic) or passive (also known as innate) (where resistance is a consequence of general adaptive processes that are not necessarily linked to a given class of antibiotic; e.g., the nonspecific barrier afforded by the outer membrane of Gram-negative bacteria) (Wikens and Wade, 2005).

Acquired resistance results from: mutations-spontaneous single or multiple changes in the bacterium's chromosomal DNA occurring at a rate of about 10^9 to 10^5 per cell, per generation cycle (a generation cycle commonly occurs every 20 minutes or so), or by the addition of new DNA most commonly by the acquisition of plasmids, transposons and integrons (Masterton, 2003, Bradbury, Richard**).**

The major advantage, in terms of dissemination of resistance, of mutations is that the progeny stably inherits them (Courvalin, 1996). Bacterial enzymes that are involved in normal physiological cell metabolism can, as a result of single or multiple mutations in their genetic determinants change their substrate spectrum and degrade antibiotics (Martinez and Baquero, 2000). Bacteria can become resistant to many antibiotics by modifying their target structures by single- or multi-step mutations, so that antibiotics cannot bind to them. These mutations usually have little or no influence on the biological activity of the gene products but render them insensitive to the inhibitory activities of a particular antimicrobial agent. Once established in a pathogen, genes encoding enzymes that catalyze covalent modifications of therapeutic agents can undergo mutations that remodel the active site of the enzyme, changing the spectrum of antibiotics that may be modified (Davies, 1994, Bradbury, Richard).

The genetic determinants of defense mechanisms may originate from bacteria such as antibiotic producing bacteria. The antibiotic producers possess defense mechanisms against their own antibiotic and these resistance genes are usually found in their chromosomes. These genes can be integrated into mobile genetic elements such as plasmids, transposons and integrons and passed on by horizontal transfer to other bacteria, thus conferring antibiotic resistance to those bacteria (Masterton, 2003).

Plasmids are extra chromosomal, double stranded DNA molecules that have been found in almost all bacterial genera of medical importance. They vary in size from 2kbp to more than 100 kbp. Plasmids are capable of autonomous replication within the bacterial cell. They encode the mechanism for their own mobilization and are

therefore excellent vehicles for transferring genes, not only to their progeny but also from one bacterium to another (Schwarz and Nobel, 1999). Plasmids generally carry genes that play a role in the bacterium's adaptation to a change in its environment, such as those involved in resistance to antibiotics, disinfectants and heavy metals (Dobrindt *et al.*, 2004). However, they also encode metabolic properties such as metabolism of carbohydrates and amino acids, virulence factors such as haemolysins and enter toxins and conjugal properties such as sex pili production and mobilization function. Plasmids may be integrated in part or in total into the chromosomal DNA or may represent vectors for transposons via conjugation or transformation (Schwarz and Nobel, 1999 Bradbury, Richard).

Transposons are double-stranded DNA elements which, in contrast to plasmids, cannot replicate autonomously. They carry transposition genes along with other genes. They have to integrate into replication-proficient vector molecules such as plasmids or chromosomal DNA for replication. They can insert more or less at random into plasmids or bacterial chromosomes (Masterton, 2003). They vary in size from 1 kbp to more than 60 kbp (Schwarz and Nobel, 1999). Transposons carry one or more additional genes most of which are genes conferring resistance to antibiotics, which can then 'jump' between the bacterial chromosome and a plasmid and vice-versa (Roy, 1999).

Integrons are another vehicle by which antibiotic resistance determinants can be passed on from one bacterium to another. Antibiotic genes are frequently trapped ingene cassettes on integrons, which provide an efficient means for capturing and exchanging various resistance genes (Van Belkum *et al*., 2001). Recruiting exogenous genes represents a rapid adaptation against antimicrobial compounds, and the integron functional platform is perfectly suited for capturing the genes that enable bacteria to survive during multiple antibiotic treatment regimens (Mazel, 2004). Integrons have been almost exclusively found in Gram-negative bacteria (Nešvera *et al*., 1998).

Integrons recognize and capture mobile gene cassettes. Gene cassettes consist of a specific recombination site and one gene that is in most known cases an

antimicrobial resistance gene (Hall and Collis, 1995). The resistance integron platforms don't allow for self-transposition. However, this defect is often complemented through association with transposons and conjugative plasmids, which can serve as vehicles for the transmission of genetic material between bacteria (Mazel, 2004, Bradbury, Richard).

Integrons possess two essential elements, located at the 5' conserved segment (CS), able to mobilize and insert gene cassettes. These are an *intI* gene encoding a site specific recombinase belonging to the integrase family and its associated primary recombination site, *attI*. Class 1 integrons also possess a quaternary ammonium compound resistance gene, located at the 3' conserved segment (Collis and Hall, 1995). Captured genes (usually antibiotic resistance genes) are part of discrete mobile cassettes that contain the protein-coding region and a 3'-associated integrase-specific recombination site known as *attic*, belonging to the family of sites known as 59-base elements (Recchia and Hall, 1995). Un-captured gene cassettes exist in their free formas circular molecules consisting of only one open reading frame and a 59-base element situated downstream. Integration of these gene cassettes involves IntI catalyzed site-specific recombination between the integron associated *attI* site and the *attC* (59-base element) recombination site associated with the incoming gene cassette (Collis *et al*., 1998). Each gene cassette has a unique 59-base element, which differs in both sequence and length (Collis *et al*., 1998).The *attC* sites vary from 19 bp to141 bp and their nucleotide sequence similarities are primarily restricted to the inverse core-site and the core-site (Mazel, 2004). The 59-base element family exhibits a common inverted repeat structure and consensus sequences at each end that consists of approximately 25 bases (Collis *et al*., 1998, Bradbury, Richard).

The outer boundaries of the 59-base element also contain the conserved seven base pair core site GTTRRRY (R = purine,Y = pyrimidine) at the recombinant cross-over point, and an inverse core site RYYYAAC at the 3' end of the inserted gene cassette (Poirel *et al*., 2001, Bradbury, Richard).

Integrons can be divided into two major groups, resistance integrons and super-integrons (Hall and Stokes, 2004). Resistance integrons carry mostly gene cassettes that encode resistance against antibiotics and disinfectants, and can be located either on chromosome or on plasmids (Fluit and Schmitz, 1999). The larger chromosomally–located integrons that contain gene cassettes with a variety of functions belong to the super-integron group. The super-integrons were initially known as class 4 integrons (Shi *et al*., 2006).

There are three different classes of resistance integron, class 1, 2 and 3, defined on the basis of homology of their integrase genes (Norrby, 2005). Each class appears capable of sharing and acquiring the same gene cassettes (Mazel, 2004). Most resistance integrons belong to class 1 and these integrons have been found in many Gram negative genera including *Pseudomonas* (Fluit and Schmitz, 2004). More than 60 different gene cassettes have been described, the majority of which encode resistance to antibiotics (Lindstedt *et al*., 2003). Class 1 integrons are the most prevalent class in clinical isolates, carrying single or multiple gene cassettes (Weldhagen, 2004, Bradbury, Richard).

Integron inserted genes encode for various antibiotic resistance mechanisms, conferring resistance to beta-lactams, aminoglycosides, sulphonamides, chloramphenicol, macrolides, rifampin, erythromycin, disinfectants and antiseptics of the quaternary ammonium compound family (Weldhagen, 2004). Class 1 integrons are frequently located on plasmids that can be transferred by conjugation (Girlich *et al*. 2001).

Class 2 integrons are embedded in the Tn7 family of transposons and consist of an integrase gene followed by gene cassettes. Class 3 integrons have been described in some isolates from Japan and are similar in structure to class 2 integrons (Fluit and Schmitz, 2004, Bradbury, Richard).

Class 1 integrons are associated with a variety of resistance gene cassettes. Class 1 integrons isolated from bacterial infections in humans often harbor gene cassettes encoding ß-lactam resistance (Fluit and Schmitz, 1999). Several classA β-lactams are encoded by integron-located gene cassettes. These include VEB, GES, IBC, PSE and CTX-M type enzymes. Two families of class B β -lactamases have been found on integrons. These are the IMP family and the VIM family. Class D β-lactams found on integron structures belong to the OXA-type family (Fluit and Schmitz, 2004).

They commonly occur on Class 1 integrons from *Pseudomonas aeruginosa*, but in rare cases have been found in *Salmonella enterica* and *Enterobacter aerogenes* (Tosini *et al*., 1998), (Ploy *et al*., 1998). OXA-type genes found on gene cassettes within integrons in *Pseudomonas aeruginosa* tend to be the secondary *bla*-gene cassette on the integron, with a class A-type *bla*-gene mostly functioning as the primary integron-borne β-lactamase (Livermore, 2002), (Poirel *et al.*, 2001). This is in contrast to oxacillinase genes found on class 1 integrons from *Enterobacteriacea*e, which tend to be carried as sole β-lactamase gene cassettes on the integron along with other co-resistance genes (Tosini *et al*., 1998), (Ploy *et al*., 1998). In the absence of an antibiotic selective pressure, the integron-borne resistance genes can be lost by the host bacterium (Rosser and Young).

Resistance to a variety of non-related compounds can be conferred simultaneously by the presence of co-resistance gene cassettes on integrons. Poirel *et al*. (2002) reported that integron-mediated drug resistance tends to favor co-selection of isolates. This allows for widespread dissemination through patients with a wide variety of clinical disciplines. It is common for gene cassettes encoding aminoglycoside-modifying enzymes to co-occur with β-lactamase gene cassettes. On Class 1 integrons these genes occur with the co-resistance genes to quaternary ammonium compounds and sulphonamides at the distal 3’ end of the integron (Poirel *et al*., 2002), (Di Conza *et al*, 2002, Bradbury, Richard).

The horizontal transfer of genetic material within and between bacteria has been extremely important in the emergence of novel antibiotic resistance traits observed worldwide (Maiden, 1998). Rapid and widespread emergence of resistance and similar patterns of resistance have been encountered in phylogenetically diverse clinical isolates on an increasing scale (Rowe-Magnus *et al*., 2002, Bradbury, Richard).

Bacteria achieve active drug resistance through four major mechanisms: the efflux of the antibiotic from the cell via a collection of membrane-associated pumping proteins, modification of the antibiotic target (e.g., through the mutation of key binding elements such as ribosomal RNA or even by reprogramming of biosynthetic pathways, such as in resistance to the glycopeptide antibiotics), and via the synthesis of modifying enzymes that selectively target and destroy the activity of antibiotics (Bradbury, Richard).

All of these mechanisms require new genetic programming by the cell in response to the presence of antibiotics. In general, the antibiotics or their action usually regulate the expression of resistance genes. Therefore, bacteria expend a considerable amount of energy and genetic space to actively resist antibiotics (Wright, 2005, Bradbury, Richard).

1.8. Membrane barrier

Bacteria use several ingenious mechanisms to develop resistance to antibiotics. These include degradation of the antibiotic (β-lactamases), inactivation of the antibiotic by enzymatic modification (aminoglycoside modifying enzymes) and altering the target of the antibiotic (Walsh, 2003). These mechanisms are all specific for a single drug or single class of drugs. There are more general mechanisms of drug resistance, in which access of the unaltered antibiotic to its target is prevented by the barrier and active transport functions of the biological membranes. The organism can surround itself with a barrier of low permeability in order to decrease the influx of

the drug into the cell and can also pump out the drug in an energy-dependent fashion (Nikaido, 1994, Bradbury, Richard).

Bacteria are unicellular organisms and their internal environment is separated from the external environment by the cytoplasmic membrane. The major permeability barrier in any membrane is the lipid bi-layer structure. Some bacteria further protect themselves by making an additional structure that surrounds the cell, outside the cytoplasmic membrane. Gram-positive bacteria surround themselves with a thick layer of peptidoglycan but this offers little protection against most antibiotics. Gram negative bacteria are surrounded by a second membrane, called an outer membrane, which functions as a very effective barrier (Nikaido, 1994). It is composed of an unusual lipid known as lipopolysaccharide (LPS).The fatty acid chains in this LPS are all saturated, therefore making the interior of the bi-layer fluid and preventing the tight packing of the hydrocarbon chains. In general, the larger number of hydrocarbon chains linked to a single head group decreases the fluidity of the lipid interior (Bradbury, Richard).

Hydrophobic molecules permeate across the outer membrane about one-hundredth the rate through the usual bi-layers (Vaara *et al*., 1990) most clinically important antibiotics show some hydrophobicity. The LPS-containing bi-layers therefore act as an efficient barrier against rapid penetration of many antibiotics (Nikaido, 1994).

However, with such an effective barrier, Gram-negative bacteria have developed a separate mechanism to bring essential nutrients into the cell. They do this by means of a special class of proteins known as porins, which produce non-specific aqueous diffusion across the membrane (Nikaido, 1994). These porins also make the influx of antibiotics almost impossible because of their narrow openings and because the openings are lined with a number of charged amino acid residues, which orient the water molecules in a fixed direction. This makes the entrance of lipophilic molecules difficult because it disturbs this energetically favorable orientation of water (Schulz, 1993).

Even with this arrangement, hydrophilic agents such as some of the newer β–lactam antibiotics can penetrate through the porins of enteric bacteria (Yoshimura and Nikaido, 1985). To overcome this, *Pseudomonas aeruginosa* lacks the typical high–permeability porins but instead has low-efficiency porins, which only allow the diffusion of small molecules at about one-hundredth the rate through classical porins (Bellido *et al*., 1992).

In order for it to take up nutrients, *P. aeruginosa* has a number of special channels that enable it to take up a specific class of compound. This makes this bacterium intrinsically resistant to most antibiotics (Hancock *et al*., 1990).

However, even the most effective permeability barrier in bacteria cannot completely shut out the influx of small molecules. Even the low permeability membrane of *P.aeruginosa* can only prolong the half-equilibration time of most antibiotics for a few minutes (Nikaido, 1989). Therefore, a second mechanism of antibiotic resistance is usually required in addition to a low permeability barrier.

1.8.1 Efflux of the antibiotic

For antibiotics to be effective they must reach their specific bacterial targets and accumulate at the concentrations that can act in some reasonable time frame. For example, antibiotics that act on the ribosome must pass through the cell membranes into the cytoplasm and then accumulate at high enough concentrations that they can block the particular susceptibility step of protein assembly (Walsh, 2000, Bradbury, Richard).

Both bacterial and eukaryotic cells typically contain an array of cytoplasmic membrane transport systems involved in vital roles such as the uptake of essential nutrients, the excretion of toxic compounds, and the maintenance of cellular homeostasis (Qinghu *et al*., 2006) Increasing numbers of such transport systems are being identified, primarily because of the explosion in the use of cloning and sequencing technology over the last 20 years (Paulsen *et al*., 1996). At least 300 gene products are proposed to transport known substrates effectively, out of which around

20-30 transport antibiotics and other drugs (Van Bambeke *et al*., 2003, Bradbury, Richard).

Both Gram-negative and Gram-positive bacteria commonly produce proteins, which act as efflux pumps for antibiotics. If the drug is pumped out faster than it can diffuse in, intra bacterial concentrations of the antibiotic are kept low and ineffectual. Therefore, the bacterial protein synthesis proceeds at largely unimpeded rates. These pumps are variants of membrane pumps that all bacteria possess in order to move lipophilic and amphi-pathic molecules in and out of the cell. Antibiotic producing microorganisms possess these pumps in order to pump antibiotics out of the cell as fast as they are produced. This acts as a protective mechanism for the microorganism and prevents it from being killed by its own chemical weapons (Walsh, 2000). Most drug efflux pumps have broad substrate specificity and, therefore may deal with a wide range of drugs of completely unrelated pharmacological classes. Drug efflux decreases the load on efflux mediated detoxification systems, thereby avoiding their saturation, while the chemical modification by enzyme-based systems, which usually increases the amphiphilicity of the drug, provides the pumps with better substrates (Van Bambeke *et al*., 2000, Bradbury, Richard).

Four protein families of efflux pumps that can function in antibiotic resistance have been described (Van Bambeke *et al.*, 2000). The first three couple drug efflux to a counter flow of protons, while the fourth uses the hydrolysis of ATP to provide the energy for active transport of the antibiotic or other foreign compounds out of the cell (Paulsen *et al*., 1996). The pumps driven by proton motive force are categorized in the major facilitator subfamily (MFS).The small multidrug regulator (SMR) family or the RND (resistance/nodulation/cell division) family, based on the projected size and the need for partner proteins and subunits. The second major category of efflux pumps, those hydrolyzing ATP, is called the ATP-binding cassette (ABC) family. The ATP-driven pumps predominate in eukaryotes, whereas the proton driven ante porters predominate in bacterial genomes (Walsh, 2003). The genes encoding these pumps can be found on plasmids, transposons or even as part of integrons, which facilitates widespread dissemination of the genes. However, several of these pumps are already encoded in microbial genomes (Van Bambeke *et al*., 2003, Bradbury, Richard).

The mechanism of transport and of substrate recognition remains largely unknown in most instances, and many of the current views are based on extrapolations from data obtained with transporters of physiological substrates. SMR, RND, and most MFS transporters use a proton gradient as the driving force. The putative method of drug transport, as established by site-directed mutagenesis of a SMR transporter, could involve the following steps: (i) exchange between the drug and a proton fixed on a charged residue; (ii) translocation of the drug by a series of conformational changes driving it through a hydrophobic pathway; and (iii) replacement of the drug by a proton in the external medium and return to the initial conformational state (Mordoch *et al*., 1999) The overall result of the transport is therefore an exchange between a drug and a proton (anti-port). As for proton ant porters, conformational change of the ABC protein is necessary for drug extrusion and probably is triggered by drug binding and ATP hydrolysis (Van Bambeke *et al*., 2000, Bradbury, Richard).

The exact mechanism for drug transport is still controversial. Among the different models that have been proposed, the two most likely ones present efflux pumps as acting either like hydrophobic 'vacuum cleaners' or like flippases. In the first model, the drug is thought to move freely into the lipid phase of the membrane, then reaching the protein and its central channel, from where it is actively expelled outwardly. In the second model, the drug is also thought to reach the protein from within the membrane, but then would be flipped to the outer layer (Van Bambeke *et al*., 2000, Bradbury, Richard).

It must be emphasized that a given antibiotic may be a substrate for different types of pumps and a given pump may extrude not only different antibiotics within the same class but also different classes of antibiotics. Finally, a single cell may possess a vast and complex arsenal of efflux pumps allowing for the extrusion of a very broad spectrum of drugs (Van Bambeke *et al*., 2000, Bradbury, Richard).

Four different efflux systems dependent on the genes *mexAB-oprM* (β-lactams), *mexXY-oprM* (aminoglycosides), *mexCD-oprJ* and *mexEF-oprN* (carbapenems and

quinolones) are known to exist, allowing extrusion of all classes of antibiotics except the polymixins (Poole and Srikumar, 2001). Genes for these efflux systems are found in all strains of *Pseudomonas aeruginosa* but are expressed at relatively low levels, under the control of regulatory genes. Mutations in these regulators can lead to high level expression and confer enhanced antibiotic resistance (Poole and Srikumar, 2001, Bradbury, Richard).

1.8.2. Clinical Impact of *Pseudomonas* Resistance

Carmeli *et al*. published several studies addressing outcomes associated with antimicrobial resistance in Gram-negative pathogens. There were no differences in mortality or length of hospital stay between patients infected with a resistant isolate at baseline and those infected with a susceptible isolate at baseline. In contrast, the emergence of resistance was associated with a greater risk of death and a longer duration of hospital stay. The emergence of resistance was also associated with an increased risk of secondary bacteremia (Carmeli, Y1999). Infection or colonization with multi-drug resistant (MDR) *P. aeruginosa* was associated with increased mortality, increased length of hospital stay and the need for more surgery and other procedures. Also, the functional capacity of the MDR *P. aeruginosa* carrying patients at discharge was poorer than that of the controls (Aloush, V2006). In patients with cystic fibrosis, infection with MDR *P.aeruginosa* was associated with accelerated progression of cystic fibrosis and increased likelihood of undergoing lung transplantation (Lechtzin N2006) Imipenem-resistant *P. aeruginosa* has been found to be associated with increased in hospital mortality rates, increase in hospitalization duration and hospital charges (Lautenbach E 2006).

1.9 Infection with *Pseudomonas aeruginosa*

1.9.1 Immune-competent patients in the community setting

P.aeruginosa causes an extremely wide range of disease in both immune-competent and immune-compromised patients. In immune-competent patients, the organism

generally requires a breach in host defenses such as a skin abrasion, or placement of a urinary catheter, in order to establish infection. In previously healthy patients, *P.aeruginosa* infection is generally limited to skin and soft tissues. Contaminated swimming pools, whirlpools and hot-tubs are a recognized cause of folliculitis and are associated with urinary tract infection in females (Stryjewski and Sexton 2003). One report described *P. aeruginosa* folliculitis consequent to the use of a contaminated loof a sponge (Bottone and Perez 1993). Folliculitis associated with depilation of the legs has also been described (Stryjewski and Sexton 2003). Otitis external (swimmer's ear) may be caused by *P. aeruginosa* following exposure to contaminated water (Kiska and Gilligan 2003 Bradbury, Richard).

While colonization of the upper respiratory tract occurs commonly, this rarely progresses to clinical disease in previously healthy individuals (Pier and Ramphal 2005). Risk factors for community acquired *P. aeruginosa* pneumonia are a history of smoking, exposure to aerosolized bacteria in water sprays or welding dust and near-drowning in fresh water or whirlpools (Stryjewski and Sexton 2003). *Pseudomonas aeruginosa* has been implicated as a cause of community-acquired gastroenteritis (Adlard, Kirov *et al*. 1998), and a cluster of community acquired sepsis cases in previously healthy infants in Taiwan was reported, associated with prior community acquired gastroenteritis (Stryjewski and Sexton 2003).

P. aeruginosa wound infections following trauma are common. Corneal ulcers in association with contact lens use and other ocular infections following trauma are well recognized, and endocarditis in intra-venous drug users has been recorded (Kiska and Gilligan 2003). Calcaneous osteomyelitis in children has been associated with penetrating injuries through footwear colonized with *P. aeruginosa* (Stryjewski and Sexton 2003; Pier and Ramphal 2005).

P. aeruginosa infections appear to be increasing in the community setting (Seigel 2008), whilst the cause of this is not completely understood, increased virulence in environmental strains through acquisition of new virulence genes (such as *exoU*) via horizontal transmission is a feasible explanation (Bradbury, Richard).

1.9.2 Immune-compromised patients in the community setting

P. aeruginosa is a common cause of infection in the immune-compromised patient, such as those with burns, diabetes mellitus or more severe immune-compromisations such as cancer or HIV.

Infection in such cases may occur through both nosocomial acquisition and by exposure in the community setting. *P. aeruginosa is* second only to *Staphylococcus aureus* as a cause of burn infections in the United States (Stryjewski and Sexton 2003). It has been shown to be the second most common cause of bacteremia subsequent to burn infections (Vostrugina, Gudaviciene *et al*. 2006). The severe and invasive progression of *P. aeruginosa* otitis external to malignant otitis external is particularly associated with the diabetic patient, with up to 94% of cases being observed in diabetics (Johnson and Ramphal 1990). Indwelling catheters and drains may easily be colonized by *P. aeruginosa*, which will form biofilms on such devices, rendering eradication difficult or impossible. In such cases, patients are often asymptomatic, but without removal of the devices, progression to true infection often results (Stryjewski and Sexton 2003). Physically disabled patients and those in long term nursing institutions are predisposed to *P. aeruginosa* infection, as such patients are often unable to personally attend to basic hygiene needs, are often catheterized, and may have concurrent physical difficulties in bladder flushing, swallowing, coughing and other protective reflexes (Stryjewski and Sexton 2003). HIV positive status predisposes to *P.aeruginosa* infection, a Spanish study finding that in 189 cases of *P. aeruginosa* bacteremia, HIV was the most common underlying defect of the immune system (Stryjewski and Sexton 2003). In those with advanced disease, chronic *P. aeruginosa* lung infection is a common and intractable infection (Asboe, Gant *et al*. 1998, Bradbury, Richard).

Transplant and chemotherapy patients are exposed to a large number of risk factors for *P.aeruginosa* infection. Such patients are given long-term immunosuppressive therapy and often concurrently have neutropenia, indwelling catheters, frequent hospital stays and repeat courses of antibiotics. Thus it is unsurprising that high rates of *P. aeruginosa* pneumonia, bacteremia and UTI are seen in these patients.

Mortality rates are 20-25% for bone marrow transplant patients with *P. aeruginosa* bacteremia (Stryjewski and Sexton 2003). Profoundly immunosuppressed patients are advised to avoid fresh vegetables, due to the probability of contamination with *P.aeruginosa* and other Gram negative bacilli, consumption of which may lead to intestinal colonization and the risk of subsequent sepsis (Correa, Tibana *et al*. 1991; Kiska and Gilligan2003). Despite these measures, that *P. aeruginosa* may be found even in bottled water (Rosenburg2003) demonstrates its ubiquity in the environment and the associated difficulty in avoiding contact with the organism. In the community setting, chronic lung disease accounts for a large amount *P. aeruginosa* mediated disease. Much of this burden is carried by CF patients (discussed below), but chronic respiratory infections with *P. aeruginosa* are also commonly seen in chronic obstructive pulmonary disease (COPD) and bronchiectasis. Predisposing factors for such disease are long term smoking or inhalation of other harmful chemicals. The damaged and dysfunctional lung becomes an ideal site for *P. aeruginosa* colonization and chronic infection. Prevalence of *P.aeruginosa* in COPD patients is 4%, increasing to approximately 13% in those with advanced disease (Maciá, Blanquer *et al*. 2005). In such cases, *P. aeruginosa* isolates often show phenotypic similarity to those recovered from the CF lung, including conversion to mucoid phenotype in up to 40% of cases (Pujana, Gallego *et al*. 1999) increased phenotypic diversification and long term colonization with a single genotype (Maciá, Blanquer *et al*. 2005, Bradbury, Richard).

1.9.3. Molecular epidemiology

P. aeruginosa is an environmental organism, and it is generally accepted that the overwhelming majority of community acquired infections are caused by environmental strains. Seven percent of healthy individuals carry *P. aeruginosa* in their throats. Other sites of colonisation include the skin (particularly in warm and moist areas such as the axilla) and the gastrointestinal tracts (variable carriage rates reported; up to 24% of healthy volunteers), where rates of carriage may be dependent upon diet (Speert 2002; Pier and Ramphal 2005). In other cases, the source of infection is exogenous, such as traumatic implantation in calcaneous osteomyelitis or

cases of contact colonisation such as swimmers ear and whirlpool folliculitis (Pier and Ramphal 2005, Bradbury, Richard).

The administration of narrow spectrum antibiotics greatly increases rates of colonisation of *P.aeruginosa*, and may select for resistant strains (Speert 2002). In hospitalised patients, where antibiotic use is greatly increased, intestinal colonisation rates of up to 60% of patients have been reported (Speert 2002). In the majority of cases *P. aeruginosa* strains imported into hospitals in the bowels of patients are considered to be the source of their infections (Speijer, Savelkoul *et al*. 1999), an observation supported by the pan mictic (multiple and diverse genotypes present) state of *P. aeruginosa* infections in most hospitals (Speert 2002; Guitierrez, Juan *et al*. 2007). Despite this, transmission of *P. aeruginosa* via environmental sources such as contaminated water and on the hands of health care workers may occur (da Silva, Filho *et al*. 2008, Bradbury, Richard).

In some cases, nosocomial outbreaks of *P. aeruginosa* infections are associated with genotypically indistinguishable strains. Reports of epidemics of MDR strains in ICUs and NSUs have been published (Deplano, Denis *et al*. 2005; Sekiguchi, Asagi *et al*. 2005), such strains will often spread rapidly within a hospital (Brisse, Milatovic *et al*. 2000)and may spread between hospitals (Guitierrez, Juan *et al*. 2007; Sekiguchi, Asagi *et al*. 2007).
Nosocomial outbreaks are not always associated with resistant strains, a study of nosocomial clinical and environmental strains in a hospital in Beirut found a single antibiotic sensitive genotype to be the predominant isolate (Matar, Chaar *et al*. 2005).

1.9.4 Infection in cystic fibrosis patients

1.9.4.1 Demographics

Cystic fibrosis (CF) is the most common lethal genetic disease of Caucasians (Tatterson, Poschet *et al*. 2001). The disease is rarely encountered in Africans, and is exceptional in those of Asian descent, but is more common in African Americans

and natives of the Middle East and Indian sub-continent (Rubin and Farber 1994; Bosque and Asensio 2003). The genetic mutations leading to CF are particularly common in people of Anglo-Saxon descent (Bosque and Asensio 2003). Tasmania has a significantly higher incidence of CF per head of population than the mainland states of Australia and most other countries of the world, with approximately 110 cases in the state (Blest 2000). This is primarily attributed to the predominantly Caucasian population with a largely Anglo-Celtic ethnic background and the low genetic diversity in the community resultant from geographic isolation (Blest 2000, Bradbury, Richard).

1.9.4.2 Epidemiology in respect to cystic fibrosis

P. aeruginosa may display a differing epidemiology in the case of CF patients compared to other types of infection. It is most probable that colonisation of the CF lung occurs following environmental exposure, and once established, a strain will generally remain present in a patient for the remainder of their life. Further genotypes may colonise an individual patient's lung and exist alongside the original colonising strain (Anthony, Rose *et al*. 2002) Person to person transmission in most cases is rare, although this may be seen in patients who spend a great deal of time in each other's company, such as siblings, and is associated with intimate contact such as kissing (Speert, Campbell *et al*. 2002; Saiman and Siegel 2004). Despite this, a recent study found that at a CF Summer camp in the Netherlands, of 80 children attending, there were 18 cases of possible transmission, and three cases of probable transmission of *P. aeruginosa* between attendees (Brimicombe, Dijkshoorn et *al*. 2008). Thus, summer camp attendance may be considered a risk factor for acquiring new *P. aeruginosa* infections or colonisation with new strains of the bacteria in CF children.

In-patient transmission of *P. aeruginosais* also considered to be a rare event, and when it does occur was commonly associated with break down in infection control techniques such as staff hand washing and sterilisation of respiratory equipment (Geddes 2001; Saiman and Siegel 2004). Interestingly, while desiccated saline

suspensions of non-mucoid *P. aeruginosa* can survive on inanimate surfaces for 24 hours, mucoid strains will survive for 48 hours. When strains are suspended in CF sputum, their survival on dry surfaces extends to 8 days (Saiman and Siegel 2004), raising the potential for *P. aeruginosa* acquisition from fomites. Mucoid isolates were found to survive longer in aerosolised droplets than non-mucoid isolates, but no significant differences were observed between clonal complexes (LES and MES) and unique CF isolates (Clifton, Fletcher *et al*. 2008, Bradbury, Richard).

CF summer camps and family camps are a well recognised source of cross-infection with respect to *B.* Cepacia (Honicky, Harden et *al*. 1993), and the potential for *P. aeruginosa* cross-infection at such camps has been investigated. The incidence of permanent conversion to an acquired genotype following attendance at a CF holiday camp was found to be only 1.9% in one study, and thus considered that the risks of cross-infection with *P. aeruginosa* were outweighed by the psychological benefits of such camps in CF patients (Hoogkamp-Korstanje, Meis et *al*. 1995, Bradbury, Richard).

1.9.4.3 *P. aeruginosa* clonal complexes in CF patients

A relatively recent and concerning development in the epidemiology of CF *P. aeruginosa* infections has been the emergence of clonal complexes of *P. aeruginosa* in both the United Kingdom and Australia. These strains have often spuriously been referred to as "clonal", a description which cannot correctly be applied without whole genome sequencing. Indeed, in many cases these strains only display only a degree of genotypic similarity beyond that attributable to random events, rather than displaying completely identical patterns upon genotypic analysis (Wiehlmann, Wagner, *et al*. 2007; Rakhimova, Wiehlmann, *et al*. 2008). Finally, such strains often display a marked degree of intra-strain phenotypic variability, due to mutation and horizontal transfer of new genes. It was therefore considered more correct to refer to such isolates as "epidemic strains". Given that such strains are almost exclusively limited to CF infections, and do not spread at an overwhelmingly rapid rate, the latter description is also strictly incorrect. Such strains are now being referred to as "CF clonal complexes of *P. aeruginosa*" (Lavenir, Sanroma, *et al.* 2008). The first report

of a potential clonal complex of *P. aeruginosa* amongst CF patients occurred in Denmark in 1984 (Pederson, Koch *et al*. 1986), this study reported an MDR clonal complex based upon serotyping and phage typing of the isolates. Later PFGE typing of these isolates showed the cluster to be composed of two genotypically distinct strains (Saiman and Siegel 2004). Römling *et al*. described the presence of a genotypically indistinguishable strain (clone C) from 12 (37%) of 32 patients (3 female siblings and 9 unrelated patients) at a CF clinic in Hannover, Germany in 1994. Two of these patients appeared to lose this strain over the 2.5 year period of the study (Römling, Fiedler *et al*. 1994). It appears that the significance of these findings may have been overlooked upon their first publication. The first report of a CF clonal complex associated with hospital CF clinics using reliable molecular epidemiology techniques was published by Cheng *et al*. (1996), describing the identification of a genotypically indistinguishable and ceftazidime resistant strain of *P. aeruginosa* in a number of CF patients at a CF clinic in Liverpool, described at the time as the "**Alder Hey strain**".

The strain formerly known as "**Alder Hey**" has since been renamed as the "**Liverpool epidemic strain**" (**LES**) LES is amongst the most studied of the currently identified CF *P. aeruginosa* clonal complexes. The strain was originally identified due to its **ceftazidime** resistant phenotype, but isolates sensitive to this antibiotic have since been identified (Scott and Pitt 2004) MDR phenotype does however predominate amongst LES isolates (Scott and Pitt 2004). This strain may act as the primary colonising strain or super-infect patients that already carry one predominant, unique genotype, and over time supplant the previously dominant strain to become the only genotype present in the lung (McCallum, Corkill *et al*. 2001, Bradbury, Richard).

Following the first description of LES, a second clonal complex was identified in 24 (16%) of 154 CF patients attending a CF clinic in Manchester (Jones, Govan *et al*. 2001). This strain also showed a greater propensity towards antimicrobial resistance when compared to genotypically unique isolates, with most isolates resistant to **ceftazidime, piperacillin, ciprofloxacin, aztreonam, imipenem, and meropenem**, and some showing additional resistance to the **aminoglycosides (gentamicin, tobramycin and amikacin**) (Jones, Govan *et al*. 2001, Bradbury, Richard).

The death in quick succession of five CF patients under the age of five years at CF clinic in Melbourne, Australia, prompted an epidemiological study of 27 patients. This identified *P. aeruginosa* clonal complex colonising all five of the deceased children as well as three other paediatric CF patients (Armstrong, Nixon *et al*. 2002). This strain was described as "*pulsotype 1*", and was shortly afterwards isolated from 10 (56%) of 18 CF patients in Sydney (Anthony, Rose *et al*. 2002). These findings lead to a study in Brisbane, which found another CF clonal complex (*pulsotype 2*) in 39 (39%) of 100 CF patients attending two centralised CF clinics (O'Carroll, Syrmis *et al*. 2004). *Pulsotype 1* was identified in 8 of the 100 patients tested (O'Carroll, Syrmis *et al*. 2004), although half of these patients had previously resided in Sydney (Armstrong, Bell *et al*. 2003) It is probable that *Pulsotype 2* represents a newly emerged strain, as typing of CF *P. aeruginosa* strains in Brisbane in 1991 by RFLP did not reveal any cross infection (Fegan, Francis *et al*. 1991). Since this time, the two strains have been re-named Australian Epidemic Strain 1 and 2 (**AES1 and AES2**).

A study in New Zealand study identified three patients with British or Australian CF clonal complexes, there was no evidence of transmission of these strains to new hosts (Schmid, Ling *et al*. 2008). However, in one CF centre a small cluster of strains existed in a number of patients above that which could be attributed to chance, and this cluster may represent a novel clonal complex (Schmid, Ling *et al*. 2008). Further studies have been performed in Turkey (Yagci, Ciragil *et al*. 2003), Brazil (Da Silva Filho, Levi *et al*. 2001; Silbert, Barth *et al*. 2001), British Columbia (Speert, Campbell *et al*. 2002) and Belgium (Van Daele, Franckx *et al*. 2005), all of which did not identify the presence of strain transmission in the tested CF populations. The Canadian study did identify 5 (3%) of 174 patients who shared a genotypically indistinguishable strain whilst having no known contact with each other. Whilst not a finding considered during the publication of the Canadian study, it may be that these five isolates represent a potential new CF clonal complex that has not yet become widespread in the Canadian CF population (Speert, Campbell *et al*. 2002, Bradbury, Richard).

In response to the presence of LES and the Manchester epidemic strain (MES),Scott and Pitt (2004).performed the most widespread genotypic study of *P. aeruginosa*

infections in CF patients to date drawing upon 1225 isolates (1 per patient) from 31 centres across England and Wales representing sampling of 20% of the entire CF populations of these areas. This study found LES in 11% of patients from 15 separate centres throughout the geographic range of the study, only 11 isolates from just 3 centres were identified as MES. A new CF clonal complex, termed "Midlands 1" was identified in 86 patients from 9 centres. This strain showed increased resistance to antimicrobials, and was the only type in the study to include a colistin (polymixin class antimicrobial) resistant isolate. FAFLP analysis showed greater heterogeneity in LES strains than midlands 1 isolates, suggesting a greater temporal space since the emergence of LES when compared to midlands1 . Also of noted in this study was the presence of "clone C" in 15 patients from 8 centres (Scott and Pitt 2004, Bradbury, Richard).

The presence of clone C in a large number of patients so far from its site of original isolation in Germany leads to interesting considerations regarding the epidemiology of this particular strain in relationship to other strains of *P. aeruginosa* and other clonal complexes thus far described. Clone C does not show the propensity towards a resistant antibiogram that is so closely associated with other clonal complexes. It has been isolated from infections other than those in the CF lung, having been found causing otitis externa in one patient and isolates showing 75% or greater similarity by PFGE have been recovered from a CF patients in Toulouse, France. the peritoneal dialysate of a non-CF patients in Birmingham, UK and in the urine of a non-CF ICU patient in Durham. also the UK. Clone C has also been widely recovered from environmental aqueous sources throughout Europe, including swimming pools, drinking water and rivers (Dinesh, Grundmann *et al*. 2003, Bradbury, Richard).

While little work has been performed thus far on the virulence of clone C, the effect on patient outcomes of infection with a number of other CF clonal complexes has been investigated. McCallum, *et al* (2002) reported the transmission of LES from an infected CF child to both of her parents, in which it caused significant disease. The father was known to have COPD, and was infected with both LES and a unique *P. aeruginosa* strain, whilst the mother was physically well apart from mild asthma (McCallum, Gallagher *et al*. 2002). Transmission of *P. aeruginosa* from CF individuals to healthy patients has not previously been described (McCallum,

Gallagher *et al*. 2002), and this suggests that in at least the case of LES, the strain is significantly more capable of colonising and causing disease in the human lung. These findings are supported by the unusual report of LES causing sinusitis in the pet cat of an LES colonised CF adult (Mohan, Fothergill *et al*. 2008, Bradbury, Richard). Further study of LES by subtractive hybridisation has found that these strains all belong to serotype 06 and elaborate pyoverdin III, an unusual form of pyoverdin. All LES strains tested possessed *exoS* (compared to a mean *exoS* positivity of only 76% in *P. aeruginosa*), are not hypermutable and all possess the PAG-1 pathogenicity island (overall *P. aeruginosa* mean positivity 81%) (Parsons, Panagea *et al*. 2002) Studies of two LES isolates and PA01 both in exponential phase and under oxidative stress by microarray identified the loss or down regulation of a number of genes and bacteriophage related clusters (Salunkhe, Smart *et al*. 2005) of particular interest amongst these results was that many of the differences in genes were associated with QS function. However, no significant results were forth coming from this analysis due to both the small sample size, and the variability within the LES isolates possession of specific genes, and response to oxidative stress (Salunkhe, Smart *et al*. 2005) Patients infected with LES showed a greater reduction in pulmonary function and BMI over four years compared to age matched controls infected with unique strains (Al-Aloul, Crawley *et al*. 2004).

Work on the phenotypic characteristics of MES has been performed. As previously discussed, these isolates are generally highly resistant to antimicrobials, but also are exclusively non-pigmented and non-motile. The presence of mucoidy in isolates is variable, while 22 out of 24 isolates belonged to an unusual pyocin type. In the first report of the existence of MES, patients harbouring this clonal complex had almost universally (23 out of 24) had recent stays (<2 years) in the Manchester hospital in which it was first isolated (Jones, Govan *et al*. 2001). A comparison of 22 patients with MES against 56 patients harbouring unique strains showed that those infected with this clonal complex had more exacerbations and required more courses of IV antibiotics, but showed no significant difference in BMI, FEV1% and FVC%. MES has not been isolated from any non-CF patients (Jones, Dodd *et al*. 2002). MES isolates suspended in saline were found to be less resistant to desiccation than unique strains or LES isolates (which showed equal resistance) (Panagea, Winstanley *et al*.

2005), however, in clinical practice such strains would be suspended in CF sputum and the resistance of these isolates to desiccation when suspended in this matrix has not been investigated. With regard to the Australian CF clonal complexes of *P. aeruginosa*, both AES1 and AES2 have been shown to have increased resistance to antimicrobials when compared to unique strains. AES1 isolates were predominantly mucoid on first isolation (Armstrong, Nixon *et al*. 2002), whilst AES2 showed no greater propensity towards a mucoid phenotype compared to unique isolates (O'Carroll, Syrmis *et al*. 2004) AES2 carriers in Brisbane were younger (mean average age 16.8 vs 24.6 for patients with unique strains), and showed poorer lung function as measured by FVC% and FEV1% (O'Carroll, Syrmis *et al*. 2004). An unpublished study by the University of Sydney using PA01 microarray chips (Affymetrix) to compare four AES1 and four matched unique isolates yielded some novel results, although the sample size was too small to reach statistical significance. The study found that QS genes in AES1 were all upregulated and that all strains of AES1 harboured the pf4 gene, which was not present in any of the unique strains.The entire PAO632-39-bacteriophage locus was absent in all AES1 isolates tested, and a putative virulence factor gene of MES, *pao724* was not present in the AES1 strains tested (Munro 2005). A similar loss of an entire bacteriophage gene locus (PAO632-48) was observed in microarray analysis of two LES strains (Salunkhe, Smart *et al*. 2005, Bradbury, Richard).

1.10 Laboratory identification

CLSI guidelines maintain that in cases of uncomplicated infection, the characteristic oxidase reaction, pigment and smell of *P. aeruginosa* is sufficient for laboratory identification (Baron 2001). However, due to the potential for severe adverse patient outcomes based upon incorrect bacterial identification further identification is required in isolates from sterile sites, (Baron 2001). In practice, the majority of laboratories in Australia employ at least one further test to identify any isolates of *P. aeruginosa*. Characteristic morphology, pigment, and a capacity to grow at 42ºC are considered sufficient for identification. Diagnostic discs impregnated with the compound C390 may be effectively used for separation of *P. aeruginosa* from other

Pseudomonas spp. (Anthony, Rose *et al*. 2002). A variety of commercial phenotypic testing kits and automated systems are available for the identification of non-fermentative Gram negative rods, these may be effectively employed to identify *P. aeruginosa* (Bradbury, Richard).

CF and other chronic respiratory *P. aeruginosa* isolates may represent a diagnostic dilemma for clinical laboratories. Many of these isolates do not conform to traditional.

P.aeruginosa phenotypes (including pigmentation variability), and may grow very slowly, limiting the effectiveness of identification methods based on biochemical properties (Miller and Gilligan 2003). In response to this, a number of PCR based applications have been developed specifically for the identification of *P. aeruginosa* and other *Pseudomonas* spp. isolates from CF respiratory samples (Spilker, Coenye *et al*. 2004; Anuj, Whiley *et al*. 2009). A review was conducted in Australia of 2, 267 bacterial isolates from the CF lung identified by routine phenotypic methods in clinical laboratories as *P. aeruginosa* using a duplex *gyrB* and *ecfX* PCR (Anuj, Whiley *et al*. 2009), followed by partial 16S rRNA sequencing of any those providing negative results. This study found a mis-identification rate of 2,3%; the most common organisms mis-identified as *P. aeruginosa* being *Achromobacter xylosoxidans, Stenotrophomonas maltophilia* and *Inquilinus limosus* (Kidd, Ramsay *et al*. 2009) . This high lights the importance of genotypic identification methods for *P. aeruginosa* from the CF lung in the routine laboratory setting (Bradbury, Richard).

1.11 Phenotypic and Genomic Variability

By far the most phenotypic variability of *P. aeruginosa* isolates is seen in chronic infections of the CF lung. Alterations in the *P. aeruginosa* phenotype in the CF airway occur as chronicity of infection develops. The most striking of these alterations is the conversion to a mucoid phenotype, an event which is associated with significantly increased morbidity in the affected patient (Lyczak, Cannon *et al*. 2002; Davidson, Currie *et al*. 2003). It has been suggested that a correlation exists between biofilm growth and mucoidy (Nivens, Ohman *et al*. 2001, Bradbury, Richard).

P. aeruginosa has an unusually large genome (~6,3 Mb) (Projan 2007). Global genome variation between *P. aeruginosa* isolates is estimated to be at a level of 11% on average (Shen, Sayeed *et al*. 2006). Between individual isolates, genomes may vary by up to 30% (Morales, Wiehlmann et *al*. 2004), representing a massive level of intra-species genome variation. It appears that very little change has occurred in core genome over the past 50 years (Morales, Wiehlmann *et al*. 2004, Bradbury, Richard).

1.12 *Pseudomonas aeruginosa* in Biofilm

The formation of biofilms is considered to be a major factor in *P. aeruginosa* persistence in the CF lung (Prince 2002). Biofilms are large masses of bacteria growing in organised structures that allow symbiosis between cells within the structure to provide greater economy in cell functions than would be possible when growing alone in a planktonic state. Biofilms may be found in the natural environment, adherent to surfaces within an extracellular polysaccharide matrix (Lewis 2001; Engel 2003) and in human infection, such as when growing on stationary mucous within the CF lung or upon the airway surface liquid (Worlitzsch, Tarran *et al*. 2002; Engel 2003). Biofilm formation occurs in four stages; attachment and replication, microcolony formation, differentiation into a structured community and release of planktonic cells (Engel 2003, Bradbury, Richard).

1.13 Quorum sensing

Many prokaryotes respond to presence in their surroundings of other cells of their species. Some prokaryotes have regulatory pathways that are controlled by the density of cells of their own kind. this is called Quorum sensing (sufficient number).
Quarum sensing is a mechanism to access population density. Many bacteria use this approach to ensure that sufficient cell numbers are present before starting activities that require a certain cell density to work effectivly. For example, a pathogenic (disease causing) bacterium that secretes a toxin will have no effect as a single cell; production of toxin by one cell alone would merely waste resourses. However, if a sufficiently large population of cells is present, the coordinated expression of toxin

may successfully cause disease. Q.S is widespread among gram-negative bacteria but is also found in gram-positive bacteria. Each species that employs quorum sensing synthesis a specific signal molecule called an Auto-inducer. This molecule diffuse freely across the cell envelop in either directions. Because of this, the autoinducer reaches high concentrations inside the cell only if these are many cell nearby, each making the same autoinducer, inside the cell, the auto-inducer binds to a specific activator protein and triggers transcription of specific genes (Michael Madigan 2011, Bradbury, Richard).

There are several different classes of auto-inducers. The first to be identified were the acyl homoserin lactones (AHL) Several different AHLs, with acyl groups of different lenghts, are found in different species of gram-negative bacteria. In addition, many gram-negative; bacteria make autoinducer2 (Al-2,cyclic furan derivative). This is apparently used as a common autoinducer between many species of bacteria. Gram positive bacteria generally use certain short peptides as auto-inducers (Michael Madigan 2011).

Q.S was first discovered as the mechanism of regulating light emission in bioluminescent bacteria. several bacteria species can emit light, including the marine bacterium *Alii vibrio fischeri,* shows bioluminescent colonies of *A.fischeri*. The light is generated by an enzyme called luciferase. The lux operons encode the proteins needed for bioluminescence. they are under control of activitor protein Lux R and are induced when the concentration of the specific *A.fischeri* AHL, N-3-oxohexanoyl homoserin lactone, becomes high enough, This AHL is synthesized by the enzyme encoded by the Luxl gene (Michael Madigan 2011, Bradbury, Richard).

Various genes are controlled by quorum sensing, including some in pathogenic bacteria, For example, *pseudomonads* use 4-hydroxyalkyl quinolines as autoinducers to induce genes involved in virulence. In *Pseudomonas aeruginosa*, for instance, quorum Sensing trigger the expression of a large number of unrelated genes when the population density becomes sufficiently high. These genes assist cells of *pseudomonas* in the transition from growing freely suspended in liquid to growing in a semisolid matrix called a biofilm. The biofilm, formed by specific polysaccharides produced by *Pesudomonas aeruginosa,* increases the pathogenicity of this organism

and prevents the penetration of antibiotics (Michael Madigan 2011,Bradbury, Richard).

The pathogenesis of *Staphylococcus aurus* involves among many of other things. The production and secretion of small extracellular peptides that damage host cells or that interfer with immune system. The genes encoding these virulence factors are under the control of a quorum sensing system that uses small peptides as auto-inducer. The regulation of these virulence genes is quite complex and require a regulatory RNA molecule as well as regulatory proteins that form a signal transduction system.

Q.S also occurs in microbial Eukaryotes. For example, in the yeast *sacharomyces cervisiae* specific aromatic alcohols are produced as autoinducers and control the transition between growth of *S.cervisiae* as single cell and as elongated filaments. Similar transition are seen in other funji, some of Q.S is mediated by the long-chain alcohol farnesol Some eukaryotes produce molecules that interfer with bacterial Q.S.Most of those known so far are furanone derivatives with halogenes attached. These mimic the AHLs or AI-2 and disrupt bacterial behavior that relies on quorum sensing. Q.S disruptors have been suggested to have possible future applications in dispersing bacterial biofilm and preventing the expression of virulence genes (Bradbury, Richard).

Within *P. aeruginosa* biofilms, cell differentiation and formation of the biofilm itself is regulated by quorum sensing (QS) cell signalling mechanisms, autoinduced by acyl homoserine lactone molecules. The QS system of *P. aeruginosa* is a complex series of positive and negative feedback pathways that consummately reflects the great complexity of gene expression within this species. The expression of genes in response to QS autoinducers is a factor of autoinducer density in the surrounding environment, and thus proportional to the density of bacterial cells secreted autoinducer molecules (De Kievit, Gillis *et al*. 2001; Engel 2003,Yildiz F,1981). As a consequence of this, planktonic cells will only begin biofilm formation when a critical density of cells is present within any given area (Dale and Park 2004). In the case of *P. aeruginosa*, biofilm formation and differentiation is controlled by the *las* and *Rhl* signalling pathways (De Kievit, Gillis *et al*. 2001; Engel 2003).The *las* pathway consists of the las R transcriptional regulator and the lasI synthetase. LasI

synthesises the N-(3-oxododecanoyl)-L-homoserine lactone (PAI-1), which also has regulation activity in virulence factor expression, the activation of *Rhl* pathway and expression of LasI itself (Engel 2003). The rhl QS pathway is composed of the RhlR transcriptional activator and the RhlI synthetase, directing production of N-butyral-L-homoserine lactone (PAI-2) (Engel 2003). The rhl QS system also plays a role in both biofilm and virulence factor regulation (Engel 2003). A third QS autoinducer in *P. aeruginosa* has recently been described; 2-heptyl-3-hydroxy-4-quinolone (PQS), positively regulated by *las* and negatively regulated by the *rhl* QS systems (Wade, Calfee *et al*. 2005). PQS is an autoinducer of its own negative regulator, LasR, and also functions in regulation of expression of genes involved in iron acquisition and oxidative stress response (Bredenbruch, Geffers *et al*. 2006), as well as a number of virulence factors, including pyocyanin and LasB (Engel 2003; Jensen, Lons *et al*. 2006).

1.14. Azithromycin as a disruptor of quorum sensing

Macrolide antibiotics have MICs to *P. aeruginosa* far above achievable levels *in vivo*. Despite this, a role for the new macrolide azithromycin has been proposed in the treatment of chronic *P. aeruginosa* lung infection. Azithromycin has been shown to have an anti-inflammatory effect and up-regulate T-helper 1 lymphocyte function, as well as disrupting QS in *P. aeruginosa* (Nguyen, Louie *et al*. 2002; Parnham 2005).As QS is essential for the formation and maintenance of biofilms, these findings have had significant implications for the treatment of chronic *P. aeruginosa* lung infections. Azithromycin has also been shown to display a bactericidal effect on *P. aeruginosa* during certain growth phases due to its interaction with the cell's outer membrane. CF patients treated over a 3 month period showed significant improvement in both FEV1 and FVC1% following treatment (Jaffé and Rosenthal 2002). The achievable level of azithromycin within the lung is only 8 mg/L (Gillis and Iglewski 2004), while the MBC of PA01 in exponential phase of growth is 128 mg/L (Imamura, Higashyama *et al*. 2005). However, in biofilm, the majority of cells are thought to be in stationary phase, and the azithromycin MBC of PA01 dropped to just 1 mg/L when the cells were in stationary phase (Imamura, Higashyama *et al*.

2005) Gillis & Iglewski found that clinically achievable levels of 8 mg/L had no effect on PA01 biofilm formation *in vitro*, whilst lower levels of 2 mg/L did delay biofilm formation (Gillis and Iglewski 2004). Sub-MIC concentrations of various macrolides have been shown to reduce the expression of virulence factors, including alginate, elastase, lecithinase, protease, leukocidin, exotoxin A, phospholipase C and pyocyanin without affecting bacterial growth rates (Tateda, Ishii *et al*. 2007). Very recent work has identified the potential for not just azithromycin, but also ceftazidime and ciprofloxacin (commonly used in the treatment of *P. aeruginosa* infections) to inhibit QS, and lead to a consequent decrease in virulence factor expression (Skindersoe, Alhede *et al*. 2008, Bradbury, Richard).

Certainly, azithromycin therapy seems to have improved patient outcomes in those with chronic *P. aeruginosa* lung infections, with the 10 year survival rate for diffuse panbronchiolitis patients rising from below 50% prior to the institution of azithromycin therapy to above 90% following its introduction (Schultz 2004). Azithromycin is now also used in the treatment of CF patients with chronic *P. aeruginosa* infections (McCormack, Bell *et al.* 2007, Bradbury, Richard).

1.15. Antimicrobial resistance

Treatment of *P. aeruginosa* with antimicrobial agents may be hampered by the large number of both inherent and acquired resistance mechanisms associated with the organism. Rates of acquired resistance to antimicrobials vary throughout the world, with higher rates seen in Europe and parts of the third world than in the United States, and nosocomial *P.aeruginosa* isolates tend to be more resistant to antimicrobials than community acquired isolates (Kiska and Gilligan 2003). CF isolates of *P. aeruginosa* demonstrate more overall resistance to antimicrobials when compared to isolates from other types of infection, and these isolates provide peculiar difficulties in antimicrobial resistance testing due to their hypermutable state. A concerning trend towards multi-drug resistance is emerging worldwide, which has grave implications for the capacity of current therapies to eradicate *P. aeruginosa* infections in the future (Kiska and Gilligan 2003, Bradbury, Richard).

1.16. Intrinsic resistance mechanisms

P. aeruginosa is resistant to a range of antimicrobials commonly used to treat Gram negative infections. This was until recently considered to be due to cell membrane impermeability, but in fact the large membrane porin OprF of *P. aeruginosa* will easily allow access of these agents into the cell. More recent work has identified the presence of the efflux pump system MexAB-OprM, which actively removes β–lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline and trimethoprim from the cell, as well as a number of dyes, detergents, inhibitors of fatty acid biosynthesis and solvents (Hirakata, Srikumar *et al*. 2002; Livermore 2002).

1.17. The chromosomal β–lactamases

P. aeruginosa possesses a chromosomal β–lactamase, AmpC, which is both induced by and hydrolyses a wide spectrum of β–lactam agents, including third generation cephalosporins and aztreonam, but not imipenem (Livermore 2001; Livermore 2002). Three AmpC types have been described in *P. aeruginosa*; CMY-1, Fox-1 and MOX-1, although with the emergence of plasmid mediated AmpC resistance more types may be found (Rice, Sahm *et al*. 2003). The chromosomal gene, *ampC* exists naturally in a repressed state, and thus levels of AmpC production are below clinically significant levels. However, exposure to specific β-lactam agents, particularly cefoxitin, ampicillin and imipenem will result in the de-repression of *ampC* and subsequent hyperproduction of the AmpC enzyme, with resultant treatment failure.

The activity of AmpC is not significantly inhibited by clavulanic acid (Rice, Sahm *et al*., 2003) but the anti-pseudomonal penicillin classes; carboxypenicillins (ticarcillin, carbenicillin) and ureidopenicillins (piperacillin), are effective despite the activity of AmpC, as while they are hydrolysed by this enzyme (carbenicillin may be resistant depending on AmpC type), they do not induce its hyperproduction (Pitt 1998) AmpC hyperproduction is a mutation which occurs relatively infrequently without induction (approximately 1 in 10^{-8})(Pitt 1998, Bradbury, Richard).

1.18. Active efflux pumps

As previously discussed, the active efflux system MexAB-OprM plays a major role in the intrinsic resistance of *P. aeruginosa* to many antimicrobial agents. This active efflux is composed of the MexB broad spectrum pump, MexA linking protein and the OprM outer membrane porin. In its natural environment, it is thought that this pump may play a role in removing toxic substances from the bacterial cytoplasm. Mutations in *nalB*, a gene of the *mexR* locus, result in high frequency up regulation of MexAB-OprM. This up regulation of active efflux will raise MICs to penicillins, cephalosporins, tetracycline and chloramphenicol. Selection of this mutation will occur *in vitro* during therapy, and it is the predominant mutation involved in resistance to ticarcillin and carbenicillin, but may also confer resistance to ceftazidime and meropenem. A much less common mutation of *nfxC* of the *mexT* locus, induced by fluoroquinolone therapy will induce up regulation of MexEF-OprN, raising MICs to both carbapenems (possibly due to co-regulation with OprD) and fluoroquinolones (Livermore 2001). A further uncommon mutation of *nfxB* upregulates MexCD-OprJ, resulting in a similar resistance phenotype to mutant MexAB-OprM (Livermore 2002, Bradbury, Richard).

A fourth mutant active efflux pump system, MexXY-OprM (syn. AmrAB) is implicated in resistance to β-lactam agents, macrolides, glycylcyclines, lincosamides, chloramphenicol, fluoroquinolones and aminoglycosides, but not imipenem (Livermore 2002). This phenotype is by far the most concerning in clinical practice, as it confers resistance to a very wide range of antimicrobial agents in one mutation. Expression of *mexXY* is regulated by MexZ, and mutation of *mexZ* gene results in up regulation of this gene. Studies have found that mutation of *mexZ in vitro* did not confer aminoglycoside resistance, and it is suggested that another as yet unknown factor may be required for complete up regulation of MexXY-OprM (Livermore 2001). Genome sequencing of *P. aeruginosa* has identified at least five other active efflux pump systems which are yet to be characterised (Livermore 2002, Bradbury, Richard).

1.18.1 Membrane changes

Changes in the membrane structure of *P. aeruginosa* to reduce permeability plays a major role in the resistance of this organism to antimicrobials. The outer membrane protein OprD is a porin for the passive uptake of amino acids, but which is also permeable to carbapenems. Loss of OprD expression raises the imipenem MIC of *P. aeruginosa* strains to clinically resistant levels, but will only reduce susceptibility to meropenem. This change does not affect the MICs of non-carbapenem antimicrobials. It has been found that MexEF-OprN is co-regulated with OprD, and thus decreased expression of OprD and up regulation of MexEF-OprN may occur simultaneously upon exposure to fluoroquinolones or less commonly, imipenem (Livermore 2001). Also notable is a significant correlation between the presence of OprR and resistance to quarternary ammonium compound disinfectants, such as cetylpyridium chloride and benzalkonium chloride (Tabata, Nagamune *et al*. 2003, Bradbury, Richard).

The operon *oprH-phoP-phoQ*, regulated by the concentration of Mg2+ ions and PhoP, codes for products involved in both aminoglycoside and polymixin resistance. These genes are expressed in conditions of low Mg2+ concentration, and it is thought that OprH may be involved in maintaining outer membrane stability, whilst PhoP and PhoQ are involved in regulation of cationic ion levels. PhoP and PhoQ have been shown to play a role in resistance to both polymixin B and aminoglycoside antibiotics. The mechanisms by which this occurs are not yet fully understood, although it is known that elevated Mg2+ decreases the sensitivity of *P.* aeruginosa to aminoglycoside antibiotics (Macfarlane, Kwasnicka *et al*. 2000, Bradbury, Richard).

1.19. Mutations in type II topoisomerase enzymes

The fluoroquinolone antibiotics (ciprofloxacin, levofloxacin) act upon the type II isomerase enzymes. These enzymes control the topological conformation of DNA during replication and transcription. In *P. aeruginosa*, fluoroquinolone resistance may occur either through multidrug efflux pumps (see previous) or alterations in the type II topoisomerase II enzymes (DNA gyrase and topoisomerase IV) which render

them resistant to the action of the fluoroquinolones. DNA gyrase is composed of two A and two B sub units, encoded by *gyrA* and *gyrB*. Topoisomerase IV bears the same heterotetramer formation as DNA gyrase, with one double sub-unit coded by *parC* and one by *parE*. Mutation in any one of these genes may result in resistance to fluoroquinolone therapy, and this mechanism is the most commonly identified cause of fluoroquinolone resistance in clinical strains of *P. aeruginosa* (Akasaka, Tanaka *et al*. 2001). Mutations in both *gyrA* alone resulted in 4 to 64 times higher MICs to fluoroquinolones when compared to wild-type, while a combination of *gyrA* and *mexR* (MexAB-OprM pump) mutations resulted in resistance levels 1,024 times higher than wild type (Nakajima, Sugimoto *et al*. 2002, Bradbury, Richard).

1.20. Aminoglycoside modifying enzymes

Three broad classes of aminoglycoside modifying enzymes have been identified in *P. aeruginosa*, these being the aminoglycoside phosphoryltransferase (APH), aminoglycoside acetyltransferase (AAC) and aminoglycoside nucleotidyltransferase (ANT) enzymes. These enzymes may be either chromosomal or mobile on plasmids and transposons. Depending on sub type and activity, they are capable of hydrolysing either a specific and limited number of antimicrobials or a broad spectrum of agents of the aminoglycoside class (Poole 2005).

The most common aminglycoside hydrolysing enzymes in *P. aeruginosa* are the ANT (2")-I enzymes, hydrolysing aminoglycosides at the 2' position. These enzymes will hydrolyse gentamicin and tobramycin, but not netilmicin or amikacin. This enzyme has been shown to be significantly more common in AES2 CF clonal complex isolates than in genotypically unique CF isolates (Syrmis, Bell *et al*. 2008). Other common enzymes are AAC (3)-I (gentamicin only) and AAC (6')-II (gentamicin, tobramycin and netilmicin) *aac(3)* and *aac(6')* genes may be found on transposons and integrons, often in association with narrow spectrum, extended spectrum or metallo β-lactamases (Poole 2005, Bradbury, Richard).

1.21. Mobile β-lactamases

Acquisition of resistance genes on mobile genetic elements is common in many bacteria, and no less so in *P. aeruginosa*. Genes coding for resistance enzymes are often found on plasmids and transposons, with some even carrying multiple resistance genes on a single integron (Walsh, Toleman *et al*. 2005). The presence of multiple resistance gene cassettes on a single mobile element is of particular concern, given the capacity of such mobile elements to create a multi-resistant *P. aeruginosa* infection literally overnight. Exposure to antimicrobial agents selects for and amplifies the presence of resistant bacterial populations within a patient, providing a source for the spread of resistance throughout a hospital. In this way, hospitals and other institutions may harbour highly resistant and difficult to treat strains of *P. aeruginosa* unless very thorough infection control procedures are adhered(Bradbury, Richard).

1.22. Narrow spectrum β-lactamases

P. aeruginosa strains commonly acquire OXA (Ambler class D) and/or PSE (syn. CARB - Ambler class A) β-lactamases (Bert, Branger *et al*. 2002; Rice, Sahm *et al*. 2003; Weldhagen, Poirel *et al*. 2003), which will hydrolyse carboxy-penicillins and ureidopenicillins, narrow spectrum cephalosporins and expanded spectrum cephalosporins, but not extended spectrum cephalosporins (Bert, Branger *et al*. 2002). These enzymes are commonly found in *P. aeruginosa*, but are considered very rare in the enterobacteriaceae. Conversely, narrow spectrum Ambler class A β-lactamases, such as TEM and SHV, commonly found in enterobacteriaceae are found only rarely in *P. aeruginosa* (Weldhagen, Poirel *et al*. 2003). A French study found that only 10% of ticarcillin resistant *P. aeruginosa* isolates (representing just 1,9% of *P. aeruginosa* isolates overall) had acquired a TEM type β-lactamase (Bert, Branger *et al*. 2002).

1.23. Extended spectrum β-lactamases

A relatively recent occurrence (1983) in the history of antimicrobial resistance was the discovery of extended spectrum β-lactamases (ESBLs) These enzymes were identified shortly after the introduction of the extended spectrum (or third generation) cephalosporins, such as ceftazidime, cefotaxime and ceftriaxone. ESBLs are narrow spectrum β-lactamase enzymes which have mutated to allow the binding of extended spectrum cephalosporins. Thus, these enzymes are often active against all penicillins, and cephalosporins below the fourth generation (cefepime and cefpirome) as well as the monobactam, aztreonam (Jiang, Zhang *et al*. 2006). Some of these enzymes show a greater affinity for specific antimicrobials, such as the CTX-M ESBLs, which more efficiently hydrolyse cefotaxime and ceftriaxone than they do ceftazidime (Rice, Sahm *et al*. 2003). The activity of ESBLs may be inhibited by exposure to clavulanic acid, sulbactam or tazobactam. Consistent with their spectra of activity, the effect of a specific inhibitor is dependent upon what type of enzyme is involved. For instance, the CTX-M enzymes are more readily inhibited by tazobactam than by clavulanic acid, whereas some TEM ESBLs are resistant to all β-lactamases inhibitors (Rice, Sahm *et al*. 2003).

Many different ESBL types have been identified in *P. aeruginosa*, some of which are associated with specific geographic regions, leading to the suggestion that these enzymes may perform a role within specific ecological niches. Although rates of ESBL positivity in *P. aeruginosa* are low compared to the enterobacteriaceae, this frequency appears to be increasing (Jiang, Zhang *et al*. 2006). It is thought that the TEM and SHV ESBLs found in *P. aeruginosa* have been acquired from enterobacteriaceae, whilst the PER and OXA classes are mutations of enzymes from *P. aeruginosa* itself. ESBL types described in *P. aeruginosa* thus far are; VEB, PER, SHV, TEM, GES, IBC and BEL class ESBLs (Weldhagen, Poirel *et al*. 2003; Bogaerts, Bauraing *et al*. 2007, Bradbury, Richard).

1.24. Mobile metallo-β-lactamases

Metallo-β-lactamases (Ambler class B) are metalloenzymes, relying upon metal ions rather than serine for catalysis of their reactions. These enzymes have a broad spectrum of activity including penicillins, cephalosporins (including the fourth generation cephalosporins) and imipenem, but not aztreonam. They are not inhibited by β-lactamase inhibitors, but will not function in the presence of ion chelators due to chelation of metal ion enzymatic co-factors. The extremely wide spectrum of action of these enzymes causes serious difficulties in clinical practice, as the use of virtually all β-lactam antimicrobials is ineffective in all organisms expressing such enzymes.

A recent and extremely disturbing event has been the emergence of multiple types of new mobile metallo-β-lactamases (MMβLs), and their spread between the enterobacteriaceae and non-fermentative Gram negative rods (Rice, Sahm *et al*. 2003). The first MMβL identified in a strain of *P. aeruginosa* was IMP-1, found to be encoded on a mobile conjugative plasmid. Since this time, at least four other types of MMβL have been identified (GIM-1, VIM-1, VIM-2, SPM-1), conferring resistance to all β-lactam agents, including imipenem, with a greater or lesser degrees of activity against aztreonam. The rapid spread of these novel resistance genes worldwide has resulted in its appearance in most countries (including Australia) (Walsh, Toleman *et al*. 2005) SPM-1 MMβL producing strains accounting for 35% of clinical carbapenem resistant isolates of *P. aeruginosa* from hospitals throughout Brazil in 2003 (Gales, Menezes *et al*. 2003). Even greater concern has been the identification of an integron carrying a partially deleted *qac* and *sul* genes, *bla*GIM-1, *aac*A4, *aad*A1 and *bla*OXA-2. Thus, in one mobile genetic element, resistance to sulphonamides, quarternary ammonia compounds, β-lactam agents and aminoglycosides, treatment with any of which would select for bacterial isolates that had acquired this integron (Walsh, Toleman *et al*. 2005). The potential for the world-wide spread of MMβL resistance and accompanying multi-drug resistance cassettes in both the enterobacteriaceae and non-fermenting Gram negative rods, in a process analogous to the rapid spread of the ESBL phenotype remains an ominous prospect.

1.25. Multi-drug resistance

Pan and multi drug resistance in *P. aeruginosa* is defined by the US CF foundation consensus guidelines as resistance to colistin or all agents (as pan drug resistance) and multi drug resistance in two or more of the following classes: β-lactams, aminoglycosides and fluoroquinolones (Armstrong, Nixon *et al*. 2012). The emergence of multi-drug resistant (MDR) strains in both CF and non-CF clinical isolates over recent years remains of great concern. The previously discussed multiple mechanisms of *P. aeruginosa* resistance and multi-drug resistance may be found in any combination within any given isolate. Multi drug resistance is more common in nosocomial isolates than in those contracted within the community, with rates of MDR isolates infection decreasing with patient age (Flamm, Weaver *et al*. 2004). Empirical treatment of *P. aeruginosa* infections and prolonged treatment with antibiotics were found to be pre-disposing factors in the emergence of MDR phenotypes (Seigel 2008). MDR isolates were found in one US study (in which multi-drug resistance was defined as resistance to three or more antimicrobial agents) to be most common in respiratory isolates from patients in ICUs (Flamm, Weaver *et al*. 2004). In many cases, treatment options become limited to single drugs, such as colistin (Kiska and Gilligan 2003). Nosocomial infections with MDR *P. aeruginosa* are resulted in severe limitation of treatment options and marked negative impacts on patient outcomes (Zavascki, Gaspareto *et al*. 2005).

1.26. Hypermutation and multi-drug resistance

MDR isolates of *P. aeruginosa* in the CF context merit separate attention to those found in a nosocomial setting. Up to 37% of CF patients carried at least one hypermutable isolate (Oliver, Levin *et al*. 2004), similar levels of hypermutation were found in a study of non-CF chronic lung infection isolates (bronchiectasis, COPD) in which 57% of patients were found to possess hypermutable isolates (Maciá, Blanquer *et al*. 2005). These figures represent the highest percentage of hypermutation in any known bacterial population (Gutierrez, Juan *et al*. 2004). By comparison, less than 1% of 103 ICU isolates investigated in one study were

hypermutable (Gutierrez, Juan *et al*. 2004). In the natural environment, a stable mutator phenotype confers great survival advantage to a bacterial strain by allowing it to better adjust to novel environmental conditions. In the case of human infection, a hypermutable state not only allows the organism to adjust to assault by the host immune system, but also to quickly develop resistance to antimicrobial agents that may be used in therapy (Gutierrez, Juan *et al*. 2004).

It has been suggested that hyper-mutation may be induced by prolonged antimicrobial therapy (as is applied to CF patients) (Morosini, Garcia-Castillo *et al*. 2005), representing the "chicken and egg" dilemma in hypermutable MDR chronic lung infection isolates. Alternately, Ciofu, *et al* performed work which suggested that hypermutation in chronic lung infections may be caused by oxidation of *Pseudomonal* DNA by reactive oxygen species liberated from polymorphonuclear leukocytes during chronic infection (Ciofu, Riis *et al*. 2005). It is possible that both mechanisms may be involved in the evolution of a hyper-mutable genotype in *P. aeruginosa* during chronic lung infection.

Hyper mutation of *P. aeruginosa* in the context of chronic lung infection isolates presents specific difficulties in treatment. Due to the prevalence of hyper mutation in these strains, it is probable that in any chronically infected lung, isolates already resistant to all or a majority of anti-pseudomonal agents exist. A study of an apparently fully sensitive PA01Δ*mutS* strain found that at concentrations of $5x10^4$ exposures to any of 11 anti-*pseudomonal* agents resulted in clinically relevant levels of resistance within 24-36 hours (Oliver, Levin *et al*. 2004). Thus, it was recommended that mono-therapy never be used in CF infections, and that levels of any single drug used in therapy should not be allowed to drop below MIC levels *in vivo*, to avoid the selection of an MDR phenotype (Oliver, Levin *et al*. 2004, Bradbury, Richard).

Hyper mutable strains will often display multiple colonial morph types, despite these colonies being of identical genotype. One study found a mean number of four morph types per CF sputum.

The same study performed antimicrobial susceptibility testing by the BSAC method and found an average of three differing anti biograms per morph type (Foweraker, Laughton *et al*. 2005). This variation in anti-bio gram is also seen within genotypes, and thus anti-biograms are considered to be a poor marker for use in epidemiological surveillance (Williams 1997). The antimicrobial susceptibility results generated by clinical laboratories based on assays of one morph type may provide misleading results to a clinician, and the efficacy of these results in guiding treatment of chronic *P. aeruginosa* CF lung infections is suspect (Bradbury, Richard).

1.27 Virulence factors of *Pseudomonas aeruginosa*

To be described as a virulence factor of an organism, a molecule must first adhere to the molecular Koch's postulates, which state that any virulence factor must i) be encoded by a gene that is associated with bacteria that cause disease, but usually absent in or inactive in strains that fail to cause disease, ii) be required for virulence, as evidenced by a loss of virulence when disrupted or a gain in virulence when expressed in an avirulent strain and iii) be usually expressed during infection (Engel 2003). Many virulence factors in *P. aeruginosa* are horizontally acquired (Engel 2003), as reflected by the great diversity in virulence factors within the species. It should be noted that most studies of virulence are performed upon laboratory control strains, and it is likely that there are novel virulence factors found in clinical strains (or even the much studied control strains) that are yet to be identified. Also to be noted is that virulence factors are likely to perform a useful function for any organism within their natural environment, and that as *P. aeruginosa* is not an obligate parasite of humans, many factors harmful to humans expressed by this organism will most probably perform a more innocuous function within the organism's natural habitat (Bradbury, Richard).

Virulence factors in *P. aeruginosa* are divided into specific groups dependent upon their mode of action or method of delivery to the host cell. Thus, these virulence factors may be described as belonging to adhesions and secreted toxins and enzymes dependent or independent of the type I secretion system (T1SS), type II secretion

system (T2SS) and type III secretion system (T3SS) these are described in detail later(Bradbury, Richard).

1.27.1 Global regulation of virulence

Between 468 and 521 genes bearing characteristics of transcriptional regulators or environmental sensors were found during the sequencing of the PA01 genome (Engel 2003). Regulation of many *P. aeruginosa* virulence factors is carried out by complex interactions with and between QS molecules (discussed previously), but QS effectors expression itself is under regulation from a number of global regulators of virulence. Global regulatory proteins identified thus far in *P.aeruginosa* include Vfr, GacA, RsaL, QscR, RsmA, mvfR, MvaT, VqsR, pprB and the sigma factors RpoS and RpoN (Dong, Zhang *et al*. 2005). To demonstrate the complexity of regulation interactions within *P. aeruginosa*, the example of one regulator alone,Vfr (virulence factor regulator), may be considered.Vfr is involved in the regulation of the *las* QS system, which in turn regulates the *rhl* and PQS QS systems.Vfr also acts directly upon *toxA* and *regA* to regulate synthesis of the toxin ExoA (Suh, Runyen-Janecky *et al*. 2002) Mutation in *vfr*alone affected the production of least 60 proteins within the cell (Suh, Runyen-Janecky *et al*. 2002). Expression of Vfr itself is dependent upon the co-factor cyclic-AMP, which is controlled by the adenylatecyclizes CyaA and CyaB (Smith, Wolfgang *et al*. 2004). The ennui of regulation in *P. aeruginosais* beyond the scope of this review, but it exemplifies the great complexity and inter-relationship between regulatory, signaling and virulence factors in the organism, an attribute that confers its tremendous phenotypic malleability, allowing successful colonization and infection in a diverse range of environmental and clinical conditions (Bradbury, Richard).

1.27.2 Cell surface virulence factors

Alginate, an exo-polysaccharide produced by all *P. aeruginosa* cells, and hyper produced by mucoid variants, was considered to have a role in adherence and invasion of host cells (Engel 2003). However, no difference in capacity to adhere to

and invade host cells has been demonstrated between non-mucoid and mucoid phenotypes (Massengale, Quinn *et al*. 2000, Bradbury, Richard).

Alginate is considered to provide protection against opsonization by complement and phagocytosis (May, Shinabarger *et al*. 1991) as well as providing a barrier to the diffusion of antimicrobials into the cell (May, Shinabarger *et al*. 1991; Zielinski, Maharaj *et al*. 1992). Further to these advantages, alginate hyper-producing mucoid strains of *P. aeruginosa* display far greater capacity to survive in aerosol droplets *in vitro* (Clifton, Fletcher *et al*. 2008, Bradbury, Richard).

Cell surface lipopolysaccharide (LPS) is involved in the adherence of *P. aeruginosa* to host cells, and subsequent internalization. *P. aeruginosa* LPS is composed of the polysaccharide O and core antigens and lipid A composed of fatty acid and phosphate groups bonded to a glucosamine disaccharide (Ernst, Adams *et al*. 2006). Truncation of the O antigen structure (to form rough LPS) may occur in the case of chronic CF and HIV infection (Asboe, Gant *et al*. 1998; Davidson, Currie *et al*. 2003; Ernst, Adams *et al*. 2006). Truncation of the O polysaccharide side chains renders the cell more susceptible to complement mediated lysis, but may provide a selective advantage by conferring immunity to detection by host O antigen specific antibodies (Davidson, Currie *et al*. 2003). Speert, *et al*. showed that change to the rough phenotype LPS could be induced in *P*. aeruginosa when grown under sub-optimal levels of nutrition, and therefore suggested that this change may be due to a lack of nutrients in the CF lung rather than interactions between the bacteria and host (Speert, Farmer *et al*. 1990, Bradbury, Richard)

The adhesion interactions of cell wall lipopolysaccharide (LPS) and CFTR in the host cell lung present a possible role of CFTR in defense against *P. aeruginosa* colonization warrant particular attention. In the normal lung, CFTR is expressed on the surface of respiratory epithelial cells (Tatterson, Poschet *et al*. 2001). It is postulated that CFTR is a cellular receptor for *P. aeruginosa* LPS, and that attachment of LPS to CFTR is followed by cellular internalization. Following epithelial cell internalization, *P. aeruginosa* would then be cleared from the airway

by epithelial detachment (Tatterson, Poschet *et al*. 2001) or epithelial cell apoptosis (Grassme, Kirschnek *et al*. 2000).

Cells expressing rough LPS have been shown to be less effectively internalized by this process (Davidson, Currie *et al*. 2003, and Bradbury Richard).

Type IV pili (TFP), found in many Gram negative organisms, and composed of a single protein; Pili-A, play a major role in the adhesion of *P. aeruginosa* to host cell membranes and inanimate surfaces, a remarkable example of which is their capacity to adhere to stainless steel (Giltner, van Schaik *et al*. 2006). Analogous to the interactions of CFTR and LPS, it is hypothesized that TFP may adhere more specifically to ΔF508 CF respiratory epithelial cells than to normal respiratory epithelium. Studies have suggested that glycoproteins on the outer membrane of ΔF508 CF respiratory epithelial cells show decreased sialation (Tatterson, Poschet *et al*. 2001). Following LPS and TFP mediated adhesion; *P. aeruginosa* has the capacity to secrete a large number of virulence factors capable of causing cell damage and death (Bradbury, Richard).

1.27.3 Secreted virulence factors

Secreted exo-products of *P. aeruginosa* account for a significant number of its known virulence factors. Secretion of virulence factors may occur by a number of different pathways, both active and passive. Virulence factors that passively diffuse across the cell membrane include the pigments pyocyanin and pyoverdin, while many other virulence factors are actively secreted by the type I, type II (sec dependent) or type III secretion systems, common to many Gram negative organisms (Bradbury, Richard).

1.27.4 Hyperactive efflux pumps

The hyperactive efflux pumps involved in MDR phenotypes of *P. aeruginosa* may also be involved in increased virulence. It has been shown that these pumps export

HSL molecules involved in the QS system, which regulates the production of many virulence factors. A Japanese study found that MexAB-OprM, MexXY-OprM active efflux systems had a direct effect on invasiveness. Knockout mutants without these efflux mechanisms showed significantly decreased invasiveness in MDCK cell lines and decreased mortality in leukemic mice when compared to wild-type strains (Hirakata, Srikumar *et al*. 2002). It was suggested that these efflux systems may actively export not only antimicrobial agents and QS molecules, but also virulence factors. Other studies have found that MexAB-OprM and MexCD-OprJ mutants demonstrate reduced expression of T2SS virulence factors (Evans, Passador *et al*. 1998; Sanchez, Linares *et al*. 2002), and that induced MexCD-OprJ mutants of PAO1 showed decreased activity of the T3SS virulence pathway (Linares, Lopez *et al*. 2005, Bradbury, Richard).

1.27.5 Phenazines, pyocyanin and other pigments

One of the most obvious and striking phenotypic traits of *P. aeruginosa* is its blue/green pigment, expressed by over 90% of isolates (Mavrodi, Bonsall *et al*. 2001; Engel 2003). This pigmentation is caused by the secretion of the phenazine compound pyocyanin (PCN), which represents a significant virulence factor in this organism. PCN is easily recovered from the ear discharges and sputum of *P. aeruginosa* infected individuals (Lau, Hassett *et al*. 2004). It also has strong antibacterial action (Engel 2003), which may provide a selective advantage to *P. aeruginosa* over other bacterial species in both its natural environment and infections. Production of pyocyanin is regulated directly by the *las* and *rhl* QS systems, and to a lesser degree the global regulator *GacA*-*GacS* and *Vfr* (Lau, Hassett et *al*. 2004). The precursor molecules, chorismic acid, coded by the *phzI* operon and phenazine-1-carboxylic acid, coded by the *phzII* operon are modified into the final three tricyclic phenazine compounds passively secreted by *P. aeruginosa*; pyocyanin, 1-hydroxyphenazine and phenazine-1-carboxamide by PhzH, PhzM and PhzS (coded by *phzH*,*phzM* and *phzS*, respectively) (Mavrodi *et al*. 2001; Finnan *et al*. 2004; Lau, Hassett *et al*. 2004, Bradbury, Richard).

Phenazine compounds exert their pathogenic action by increasing intracellular oxidative stress through intracellular redox cycling of reducing agents and oxygen, producing superoxide and hydrogen peroxide (Mavrodi, Bonsall *et al*. 2001). By this mechanism, phenazines can inhibit mitochondrial activity and superoxide production in neutrophils and macrophages. Inhibition of cell proliferation and cytokine secretion is also observed (Engel 2003). Pyocyanin may be recovered in high concentrations from the respiratory tract of CF patients, and is believed to be involved with lung damage through interference with ion transport mechanisms and inhibition of cilliary beating as well as interference with mucous secretion through alteration of intracellular concentrations of Ca2+ (Mavrodi, Bonsall et *al*. 2001, Bradbury, Richard).

P. aeruginosa may produce a number of pigments other than pyocyanin, including pyoverdin (yellow), pyorubrin (red) and pyomelanin (brown/black). The most clinically significant of these appears to be pyoverdin, expressed by 70% of clinical isolates. Pyoverdin acts as a siderophore, expression of which is regulated by its own concentration, but which also regulates the production exotoxin A (Engel 2003). Pyorubrin is a water soluble pigment resulting in the production of rust colored colonies. This pigment is found in only 2% of clinical isolates, primarily those from urine and CF cultures. Pyomelanin is only seen in 1% of clinical isolates, the dark brown/black colored colonies resulting from pyomelanin production have provided difficulties in identification to in experienced laboratory staff (Pitt 1998, Bradbury, Richard).

1.27.6 Cyanide

Cyanide has long been accepted as a potent toxin, being a powerful inhibitor of cytochrome *o*xidase, the terminal component of respiratory metabolism (Finnan, Morrissey et *al*. 2004). Thus, those cyanogen properties of *P. aeruginosa* provide it with a potent virulence factor in organisms that obtain energy through aerobic respiration. It has been suggested that HCN production is the primary virulence factor involved in the Cenorhabditus elegans virulence model (Gallagher and Manoil

2001). Cyanide synthesis is coded by three separate genes; *hcnA*, *hcnB* and *hcnC* (Ramette, Frapolli *et al*. 2003), each coding for separate sub units of the enzyme (Laville, Bulmer *et al*. 1998). Cyanide production by *P. aeruginosa* is controlled by the RsmA/RsmZ system (Heurlier, Williams *et al*. 2004), and occurs primarily when the organism is grown in micro-aerophilic conditions (Laville, Bulmer *et al*. 1998; Sanderson, Wescombe *et al*. 2008, Bradbury, Richard).

1.27.7 Rhamnolipid and cytotoxic lectins

Rhamnolipid is a rhamnose containing a glycolipid bio surfactant which solubilizes phospholipids in the lung surfactant, increasing their accessibility to the type II secreted phospholipase C enzymes (Engel 2003). Rhamnolipid is a heat stable haemolysin (Pitt 1998), also acting as a surfactant for swarming motility (Kohler, Curty *et al*. 2000). Studies utilizing purified rhamnolipid have found it to induce cytotoxic blebbing and decrease phagocytic ability in macrophages, inhibit ciliary beating in respiratory epithelial cells and rapidly lyse the environmental slime mold Dictyostelium discoideum (Engel 2003, Bradbury, Richard).

Two cytotoxic lections are associated with *P*. aeruginosa; the galactose specific lecA (*lecA*, synonym PA-IL) and the fucose specific lecB (*lecB*, synonym PA-IIL). These lectins act as both adhesions and cytotoxins in the respiratory tract, and are also required for QS and rpoS σ factor function (Engel 2003; Tielker, Hacker *et al*. 2005). LecB appears to have a function in biofilm formation as lecB deficient mutants' show impaired capacity to form biofilms (Tielker, Hacker *et al*. 2005). Expression of rhamnolipid and lecA is positively controlled by the Rhl QS pathway (Engel 2003), and is negatively controlled by small ribosome binding protein RsmA (Heurlier, William *et al*. 2004).

1.27.8 The type I secretion system

1.27.9Alkaline protease

Alkaline protease (Apr, coded by *apr*) represents the only toxin of *P. aeruginosa* transported out of the cell by the type I secretion system, a sec independent active transport mechanism to transfer molecules from the inner to the outer membrane of a cell. Alkaline protease has a wide range of substrates, including collagen, C1q and C3 of the complement pathway, serum protease inhibitors, fibrin, fibrinogen, laminen and elastin (Pitt 1998; Engel 2003). The ability of Apr to hydrolyze pulmonary elastin is markedly inferior to that of the elastase enzyme, secreted by the type IIsecretion system (Pitt 1998).

1.27.10The type II secretion system

The type two secretion systems (T2SS) apparatus is coded for by the *xcp* cluster. The apparatus itself transports molecules from the inner cell membrane using a sec dependent mechanism (Bradbury, Richard).

Molecules are excreted through the outer membrane by means of an outer membrane type II secretion apparatus similar to, and sharing components with the TFP and some DNA uptake systems. The expression of both T2SS secreted factors and *xcp* itself is controlled by cell density dependent QS molecules (Lee, Smith *et al*. 2005, and Bradbury Richard).

1.27.11 Elastolytic Enzymes

Elastase (syn: pulmonary elastase, LasB) is the most powerful proteolytic enzyme secreted by *P.aeruginosa*, accounting for 90% of its proteolytic activity (Pitt 1998; Engel 2003, Bradbury, Richard).

Elastase begins as a 53.6 kDa pre-protein, which is then cleaved in the periplasm during transport across the cell membrane to a 33 kDa zinc metallo-protease with pH

optima of 7-8 (Pitt 1998; Engel2003). The enzyme has a wide range of substrates, including elements of connective tissue such as elastin, collagen, fibronectin and laminen, as well as immune and host defense molecules such as Fibrin, gastric mucin, transferrin, α-1 proteinase inhibitors, IgG, γ-interferon and components of complement pathway. Elastase is coded by *lasB*, the expression of which is controlled through the las QS pathway (Engel 2003), and is produced in greatest quantity when cells are in the late logarithmic phase of growth or at high cell density (Finnan, Morrissey *et al*. 2004).

P. aeruginosa also produces a second elastolytic enzyme, the serine protease LasA (coded by *lasA*). This enzyme was thought to nick elastin, exposing active sites for proteolysis by LasB (Engel 2003). Subsequent studies have however shown that the enzyme is capable of elastolytic activity independent of LasB and Apr (Toder, Ferrell *et al*. 1994).

1.27.12 Exotoxin A

ExotoxinA (ExoA, *toxA*) is a 66 kDA protein acts as a major virulence factor of *P. aeruginosa*, analogous in action to that of diphtheria toxin. ExoA is a highly virulent protein, exhibiting and LD50 of 2.5 mg/kg in mice, it has been shown that Δ*toxA* mutants are less virulent than wild type strains, and that vaccination against ExoA confers partial immunity to *P. aeruginosa* infection in animals (Engel 2003). Injection of purified ExoA results in leucopaenia, hepatic necrosis, hypotension and shock when injected into test animals. On a microscopic level, collagen is disrupted, proteoglycan ground substance is lost and widespread endothelial and epithelial cell death is observed (Pitt 1998).

1.27.13 Phospholipase C and lipases

P.aeruginosais known to produce two heat labile phospholipase C enzymes; Plc-H (coded by *plcS*) and Plc-N (coded by *plcN*) (Pitt 1998; Engel 2003).Mutants deficient in Plc-H and Plc-N display decreased alveolar destruction in rabbit pneumonia

models, decreased virulence in a Cenorhabditus elegans model, and a *plcH* mutant showed decreased virulence in a burnt mouse model when compared to wild-type strains (Engel 2003). The release of diacyl glycerol as a bi-product of lecithin hydrolysis is most probably involved in the inflammatory reaction to *P*.aeruginosa infection, as this compound is further reduced to the powerful inflammatory mediate or arachidonic acid by bacterial or host cell lipases (Pitt 1998, Bradbury, Richard).

P. aeruginosa excretes two lipase molecules, LipA a pro-enzyme of 30 kDa, and a monomeric lipase of 29 kDa, LipC. The lipases of *P.aeruginosa* are active against a variety of fats as well as tween 20 and tween 80 (Pitt 1998). Lipase has been found to inhibit monocyte activity *in vitro* and may be a promoter of the inflammatory response *in vivo* (Engel 2003). LipA is secreted during late logarithmic growth phase and appears to be bound to LPS (Pitt 1998, Bradbury, Richard).

1.27.14The type III secretion system

The type III secretion system (T3SS) of *Pseudomonas aeruginosa* is a sec independent molecular export system (Engel 2003). The T3SS requires direct contact with host cells to function, effect molecules being actively translocated from the prokaryotic to the eukaryotic cell cytosol by means of a complex secretory apparatus (Miyata, Casey *et al*. 2003). Rates of T3SS expression are greater than 80% in acute *P. aeruginosa* infections, while expression occurs to a much lesser degree in chronic CF respiratory infections (Soong, Parker *et al*. 2007, Bradbury, Richard).

The T3SS of *P. aeruginosa* is composed of 5 classes of protein; the secretion apparatus, factors requiring translocation, effecter proteins, chaperones and transcriptional regulatory proteins.

Twenty or more proteins, coded by three contiguous operons, form a flagella related "needle "apparatus that creates a translocation channel, allowing transport of molecules across the bacterial cell membrane. Following attachment of the needle apparatus to the host cell, the hydrophobic proteins PopB and PopD, excreted by the

T3SS, form pores in the host cell lipid bilayer, allowing unimpeded transport of effecter proteins from the bacterial cytoplasm into the host cell cytoplasm (Engel 2003).

The regulatory protein ExsA is required for optimal transcription of both the translocation and effecter molecules of the T3SS (Engel 2003; Smith, Wolfgang *et al*. 2004), and ExsA has been shown to be required for full virulence in animal models of acute pneumonia (Shaver and Hauser2004, Bradbury, Richard).

Expression of the T3SS is calcium ion concentration dependent (Kim, Ahn *et al*. 2005), and may be negatively regulated by the Rhl QS pathway (Bleves, Soscia *et al*. 2005). However, more recent work has suggested that whilst T3SS expression is cell density dependent; this faculty is independent of QS signaling (Shen, Filopon *et al*. 2008). Furthermore, it is suggested that the negative regulation of T3SS is not dependent on QS signaling, but is controlled by the expression of the activity of tryptophan synthetase and the presence of its substrate, tryptophan. This finding is not in conflict with the previously findings regarding a correlation between QS signaling molecule prevalence and negative regulation of T3SS, as tryptophan is a precursor molecule for a number of such signaling molecules (Shen, Filopon *et al*. 2008). Significantly, the presence of CD95 on host cell macrophages appears to mediate host cell apoptosis by the T3SS (Engel 2003).Suggesting that this may be a receptor site for the T3SS apparatus. Also of note is that antibodies to PopB and the T3SS effecter exo-enzyme S are detectable by ELISA in the serum of CF patients at the time of colonization with *P. aeruginosa*, and screening for these antibodies has been suggested as a method of early detection of this important clinical event (Corech, Rao *et al*. 2005, Bradbury, Richard).

While the translocation machinery of the T3SS is highly conserved in *P. aeruginosa*, presence or absence the four identified T3SS effecter genes is diverse. The protein products of these genes are translocated across the T3SS secretory apparatus bound to chaperones, which maintain their stability during transport (Engel 2003). Four T3SS effecter enzymes have been identified thus far, these being exoenzymes S, T, Y and U (ExoS ExoT, ExoY and ExoU) (Finnan, Morrissey *et al*. 2004). Different

strains will display differing combinations of T3SS effecter enzyme genes. While most strains possess *exoT*, the presence or absence of *exo S*, *exoY* and *exo U* is variable between strains (Engel 2003, Bradbury, Richard).

When compared to isolates from other sources, CF strains appear to be significantly more likely to carry *exoS*, but not *exoU* (Engel 2003). It has also been asserted that *exoS* and *exoU* are mutually exclusive (Lee, Smith *et al*. 2005; Vance, Rietsch *et al*. 2005), despite these genes being located at different points along the *P. aeruginosa* genome. However, Finnan, *et al* identified eleven CF clinical strains and one isolated from a plant rhizosphere that were PCR positive for both *exoU* and *exoS* in conjunction (Finnan, Morrissey *et al*. 2004) and the laboratory control strains PA99 has been shown to express both ExoU and ExoS simultaneously (Shaver and Hauser 2004, Bradbury, Richard).

Further to this, a study of *exoU* and *exoS* prevalence found that 9% of 45 isolates of *P. aeruginosa* carried both *exoU* and *exoS* concurrently (Wong-Beringer, Wiener-Kronish *et al*. 2007). As *P.aeruginosa* strains deficient in all four known T3SS effecter enzymes are capable of T3SS mediated induction of apoptosis in host (Bradbury,Richard).

1.27.15. ExoT and ExoS

The first identified, and most studied, of the T3SS effecter enzymes, ExoT and ExoS share 75% homology at the amino t cells, it is probable that more as yet unidentified T3SS effectors exist (Engel 2003).

The amino terminal end of the proteins target G-protein activating protein, while the carboxyl terminal ends are ADP ribosylases (Vance, Rietsch *et al*. 2005). The N-terminal targets Rho-like GTP ases, resulting in actin cytoskeleton re-arrangements with consequent host cell rounding, host cell signaling disruption, inhibition of internalization and impaired wound healing (Engel 2003; Vance, Rietsch *et al*. 2005). The C-terminal ends of both enzymes show ADP ribosyltransferase (ADPRT) activity, with ExoT showing only 0.2% of the ADPRT activity of ExoS (Engel

2003). ExoS C-terminal domains target small Ras-like proteins Ra1 and Ra5, inhibiting DNA synthesis and internalization, and inducing apoptosis (Engel 2003; Vance, Rietsch *et al*. 2005). The C-terminal domains of ExoT target CrkI and CrkII, host kinases involved in focal adhesion and phagocytosis (Vance, Rietsch *et al*. 2005, Bradbury, Richard).

It has been found that ExoS is the major cytotoxin involved in colonization, invasion and dissemination during infection, while ExoT protects cultured cells from T3SS dependent lysis *invitro* (Lee, Smith *et al*. 2005; Soong, Parker *et al*. 2007) It is suggested that ExoT may counteract any damage caused to the host cell by insertion of the T3SS needle apparatus, analogous to the role of YopE in the *Yesinia spp*. T3SS (Lee, Smith *et al*. 2005) ExoT has been found to play only a minor role in the virulence of *P. aeruginosa* in the lung, although it has been associated with dissemination of disease from the lung to the liver in mice (Shaver and Hauser 2004), and may possess anti-internalization activity (Soong, Parker *et al*. 2007) Despite its low cytotoxicity in comparison to ExoS, the expression of ExoT alone is sufficient to induce death in *Galleriamelonella* (Miyata, Casey *et al*. 2003) and to induce apoptosis in HeLa cells via activation of the mitochondrial apoptosis pathway (Shafikhani, Morales *et al*. 2008) More than 90% of *P.aeruginosa* strains carry *exoT* (Lin, Huang *et al*. 2006; Shafikhani, Morales *et al*. 2008), whilst approximately two thirds of strains carry *exoS* (Jain, Ramirez *et al*. 2004; Wong-Beringer,Wiener-Kronish *et al*. 2007, Bradbury Richard).

1.27.16 ExoY and ExoU

Approximately 89% of *P. aeruginosa* strains carry *exoY* (Vance, Rietsch *et al*. 2005), though it is not able that only 70% of *P. aeruginosa* strains isolated from urine have been found to harbor *exoY* (Lin, Huang *et al*. 2006). The structure and function of this genes product; ExoY, is still relatively unknown, it has been shown to be an adenylate cyclase of unknown molecular weight, requiring a presently unidentified host cell protein for activity (Engel 2003). While studies in mouse pneumonia models have shown little ExoY mediated pathology (Lee, Smith *et al*. 2005),other

studies in MDCK cell culture have attributed significant cytotoxic effect to ExoY (Lin, Huang *et al*. 2006, Bradbury, Richard).

In contrast, the fourth and most recently described exoenzyme of the *P. aeruginosa* T3SS, ExoU, shows marked cytotoxic capabilities. ExoU is a phospholipase with remarkably rapid and fulminate cytotoxic effect (Engel 2003; Finnan, Morrissey *et al*. 2004; Vance, Rietsch *et al*. 2005). One study found that ExoU causes significant cytotoxicity in MDCK cell culture, but that it was not associated with colonization and invasion in BALB/c mice (Lin, Huang *et al*. 2006, Bradbury, Richard).
Deletion of *exoU* has been shown to severely limit the toxicity of *P. aeruginosa* strains in the lung, and the enzyme has been implicated as an agent associated with septic shock and increased disease severity and mortality in pneumonia (Engel 2003; Schulert, Feltman *et al*. 2003; Vance,Rietsch *et al*. 2005; Wong-Beringer, Wiener-Kronish *et al*. 2007) The prevalence of *exoU* among clinical *P*. aeruginosa isolates was found by one study to be 27% (Wong-Beringer, Wiener-Kronish *et al*. 2007). The prevalence of *exoU* appears to vary depending on the site of infection, with approximately one third of non-CF respiratory isolates possessing the gene, whilst twice this frequency has been observed in isolates recovered from wound infections (Wong-Beringer, Wiener-Kronish *et al*. 2007), suggesting that exoU serves particular functions, depending on disease setting.

1.27.17 Fluoroquinolone resistance and exoU

It is suggested that fluoroquinolone resistance may linked to the increased expression of T3SS virulence (Wong-Beringer, Wiener-Kronish *et al*. 2007). It is postulated that mutations conferring resistance to fluoroquinolones develop either through exposure to fluoroquinolone antibiotics, or by genetic recombination of resistance and virulence genes. Alternatively, the effect of DNA gyrase mutations (resulting in resistance to fluoroquinolone antibiotics) on DNA super coiling may alter T3SS expression. The latter effect has been documented in previous studies of other bacteria possessing a T3SS (Wong-Beringer, Wiener-Kronish *et al*. 2007).Studies previous to this have established a link between multi-drug resistance and expression

of *exoU* (Zaborina, Kohler *et al*.2006). Importantly, a significant correlation of *exoU* positivity and *gyrA* mutation has been reported (Wong-Beringer, Wiener-Kronish *et al*. 2007). It is known that *exoU* is acquired on a genomic island through horizontal gene transfer, and that this acquisition has an advantageous role in cell fitness within differing environments, and the same selective process may apply to development of the *gyrA* mutation (Wong-Beringer, Wiener-Kronish *et al*. 2007, Bradbury, Richard).

1.27.18The type III secretion as a dynamic system

During the course of this review, the concept of virulence as a complex, dynamic and irrevocably inter twined system of multiple effectors and regulators should become clear. *P. aeruginosa* T3SSvirulence needs to be considered in the context of the other virulence mechanisms to allow abroad overview of the complex interactions and appreciation of how they together convey the unique environmental and clinical versatility associated with this organism (Bradbury, Richard).

1.27.19 Action of type III secretion system effecter enzymes in combination

There has been limited study of T3SS in the clinical setting. Much work has been performed using *P. aeruginosa* control strains, and to a lesser degree clinical isolates with known genotypes of T3SS effecter enzymes in both wild type and with T3SS gene knockout mutants. It has been suggested that strains expressing *exoU* are predominantly cytolytic, whilst strains expressing *exoS* are invasive. Rounding of epithelial cell lines has been found to depend upon ExoS and ExoT, but lysis of macrophages may occur without the involvement of any of the T3SS effecter enzyme genes. This suggests that cell lysis through membrane puncture by the T3SS needle apparatus or by an as yet unidentified T3SS effecter molecule (Vance, Rietsch *et al*. 2005, and Bradbury Richard).

Work on T3SS knockout mutants of PAK, PA103 and PA14 in CHO cell culture and BALB/c mouse models suggested that ExoS was more responsible for cytotoxicity than ExoU, whilst also demonstrating a possible protective role for ExoT (Lee, Smith

et al. 2005) Conversely, another study employing T3SS mutants of PA103 and PA99 in a mouse model of pneumonia found that the expression of ExoU had a very significant impact on disease severity. This same study showed ExoT as having only a minimal effect. Strains expressing only ExoS showed disease severity in between ExoU and ExoT expressing mutants (Shaver and Hauser 2004).A study employing a *G.mellonella* caterpillar host virulence model with wild type and mutants of PA14 with deleted effecter and translocation apparatus genes was carried out by Miyata, *et al*. This found that ExoS and ExoY were not required for disease in the presence of ExoU, and that either ExoU or ExoT alone was sufficient to induce death (Miyata, Casey *et al*. 2003. A Δ*pscD* mutant (unable to construct translocation needle apparatus) showed greatly attenuated virulence in comparison to wild type (Miyata, Casey *et al*. 2003), further suggesting that the T3SS needle apparatus itself is capable of mediating host cell damage, or that an as yet unrecognized T3SS effecter molecule exists. A further explanation for this effect could be that lack of transcription of the T3SS effecter genes may up-regulate expression of one of the many other virulence factors associated with *P.aeruginosa*.

1.27.20 ExoS and regulation of the type III secretion system

Expression of the T3SS effecter proteins is positively regulated by the presence of the translation promoter ExsA, whilst the activity of the T3SS translocation needle apparatus is controlled by calcium ion concentration.

1.27.21 Putative virulence factors

The product of the *nan1* gene of PA01 has been suggested as a potential virulence factor in *P.aeruginosa*. While the role of this product is unknown, it appears to encode a sialidase, which may liberate sialic acid from gangliosides and thereby increase the availability of the attachment site at sialo-GM1 on respiratory epithelial cells. The gene has a significantly different G+C content to the majority of other PA01 genes, and thus most probably was acquired by horizontal transfer from another organism (Lanotte, Watt *et al*. 2004). Lanotte, *et al.* also found that the nan1

gene was most prevalent in isolates from the CF lung and those from plants (Lanotte, Watt *et al*. 2004, Bradbury, Richard) .

A newly discovered virulence factor transportation mechanism is the type VI secretion system (T6SS). This is a sec-independent mechanism for the transportation of effecter proteins across the cell membrane. Although this mechanism was only recently discovered, and there is little knowledge of its mechanisms or effecter proteins, genes corresponding to T6SS machinery have been found in *P. aeruginosa*, and serine-threonine phosphorylation has been proposed as the triggering mechanism for T6SS activity (Shrivastava and Mande 2008, Bradbury, Richard).

1.27.22 Temporal changes in virulence

Of particular importance when considering differing types of P. aeruginosa infection is the degree of expression rather than the presence or absence of virulence factors. Jain, *et al* carried out an extensive study of the expression of T3SS effectors in nearly 500 clinical CF isolates of *P.aeruginosa*. This study found that only 12% of isolates from chronically infected CF patients expressed ExoS, ExoU or ExoT (Jain, Ramirez *et al*. 2004). This finding correlated with those of Dacheux, *et al*. who found only 29% of 28 CF isolates from France expressed T3SS proteins (Dacheux, Toussaint *et al.* 2000) and Roy-Burman, who found only 41% of 37 CF isolates from the United States secreted one or more T3SS effecter proteins (Roy-Burman, Savel *et al*. 2001, Bradbury, Richard).

The study by Jain, *et al* found that 90% of environmental isolates (the presumed source of CF isolates) expressed T3SS effecter proteins. In newly infected CF patients, only 49% of isolates expressed ExoS, ExoU or ExoT, with this percentage dropping to 8% in chronically infected children and to only 4% in chronically infected adults (Jain, Ramirez *et al*. 2004). Lee, *et al.* compared isolates collected from CF patients soon after colonization with genotypically identical isolates collected years later. Early isolates (except highly mucoid strains) showed significant

cytotoxicity, while later isolates where shown not to result in cytotoxicity in CHO cells (Lee, Smith *et al*. 2005).

A study of chronic CF isolates found that this lack of expression of T3SS effectors was irreversible (strains termed non-inducible) (Filopon, Merieau *et al*. 2006), suggesting that mutations had occurred in regulator genes rather than the effecter genes themselves. Smith, *et al.*carried out whole genome analysis on genotypically identical *P.aeruginosa* strains collected from the same CF patient 8 years apart and found 24 mutations in virulence factor genes, including one in *exsA*, which acts as a transcriptional regulator of theT3SS.In addition, sequencing of this gene in 91 isolates taken from 29 CF patients found mutations in *exsA* in eight isolates, with six separate patients' harboring *exsA* mutant strains (Smith, Buckley *et al*. 2006, Bradbury, Richard).

1.27.23 Eukaryotic virulence models

In recent years, attention has been focused on whole eukaryotic cell models of virulence in *P.aeruginosa*. Currently, methods for the measurement of virulence may employ amoebae such as *Acanthamoeba*, the slime mold Dictyostelium disodium, the nematode *Caenorhabditis elegans*, the plant *Arabidopsis thaliana* and the fruit fly *Drosophila melanogaster* (Pradel and Ewbank 2004). Such models provide an opportunity to measure the phenotypic virulence of an organism interacting with a eukaryotic host, and allow such work to be performed without the ethical concerns with mammalian models.

One such study compared the virulence properties of *P. aeruginosa* strains recovered from burn wounds and CF sputum in a *Drosophila melanogaster* model. Burns isolates were reliable lethal in both fly feeding (expected not to involve T3SS virulence) and fly nicking (expected to involved T3SS virulence) experiments. However, CF isolates showed great variability in their virulence towards the host using this model. Interestingly, multiple morph types of *P. aeruginosa* recovered from a single CF patient displayed great variance in their respective capacities to cause fly death in the models employed (Lutter, Faria *et al*. 2008, and Bradbury Richard).

The *D. discoideum* models presently employed involve simple temporal killing assays; looking at the degree of killing affected by *P. aeruginosa* in mixed culture or *P. aeruginosa* culture supernatants at increasing concentrations over time (Cosson, Zulianello *et al*. 2002). These methods were found to show good correlation with mammalian host models, and certainly provide more information regarding virulence as a complex entity than simple single virulence factor tests.

The first published use of a *D. discoideum* virulence assay in the analysis of *P. aeruginosa* virulence was by Cosson, *et al* in 2002 (Cosson, Zulianello *et al*. 2002). This paper described assays involving the co-culture of Klebsiella pneumonia with P. *aeruginosa* and *D. Discoideum* wild-type strain DH1-10 on solid agar, culture of DH1-10 alone on *P. aeruginosa* lawn plate's and the effect of *P. aeruginosa* cell culture supernatants on a *D.* discoideum and *K. pneumonia* co-culture plate. It was found that wild-type strains of PAO1 did not allow the growth of Discoideum plaques. By repeating the assays using virulence gene knockout mutants of the samePAO1 strain, virulence factors expressed through the *rhl* QS pathway, and specifically rhamnolipid were found to be major virulence factors in *D. discoideum*. This study also identified that the presence of multi-drug resistance efflux pump, MexEF-OprN significantly reduced the virulence of *P. aeruginosa*, whilst the presence of the MexAB-OprM and MexCD-OprJ pumps had no effect on virulence. It was found that the degree of virulence of *P. aeruginosa* in the *D.discoideum* virulence assay could be used to predict virulence of the bacterial strains tested in rats (Cosson, Zulianello *et al*. 2002, and Bradbury Richard).

Almost simultaneously, a study was published by Pukatzki, *et al.* involving wild-type and mutants of PA14 and PA103, and their interactions with *D.* discoideumAX3. This found that AX3 did not grow on lawns of wild-type PA14 or PA103, whilst respective Δ*lasR* and Δ*exoU* mutants were permissive to plaque formation. Note that *lasR* is a precursor of the *rhl* QS pathway. It also found that pyocyanin production did not affect *P. aeruginosa* virulence in *D. discoideum*. Importantly, this study showed that the lack of plaque formation was due to killing of *D. discoideum*, and

not starvation and that the lack of plaque formation in wild-type strains was not due to inhibited phagocytosis (Pukatzki, Kessin *et al*. 2002, and Bradbury Richard).

Only two further studies of *P. aeruginosa* virulence in *D. discoideum* have been performed; both utilizing modifications of the original virulence assay method published by Cosson *et al.* One study used microarray to determine changed in host transcription shortly after exposure to PA14and PAO1. This study found large and significant changes in the up-regulation and down-regulation of *D. discoideum* AX4 gene expression when exposed to *P. aeruginosa* compared to controls grown with *Klebsiella aerogenes*. It also found that strain AX4 would feed and grow on stationary phase cultures of PAO1, a finding not observed in PA14. Thus, it was asserted that PAO1 virulence in *D. discoideum* requires active bacterial cell growth. Indeed, this study found that virulence was generally increased in PA14 when compared to PAO1. One obvious source of error in this study was that a *K. aerogenes* co-culture test was used, and thus the virulence interactions of *K. aerogenes* with both *P. aeruginosa* and *D. discoideum* could not be excluded from the analysis (Carilla-Latorre, Calvo-Garrido *et al*. 2008, and Bradbury Richard).

The second study discussed identified three new genes involved in the virulence pathways of *P.aeruginosa*; *trp, pchH* and *pchI* by examining the permissiveness of mutagenized strains of a clinical isolate of the bacteria in a *D. discoideum* DH1-10 model. This study also questioned the superiority of animal models compared to the *Dictyostelium* model due to the inability of changes in the virulence of *P. aeruginosa* mutant strains to be detected by Drosophila when ingested; inoculation of the fly by pricking with a needle was required to detect virulence. This study found the *D. discoideum* model to be comparable with a mouse model of pneumonia. Once again, the robustness of the *D. discoideum* virulence assay as a model for the identification of new virulence genes in *P. aeruginosa* was demonstrated (Alibaud, Kohler *et al*. 2008)

Despite the obviously significant findings of these studies, and the demonstrated usefulness of the *D.discoideum* assay in analyzing the virulence of *P. aeruginosa*, this method has never to date been used to compare the virulence of clinical strains

of *P. aeruginosa*. Potentially, this is because all wild-type control strains of the organism thus far analyzed have been lethal to *D. discoideum* in co-culture. However, another amoeboid model previously found great variation in the virulence of clinical and environmental strains of *P. aeruginosa* (Fanner, Richet *et al*. 2006, and Bradbury Richard).

The virulence of clinical and environmental strains of *P. aeruginosa* has been successfully assayed in co-culture with *Acanthamoeba polyphagia* strain link AP-1. This assay employed culture of *A.* polyphagain the center of lawns of 83 community environmental and 69 hospital clinical isolates of *P. aeruginosa*. The majority (75%) of the clinical isolates were isolated from blood culture, with the balance being from BAL specimens, it is unknown if CF isolates were included in the BAL isolates. The distribution of size in plaques formed was bimodal, and a resistant/sensitive cut off was devised based upon this. Environmental strains were found to be significantly less virulent than the clinical isolates in this assay. Whilst no significant trend between resistance to any one of eight antimicrobials tested and virulence noted in the isolates, it was found that isolates resistant to only one antibiotic appeared less virulent than fully sensitive strains, whilst those resistant to at least four antibiotics were significantly more virulent. One exception to this rule involved two genotypically indistinguishable BAL isolates recovered over a period of four weeks from the same patient, and assumed to represent the same infection, which over this time increased antimicrobial resistance, but decreased virulence towards *A. Polyphaga* (Fenner, Richet *et al*. 2006, and Bradbury Richard).

There is great versatility and potential of such eukaryotic/prokaryotic models in the study of virulence. The aqueous environmental natures of *P. aeruginosa* would allow it many opportunities for contact with slime molds and other amoebae in its natural habitat. Moreover, adaptation for interaction with such amoebae may then be reflected in the interaction of *P. aeruginosa* with higher eukaryotic cells, such as the human macrophage, as has been previously recorded in other organisms such as Legionella pneumophila. *P. aeruginosa* has not found to be a good food source for *Acanthamoeba castellanii* and at high bacterial concentrations will kill this amoeba. However, *P. aeruginosa* and *Acanthamoeba* species have been recovered co-existing

in contact lens fluid and amoeba resistant *P. aeruginosa* strain surviving within free-living amoebae have been recovered from the environment (Grueb and Raoult 2004, Bradbury, Richard).

1.28. Characteristics of Typing Methods

"Typing methods fall into two broad categories: phenotypic methods and genotypic methods. Phenotypic methods are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacterio-phage types, and antigens present on the cell's surface, and antimicrobial susceptibility profiles all are examples of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to vary, based on changes in growth conditions, growth phase, and spontaneous mutation" (Tenover FC 1997, Bradbury, Richard).

"Genotypic methods are those that are based on an analysis of the genetic structure of an organism and include polymorphisms in DNA restriction patterns based on cleavage of the chromosome by enzymes that cleave the DNA into hundreds of fragments (frequent cutters) or into 10 to 30 fragments (infrequent cutters), and the presence or absence of extra chromosomal DNA ((Tenover F, Bradbury, Richard)
Genotypic methods are less subject to natural variation, although they can be affected by insertions or deletions of DNA into the chromosome, the gain or loss of extra chromosomal DNA, or random mutations that may create or eliminate restriction endonuclease sites. All typing systems can be characterized in terms of type ability, reproducibility, discriminatory power, ease of performance, and ease of interpretation the characteristics of a number of typing methods are presented in. Type ability refers to the ability of a technique to assign an unambiguous result (type) to each isolate. Although non-type able isolates are more common with phenotypic methods, they have been recognized with most methods. For example, with PFGE, a technique that is almost uniformly applicable to bacteria, "some strains of *Clostridium difficile* remain non-type able because the chromosomal DNA is degraded, presumably by endogenous nucleases, before it can be cleaved properly by

the restriction endonucleases used in the PFGE protocol. A reproducible method is one that yields the same results upon repeat testing of a bacterial strain. In the context of an epidemiological study, this means that the same strain recovered from epidemiologically linked patients will give the identical (or nearly identical) typing result. Poor reproducibility may reflect technical variation in the method or biologic variation occurring during *in vivo* or *in vitro* passage of the organisms to be examined. Over time (a few weeks to years, depending on the species), the typing patterns produced by DNA-based methods, such as PFGE and AP-PCR, will show some minor, natural variation. Thus, when analyzing results, it is important to consider the length of time. The discriminatory power of a technique refers to its ability to differentiate among epidemiologically unrelated isolates, ideally assigning each to a different type. Traditional phenotypic methods, such as anti-bio-gram typing, serotyping, and bio- typing, frequently show lower discriminatory power than newer molecular methods. Ease of performance reflects the cost of specialized reagents and equipment, the technical complexity of a method, and the effort required to learn and to implement the technique in the laboratory. Most molecular methods require purchase of new equipment, some of which is costly ($4,000-$20,000). However, these methods are learned easily and are widely applicable to a variety of species. Many traditional methods also involve considerable costs in labor and materials, but are restricted to a single or relatively few species.

For example, bacteriophage typing, which is used primarily for *S. aureus* and a few other bacterial species, requires the maintenance of bacteriophage stocks that constantly must be replenished and titered, a process that is both time-consuming and labor-intensive. Finally, ease of interpretation refers to the effort and experience required to obtain useful, reliable typing information using a particular method. At present, the interpretation of the results of molecular methods remains an area of active discussion. However, this is in contrast to methods such as bacteriophage typing and pyocin typing, which require significant expertise to perform and interpret and often still yield ambiguous results".

1.28.1. Indicator Techniques (Phenotypic Methods) Biotyping

"In the 1960s and early 1970s, identification of bacterial species frequently was undertaken using racks of tubes representing a variety of biochemical tests, and the variability of certain tests, such as indole, H2S, or pigment production, served as markers for particular strains. Thus, biotyping emerged as a useful tool for epidemiological investigations. Today, identification of bacterial species normally is accomplished by using a combination of biochemical and immunologic tests, many of which now are performed using commercial kits or automated devices. However, biotyping using automated methods relies on a variety of novel substrates, and some of these tests, such as carbohydrate fermentations, are highly variable even within isolates of the same strain. Thus, biotyping, like most phenotypic methods, has only modest reproducibility, because microorganisms can alter unpredictably the expression of many cellular products. Moreover, contemporary biotyping typically has poor discriminatory power and cannot differentiate among some of the current nosocomial problem pathogens, such as enterococci, where biochemical diversity is uncommon. Occasionally, outbreaks are observed that are caused by bacterial strains that represent unusual species or unusual biotypes of common species, for example, H2S-producing isolates of *Escherichia coli*. In such situations, additional typing techniques may not be needed. However, even clusters of unusual isolates may not always indicate a common source outbreak, as indicated by a recent report in which four isolates of *Leptotrichia bucolic*, an unusual anaerobic gram-negative bacillus, recovered from blood cultures of four different bone marrow transplant patients, were found to be unrelated by PFGE and fatty-acid profile analysis. The investigation of the suspected outbreak revealed that each of the patients had undergone dental manipulations prior to developing bacteremia. In addition, all of the patients had been placed on prophylactic antimicrobial agents to which the *L buccalis* isolates were resistant. Thus, each neutropenic patient developed bacteremia with his own endogenous strain of *L buccalis*, which served as an opportunistic pathogen. None the less, it should be noted that outbreaks can, in some cases, be caused by multiple pathogens. Antimicrobial susceptibility patterns also have relatively poor discriminatory power; because antimicrobial resistance is under

tremendous selective pressure in health care institutions and often is associated with mobile genetic elements (e.g., transposons and plasmids). Changes in antibiograms also may reflect spontaneous point mutations, such as seen with fluoroquinolones. Thus, isolates that are epidemiologically related and otherwise genetically indistinguishable may manifest different antimicrobial susceptibilities due to acquisition of new genetic material over time or the loss of plasmids. Conversely, unrelated isolates may have indistinguishable resistance profiles, which may represent acquisition of the same plasmid by multiple species (a "plasmid outbreak"). Serotyping, a mono molecular method, uses a series of antibodies to detect different antigenic determinants on the surface of the bacterial cell. Serotyping is one of the classic strain typing techniques that have been used over the years for epidemiological studies of many species of bacteria. It remains a key method for typing isolates of *Salmonella, Shigella,* and *pneumococci*. However, maintaining stocks of typing sera (including the >2,200 antisera required for definitive *Salmonella* typing) is a major limitation of this method. Because of the association of certain *Salmonella* serotypes with food borne disease, and the association between specific *pneumococcal* serotypes and invasive disease, particularly in children, serotyping continues to be valuable typing technique. Nonetheless, PFGE has been shown to resolve distinct clonal strains within individual serotypes of both *Salmonella* and *pneumococci*, thus indicating that it is a more discriminatory typing tool" (Tenover FC 1997,Bradbury, Richard).

GENOTYPIC METHODS "Over the last several years, six molecular techniques have emerged as the methods of choice for typing bacterial isolates. They are; restriction endonuclease analysis (REA) of plasmid DNA; REA of chromosomal DNA using frequent cutting enzymes and conventional electrophoresis; restriction fragment-length polymorphism (RFLP typing) analysis using DNA probes; PFGE; and AP-PCR and other related nucleic acid amplification-based typing methods. In addition, PCR-DNA sequencing methods are just beginning to be utilized. Plasmid Finger-printing Plasmid fingerprinting was the first molecular method to be used as a bacterial typing tool. Plasmids are extra chromosomal DNA elements that are present in most clinical isolates and can be identified readily by simple cell lysis procedures

followed by agarose gel electrophoresis of the lysates. The number and size of the plasmids present is used as the basis for strain identification. This strain typing technique has been used successfully for analysis of outbreaks of nosocomial infections and community-acquired infections caused by a variety of species of gram-negative rods. REA of Plasmid DNA some strains of bacteria contain only a single large plasmid, often in the size range of 100 to 150 kilo bases" (kb).

"Because it is difficult to differentiate plasmids in this size range, especially those that vary by only 10 kb to 15 kb, some investigators have added a restriction endonuclease digestion step to try to increase the discriminatory power of agarose gel electrophoresis. While this can be helpful, large plasmids produce many restriction fragments, which can make interpretation more difficult, especially when multiple large plasmids are present. Thus, for gram-negative rods, the REA step no longer is performed in most laboratories. However, for analysis of *staphylococci*, where the plasmids typically are <50 kb, REA appears to increase the discriminatory power of the analysis, because the number of restriction fragments generated usually is <20.33. Digestion also makes the patterns of the restriction fragments produced from *staphylococcal* plasmids easier to analyze than the undigested profiles, which often show multiple forms for plasmids of less than 15 kb, because circular and linear forms of the plasmid migrate at different rates than the covalently closed circular form. Plasmid fingerprinting is technically simple to perform and requires relatively inexpensive equipment ($1,500-$3,000) at this time; the method is used primarily as an alternative technique for *staphylococcal* isolates, which frequently carry multiple plasmids, and for selected species of *Enterobacteriaceae*, which often have large distinctive plasmids. When applying the plasmid Fingerprinting technique, investigators must be aware of two confounding factors. First, it is possible that plasmids can spread to multiple species of bacteria, causing a plasmid outbreak in which unusual anti-biograms are recognized in multiple species. This has been recognized both in gram-negative rods and in *staphylococci*. Second, it is important to appreciate that the structure of individual plasmids and the plasmid content of a particular strain may vary overtime. This variability reflects two factors: over time, plasmids can be lost spontaneously or acquired from other organisms, and

plasmids frequently carry smaller mobile genetic elements (transposons and insertion sequences) that promote duplications and deletions of DNA segments. Both plasmids and transposons often include antimicrobial resistance determinants and thus are subject to considerable selective pressure within hospitals due to antimicrobial agent use. In general, plasmid finger-printings most useful for epidemiological studies that is limited both temporally and geographically. In selected instances, plasmid finger-printing may complement other techniques, such as PFGE analysis, by providing a basis for differentiating isolates that are related genotypically but are separated epidemiologically by moderate time periods, such as several months"(Tenover F).

Gel Electrophoresis Techniques for Analysis of Chromosomal DNA: "There are two methods of typing organisms based on fragment patterns produced by cleaving chromosomal DNA with restriction endonucleases. The first method, often referred to as conventional electrophoresis, uses a restriction enzyme that cuts the chromosome into hundreds of pieces (frequent cutter), followed by standard agarose gel electrophoresis. Fragments that are 25 kb to0.5 kb are resolved into a discernible banding pattern, although a single band may contain fragments of similar size from several different areas of the chromosome. Larger fragments coalesce at the top of the gel or do not migrate into the gel. The second method, PFGE, uses enzymes that cut chromosomal DNA infrequently, generating from 10 to 30 bands, followed by a novel form of electrophoresis that can separate fragments from 1 kb up to 1,000 kb" (1 Mega base) (Tenover F 1997, Bradbury, Richard)

"Each method, and a variation of the conventional electrophoresis method, is described in greater detail below REA of Chromosomal DNA with Frequent Cutting Enzymes and Conventional Electrophoresis Each restriction endonuclease cleaves DNA at a particular sequence of Nucleotides that maybe repeated numerous times around the chromosome. The number and size of the restriction fragments generated by digesting a given piece of DNA reflects the frequency and distribution of the restriction sites. In conventional REA, endonucleases with frequently occurring sites in the bacterial genome are used to digest total DNA (plasmid and chromosome), there by generating hundreds of fragments ranging from approximately 0.5 to 50 kb

in length.Such fragments can be separated by size using agarose gel electrophoresis, and the pattern can be detected by staining the gel with ethidium bromide (or other dyes) and photographing under Ultraviolet light. Different strains of the same bacterial species have different REA profiles (depicted as a series of bands on agarose gels) because of variations in their DNA sequences. All isolates are type able by REA; however, it can be very difficult to interpret the complex profiles, which consist of hundreds of bands that may be indistinct or overlapping. Although the approach has been applied to many species, at this time, its primary use is as an alternative technique for analyzing *C.difficile.*

RFLP Analysis using DNA probes: In this technique, chromosomal restriction digests produced by frequent cutting enzymes are separated by conventional agarose gel electrophoresis, as described above, and then the DNA fragments are transferred onto a nitrocellulose or nylon membrane" (Tenover F 1997, Bradbury, Richard).

"The DNA on the membrane then is hybridized with a specific chemically or radioactively labeled piece of DNA or RNA (a probe), which binds to the relatively few fragments on the membrane that have complementary nucleic acid sequences. Variations in the number and size of the fragments detected by hybridization referred to as RFLPs. One common typing method that uses chromosomal DNA preparations and a ribosomal RNA probe is ribotyping. Because all bacterial isolates have one or more chromosomal rRNA operons distributed around the chromosome, and because those sequences are highly conserved, essentially all bacterial isolates can be typed using probes directed to the DNA sequences that encode the rRNA loci using a single rRNA probe. However, enthusiasm for this system has diminished, because the approach has proven to be only moderately discriminatory" (Tenover F, Richard).

"Restriction fragment-length polymorphism analysis using the DNA insertion element currently is the method of choice for typing isolates of *Mycobacterium tuberculosis*. An insertion element is a piece of DNA with a defined structure that is able to move independently and to insert in multiple locations in plasmids or chromosomal locations, but does not contain antimicrobial resistance genes or genes involved in pathogenesis. IS 6110 is present in essentially all isolates of *M.*

tuberculosis, and, because insertion sequences are mobile, the number and chromosomal locations of the insertions vary greatly from strain to strain. The approach has proven reliable and discriminatory and, in most cases, can distinguish successfully sporadic and clustered cases of tuberculosis. However, for isolates with fewer than five copies of IS6110, the method has relatively poor discriminatory power and must be supplemented by studies using other probes" (Tenover FC 1997, Bradbury, Richard).

"PFGE Pulsed-field gel electrophoresis was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms. Subsequently, PFGE has proven to be a highly effective molecular typing technique for many different bacterial species, in this method, the bacterial genome, which typically is 2,000 to 5,000 kb pairs in size, is digested with a restriction enzyme that has relatively few recognition sites and thus generates approximately 10 to 30 restriction fragments ranging from 10 to 800 kb" (Tenover FC 1997, Bradbury, Richard).

"Essentially all of these fragments can be resolved as a pattern of distinct bands by PFGE, using especially designed chamber that positions the agarose gel between three sets of electrodes that form a hexagon around the gel. Instead of applying an electric current to the gel in a single direction, as is done in conventional electrophoresis, in PFGE, the current is applied first in one direction from one set of electrodes, then shifts to the second set of electrodes for a short period of time (a pulse), and then shifts to the third set of electrodes. Thus, the electric field that causes the DNA to migrate in the gel is provided in pulses that alternate from three sets of electrodes. This causes the DNA to wiggle through the gel, and the back-and-forth movement results in the higher level of fragment resolution seen with the technique. All species are type able by PFGE, although the isolation of intact chromosomal DNA is technically difficult for some species. As noted above, the chromosomal DNA of some strains of *C. difficile* spontaneously degrades during the cell lysis procedure, making this typing approach impractical. PFGE has been applied successfully to a wide range of bacterial species, both gram-positive.

(e.g, *staphylococci*, *enterococci*, and *mycobacterium*) and gram-negative (e.g., *E coli*, other Enterobacteriaceae, and *Pseudomonad*).In general, PFGE is one of the most reproducible and highly discriminatory typing techniques available and is currently the typing method of choice for many species. The major difficulties associated with PFGE relate to the technical demands of the procedure and initial cost of the equipment. Preparation of suitable genomic DNA requires 1 to 3 days, depending on the organisms tested, and the equipment required (including the electrophoresis apparatus and Trans illuminator) costs between $10,000 and $20,000. However, once the method is operational in a laboratory, it can be applied readily to a wide range of species with only minimal modifications" (Tenover F 1997, Bradbury, Richard) .

The interpretation of PFGE gels is relatively straight forward, and consensus guidelines for Correlating variations in restriction profiles with epidemiological relatedness were published recently.

Typing Methods Using PCR: "Polymerase chain reaction, which has been used for several years for the direct detection of many types of infectious agents in clinical samples, has been adapted for use as a typing tool. The hall-mark of PCR is the ability to produce literally millions of copies of a particular DNA segment with high fidelity within 3 to 4 hours' time. The procedure requires template DNA (or RNA if a reverse transcriptase is used initially), which may be present in the sample in minute quantities; two oligonucleotide primers, which flank the sequences on the template DNA to be amplified (thus defining the starting points for DNA polymerase activity); and a heat-stable DNA polymerase. Efficient amplification is accomplished readily for templates of less than 2,000 base pairs, although templates as large as 35 kb now can be amplified by using newer polymerases. A typical PCR assay requires approximately 3 hours to complete 30 cycles, where each cycle consists of a heat denaturation phase, in which double-stranded DNA is melted into single strands; an annealing phase, in which the primers bind to the target sequences on the single strands; and an extension phase, in which DNA synthesis proceeds from the primers along each strand of the template DNA, there by generating two new double-stranded copies of the original template". "After 30 such cycles, a single initial copy of

template DNA theoretically can be amplified to 1 billion copies. “Arbitrarily Primed PCR also referred to as the randomly amplified polymorphic DNA assay is a variation of the PCR technique employing a single short (typically 10 base pairs) primer that is not targeted to amplify any specific bacterial DNA sequence. Rather, at low annealing temperatures, the primer will hybridize at multiple random chromosomal locations and initiate DNA synthesis. If one copy of the primer binds to one strand of DNA, and another copy of the primer binds on the opposite strand of DNA but in proximity of the first primer, a DNA fragment will be synthesized and amplification of that fragment will occur. The resulting PCR products will represent a variety of different-sized DNA fragments that are visualized by agarose gel electrophoresis. This approach has remarkable general applicability and has been applied to typing eukaryotic species as well as many bacterial species. The reproducibility and discriminatory power of AP-PCR is a subject of active discussion and investigation. Recent reports have described specific primers and conditions for analyzing isolates of *C. difficile* and *S. aureus,* and have provided formal assessments of the procedure. These studies have indicated that, although AP-PCR is appreciably faster than other typing systems (often 20 to 30 organisms can be completed in a single day), the method is much more susceptible to technical variation than is routine PCR employing primers directed at known sequences. In AP-PCR there can be substantial variations in the efficiency with which the primers initiate DNA synthesis at a particular site, depending on even slight variations in the pH or ionic strength of the buffers used, the temperature of the reaction, or the source of the DNA polymerase. Consequently, on independent amplifications of the same strain, the Number of copies generated from a particular locus can be appreciably different, yielding wide Variations in the intensities of individual fragments. These factors can make it difficult to obtain reproducible patterns and interpretations with AP-PCR, particularly when attempting to compare isolates tested on different days. At this time, the most reliable results are obtained when a set of isolates is tested in a single amplification reaction and analyzed on a single electrophoretic gel. The comparison of AP-PCR fragment patterns obtained by testing the same isolates in different laboratories often is problematic, as was shown recently in a multicenter study. Furthermore, standard guidelines for interpretation of AP-PCR are not yet available,

and the general principles that are emerging for the interpretation of other molecular typing data, such as for PFGE, can-not be applied readily PCR. DNA sequencing although infrequently used at this time, DNA sequencing of hyper variable gene sequences has shown promise as a typing tool. While this technique currently is not feasible for most laboratories, the availability of less expensive DNA sequencing machines may make this technique more accessible in the future" (Tenover F 1997).

1.28.2. When to use strain typing

"Bacterial strain typing data are most effective when they are collected, analyzed, and integrated into the results of an epidemiological investigation. The preferred typing methods for various organisms are presented. The hospital epidemiologist should initiate strain typing studies in consultation with the hospital infection control laboratory or the hospital microbiology staff when investigating a potential outbreak of an infectious disease. This may be triggered by a noticeable increase in the rate of isolation of a particular pathogen, a cluster of infections on a particular ward, or the recognition in the clinical microbiology laboratory of multiple isolates with an unusual biotype or anti-biogram " (Tenover FC1997, Bradbury, Richard)

"Strain typing data should supplement, and not replace, a carefully conducted epidemiological investigation. In some cases, typing data can effectively rule out an outbreak and thus avoid the need for an extensive epidemiological investigation. In other cases, strain typing data may reveal the presence of outbreaks caused by more than one strain. However, undue reliance on strain typing in the absence of epidemiological data is an inefficient use of laboratory resources".

1.28.3. Sets of isolates most appropriate for typing studies

"Sets of epidemiologically related isolates representing a putative outbreak spanning a period of 1 to 3 months are appropriate for organisms strain typing studies. This implies that the infection control staff and hospital epidemiologist already have made a preliminary assessment and have determined the need for strain typing data. One

should not test sporadic isolates, except as epidemiologically unrelated controls (see below). The results of testing sporadic isolates often are misleading and frequently lead to wasted resources in both the microbiology laboratory and the infection control service. Most of the techniques described above can be applied effectively to 10 to 30 isolates per day, especially in dedicated infection control laboratories. Control strains in attempting to detect single-strain outbreaks involving species that may be endemic, e.g., MRSA or multiply resistant gram-negative bacilli, it is important to include epidemiologically unrelated isolates or historical controls to help differentiate endemic problems from new outbreaks. Outbreaks caused by more than one organism (which occur with considerably lower frequency than those caused by single strains) are much more complex to analyze and require large numbers of additional control strains in order to recognize the outbreak strains. Although endemic strains may cause clusters of infections, the inclusion of historical controls from previous out-break can help determine whether the recent isolates are likely to represent a new outbreak. Additional controls include the testing of well-characterized strains, such as *S.aureus* NCTC 8325, E.coli MG1655, and *Enterococcus facials* OG1RF, 11 to ensure that each step of the typing method is working properly" (Tenover FC 2004, Bradbury, Richard).

1.28.4. Interpretation

"Interpretation of strain typing results is facilitated greatly by an appreciation of the molecular basis of genetic variability of bacteria and the technical factors that can affect results. Three assumptions usually are made: isolates representing the outbreak strain are the recent progeny of a single (or common) precursor; such isolates will have the same genotype; and, epidemiologically unrelated strains will have different genotypes. Ideally, strain typing will provide a clear, objective basis for identifying the outbreak strain and distinguishing it from epidemiologically unrelated isolates. In practice, the interpretation of typing data is complicated by the fact that isolates from an ongoing outbreak may demonstrate some, albeit typically limited, genetic variability. The purpose of interpretive criteria is to establish a guide for distinguishing true differences in strains from the natural genetic variation that

occurs over time within a given strain. For illustration, assume that a set of up to 20 putative outbreak isolates has been typed and that the analysis has detected a subset of isolates with a common (modal) type, which is presumed to represent the outbreak strain. Typically, among the other isolates in the set, some have similar types (as represented, for example, by a few band changes in a PFGE pattern) and some are distinctly different types (distinctive PFGE patterns) The interpretative criteria should provide consistent objective guidelines for correlating the level of variation observed between an individual isolate and the putative outbreak strain with an estimate of the likelihood that the isolate is, in fact, part of the outbreak. To provide a generally applicable approach, this correlation focuses on the number of genetic events required to generate the observed typing variation, rather than on the types of specific changes observed in a particular typing system. In the example cited, there is a group of isolates that produce identical typing patterns, i.e., the presumed outbreak strain. Because only a small portion of the organisms' genetic complement is undergoing analysis, isolates that give identical results are classified as "indistinguishable, "not "identical. "A more detailed analysis theoretically could uncover differences in the isolates that appeared to give identical patterns but that were epidemiologically unrelated. However, when a set of epidemiologically linked isolates are analyzed, this is unlikely to occur "(Tenover F 2004, Bradbury, Richard).

Classifying Minor Typing Pattern Variations "The next consideration is how to classify those isolates with minor typing pattern variations. A number of studies using PFGE and other typing methods indicate that single genetic events (i.e., random mutations that may destroy or create a new restriction endonuclease site, or deletions or insertions of new DNA such as plasmids, bacteriophages, or insertion sequences) occur unpredictably even within the time span of a well-defined outbreak" (1-3months).

Therefore, "interpretive criteria need to accommodate such natural variation. The criteria for interpreting strain typing results are given in, with their epidemiological corollaries. The changes are depicted in cartoon form in. When differences are encountered in the strain typing results, it is important to attempt to ascertain what

type of genetic event may have led to the differences. For example, if a single band increases in size, could an insertion of new DNA, such as a bacteriophage or transposon, account for the change in size? Would a random mutation in a restriction endonuclease site account for the appearance of two smaller bands where a single larger band had been seen with other epidemiologically related isolates? Such questions often can be answered by careful analysis of the patterns. If two genetic events have occurred and are recognized through the differences in fragment patterns, the epidemiological interpretation falls into a gray zone. The results may indicate that the isolates are related; especially if they were collected over a long period of time (3-6 months), but there also is a possibility that the strains are unrelated, and the similarity was a result of chance. In such cases, additional information, such as the use of a second strain typing method or supplementary epidemiological analysis, should be sought. This often will help to clarify the relationship among the isolates examined. Finally, isolates that differ by more than three genetic events represent genetically different strains and should be considered unrelated. These general principles have been applied directly to the development of criteria for interpreting PFGE".

1.29 Pulsed Field Gel Electrophoresis

PFGE is a technique used by scientists to generate a DNA fingerprint for a bacterial isolate.As large DNA particles easily break, also due to their great viscosity, pipetting of them is difficult. For this reason, DNA and microrganism are embedded in agarose gel and affected with restriction endonuclease enzymes.

PFGE uses molecular scissors, called restriction enzymes, to cut bacterial DNA at certain locations known as restriction sites. These molecular scissors are selected to generate a small number of DNA pieces that can be separated based on size. Usually these DNA pieces, or restriction fragments, are large and need to be specially treated and separated to generate a DNA fingerprint. First the bacteria are loaded into an agarose suspension, similar to gelatin, and then the bacterial cell is opened with lysozyme and proteinase K to release the DNA. Once the DNA is released then the

agarose and DNA suspension, also known as a plug, is treated with restriction enzymes. The treated plugs are then loaded onto an agarose gel and the restriction fragments are separated based on size using an electric field. What makes PFGE different from other methods? How scientists usually separate DNA is because PFGE can separate several large restriction fragments.

To do this an electric field that constantly changes direction is used to generate a DNA fingerprint *(CDC* Pulsenet website).

Advantages of PFGE

- "PFGE sub typing has been successfully applied to the sub typing of many pathogenic bacteria and has high concordance with epidemiological relatedness.
- PFGE has been repeatedly shown to be more discriminating than methods such as ribotyping or multi-locus sequence typing for many bacteria.
- PFGE in the same basic format can be applied as a universal generic method for sub typing of bacteria. Only the choice of the restriction enzyme and conditions for electrophoresis need to be optimized for each species.
- DNA restriction patterns generated by PFGE are stable and reproducible.

Limitations of PFGE

- Time consuming
- Requires a trained and skilled technician
- Does not discriminate between all unrelated isolates
- Pattern results vary slightly between technicians
- Can't optimize separation in every part of the gel at the same time
- Don't really know if bands of same size are same pieces of DNA
- Bands are not independent
- Change in one restriction site can mean more than one band change
- "Relatedness" should be used as a guide, not true phylogenetic measure
- Some strains cannot be typed by PFGE"

Parallel with development of technology, variety of Epidemiological classification methods has been introduced. Despite introducing variety of morphological classification methods, due to their non-reliability, DNA based classification methods have been introduced during recent years. Pulsed field gel Electrophoresis (PFGE) due to its high sensitivity, reproducibility, specificity has been well known and accepted to Epidemiologists.

PFGE, due to its results simplicity of interpretation has been used in MRSA (Methicillin resistant *Staphylococcus*) and other bacterial differentiation. Due to its reproducibility and its application for bacteria and fungus, its uses increase daily. Classification due to PFGE has great importance in nosocomial infection. Because we can determine origin of drug resistant strain, its eradication, non-eradication, recurrence or re-infection with similar strain. Its application is merely for hospital infection and epidemiological infection. It does not have any value for detection of bacteria for clinicians.

Similar to other classification methods, in this method we compare profile with control profile or library profile and determine existence of relatedness with control strain.

Theoretically, DNA particles greater than 30-50 kilo base aggregate in one point in electrophoresis and move with same speed on the electrophoresis in agarose gel, So for their isolation they must be degraded to small particles and be affected with different directional Electricity. Pressuring particles with different directional electricity and its direction continuously changing is the base of PFGE electrophoresis.

Small particle will move faster than large particles. And different directional electricity will differentiate bands from each other. In other electrophoresis application of constant electric field will isolate proteins from each other. While in DNA, it cannot isolate 30-50 kb particles. in 1970 they were using low voltage and low gradient agarose gel for isolating DNA bands with less success, while in 1984 David Schwartz performed DNA electrophoresis with periodic electricity with

directional changing, he succeed to isolate DNA bands from 20 Kb till 10Mb. Different kind of PFGE equipment has been produced.
Now with PFGE development all kind of genomes (Bacteria, Fungus, and yeast) can be differentiated and their profile can be investigated through molecular biologists.
At the beginning PFGE apparatus was using two homogeny and heterogenic fields for isolating of DNA particles.

Under normal electrophoresis, large nucleic acid particles (above 30-50 kb) migrate at similar rates, regardless of size. By changing the direction of the electric field at a certain frequency (thus the name Pulsed-field gel electrophoresis), much greater size resolution can be obtained.

The smaller nucleic acid pieces are able to re-orient to the new field more quickly than are larger ones. This delay in re-orientation means larger pieces end up migrating down the gel slower than smaller ones. This method is used as a molecular typing method: genomic DNA is cut by restriction enzymes and then run through a PFGE gel giving a specific profile for a specific strain (Chu G1991).

ORFGE (octagonal field alteration gel electrophoresis is using two non-homogeny fields; its major problem was its straight line movement. That its band comparison with pattern band was very complicated, for solving this problem TAFE (transverse alternating field electrophoresis) was developed, in which homogenous electric field was used in full of its surface. It had two disadvantages, Fluid samples cannot be used in it, molecules were not migrating with constant electricity, and also redirecting of band was not possible (Durmaz, R).
With modification of TAFE, ST/RIDE (Simultaneous tangential/rectangular inversion decussate electrophoresis was introduced. With this apparatus aggregation of bands was decreasing to minimum amount and it was suitable for re-directing.
FIGE (Field inversion gel electrophoresis) was developed with using mono-directional constant electric those periodically directing particles. It was the easiest apparatus from PFGE (Durmaz, R).

ZIFE (Zero integrated field electrophoresis) was discovered. It was working with same mechanism. Slower movement of particles and larger DNA particles isolation was its advantageous to FIGE (Durmaz, R).

CHEF (Contour clamped homogenous electric field) in which with using several electrodes, straight line movement of particles in a homogeny electrical field is supported (Durmaz, R).

RGE (Rotating gel Electrophoresis) system, gel move on a platform and electricity constantly pass and isolate particles.

PFGE, in nowadays using Pulsed field gel electrophoresis, gel remain constant and Electric field direction change and DNA particles are isolated (Durmaz, R).

Variety of apparatus has been developed, they are categorized in two main class. First has been produced for FIGE that is simplest. In this model, Electric direction is changed periodically. DNA particles with 180 degree changing direction of electricity go back. System can be work with simple directional changing can be produced. ZIFE (Zero integrated field electrophoresis) is work with first class kind of systems. In comparison of FIGE, ZIGE work slower than FIGE and isolate greater size of DNA particles (Durmaz, R).

Second class of equipment, are those, in which direction of electricity change between 96-120 degrees (Durmaz, R).

They cause forward movement of DNA in a zigzag form. Their speed are faster than FIGE, they isolate greater DNA size particles. CHEF, TAFE, ST/RIDE and RGE use such class of apparatus. PACE (Programmable autonomously controlled electrophoresis) can adjust degree of Electricity direction, voltage amount, time for changing voltage, they have special electrodes, need to special kind of controlling computerized software programs (Durmaz, R).

At first non-homogeny Electric field was used and comparing band cannot be easy. With using FIGE homogeny Electric field and parallel electrodes with 180 degree angle, DNA particles under 1 Mb successfully was isolated. For isolating larger DNA particle less angles was suggested. Angles between 96-140 degrees (optimally 120 degree angle) successfully separated larger DNA particles. Less than 96 degrees was not suitable for isolation, so 96 degree is low threshold of PFGE angle. TAFE, ST/RIDE due to vertical electrodes insertion in the gel and geometry laws, support

straight band pattern production. CHEF electronically change direction of electric field with its electrodes, in RGE electric field is constant, but with changing of gel direction, direction of DNA changes. Different rotation of gel in electrophoresis tank cause different angles electric movement (Durmaz, R).

Voltage, angle, time of electric can be adjusted in all of apparatus.

As large DNA particles easily break, also due to their great viscosity, pipetting of them is difficult. For this reason, DNA and organism are embedded in agarose gel and affected with restriction endonuclease enzymes.

Specifically for each kind of bacteria special PFGE protocol has been introduced. They clarify voltage, angle, time of electric follow, times of direction changing, Buffer concentration, etc.

Lower angles; accelerate DNA particles isolation.96-105 degrees are used for fast isolation of great DNA particles (Durmaz, R).

Generally Tris Acetate EDTA(TAE) and Tris Boric Acid (TBE) (1xTAE:40mM Tris Acetate,1mM EDTA, pH 8.0; 1xTBE; 89 mM Tris, 89 mM boric Acid, 2 mM EDTA, pH 8.0) are used for PFGE. Heating of buffer, offer better results.

Lower ionic buffers increase speed of DNA movements in a research in RGE 0.25x TAE and TBE increase DNA movement 40-50%. Buffers less than 0.25 percentage decreases DNA movement.

Agarose: Type of agarose is very important for PFGE isolation. Low "EEO" Electro Endosmosis agaros both facilitate acceleration and suitable isolation. Concentration of agarose has great effect on above two factors. High concentration agarose increase resolution of bands, despite decreasing speed of DNA movement. Mostly 0.8-1.2 percent agarose are used for PFGE. Low EEO agarose, despite decrease isolation percentage increase resolution of bands.

Temperature of Environment should remain constant. Increasing environmental temperature, however may increase DNA particles movement speed, but definitely will decrease resolution of isolated bands (Durmaz, R).

PFGE, from its discovery, has proved that is a valuable method for classification. In future, it is planned to load till 10 Mb DNA particles. For reaching it, modification of some methods is predicted (Durmaz, R).

PFGE involves embedding organisms in agarose, lysing the organisms *in situ,* and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently. Slices of agarose containing the chromosomal DNA fragments are inserted into the wells of an agarose gel, and the restriction fragments are resolved into a pattern of discrete bands in the gel by an apparatus that switches the direction of current according to a predetermined pattern. The DNA restriction patterns of the isolates are then compared with one another to determine their relatedness. Currently, there are no standardized criteria for analyzing the fragment patterns. Consequently, different investigators viewing the same PFGE results may come to quite different conclusions as to which isolates should be designated as outbreak related and which should be designated as non-outbreak related (Durmaz, R).

Pulsed-field gel electrophoresis is one of the key technological advances of the past ten years that has made the mapping of genomes of whole organisms possible. In conventional electrophoresis, the mobility of DNA at almost any practical values of the field strength is essentially independent of mol *wt* above appromax 30 kbp. Therefore, large (≥50kbp) fragments of DNA required for mapping the genomes of entire organisms could not be separated prior to the introduction of these new electrophoresis techniques (Durmaz, R).

PFGE, in the earliest incarnation introduced by Schwartz and Cantor, used two sets of electrodes in a square configuration to generate highly inhomogeneous electric fields. The voltage was periodically switched from one set to the other, swinging the field direction by an obtuse angle back and forth. In their original work, Schwartz and Cantor proposed that electric-field gradients were responsible for the dramatically improved separation of DNAs in the size range of 50 kbp to nearly 2Mbp.in subsequent work by Chu, Vollrath and Davis, a hexagonal array of voltage-clamped electrodes was used to generate homogenous alternating field with a constant reorientation angle of 120 degree, an approach they denoted Contour-clamped Homogenous Electrophoresis (CHEF) (Durmaz, R).

Separations of high-mol-wt DNA fragments qualitatively similar or superior to those of Schwartz-Cantor approach were obtained using the CHEF system; thus, it became clear that in homogenous fields were not required in order to effect electrophoretic separation of large DNAs (Durmaz, R).

At about the same time, Carle, Frank, and Olson introduced another development they showed that periodically inverting the field in conventional two-electrode gel electrophoresis experiment (but using a duty cycle greater than 50%, so that leads to a striking improvement in the resolution of DNAs within a restricted size range. Moreover, the overall dependence of mobility on size was generally observed to be a no monotonic function of DNA length; the mobility of some of DNA fragments in these experiments were reduced by 1-2 orders of magnitude relative to their steady field values. The expected decreasing mobility-to-mol wt relationship was retained for molecules smaller than this critical mol wt, whereas mobility increased with mol wt for DNA larger than the critical value. Above a particular DNA mol wt larger than the value at the mobility minimum, DNA mobility was once again independent of mol wt, Carle, Frank ,and Olson called this intermediate range of DNA lengths the "windows "of improved resolution (Durmaz, R).

A quantitative understanding of the effects rapid changes in field strength or direction on mobility remains largely incomplete, owing to the relative simplicity of the models used to describe gel electrophoresis. In this review; we discuss principles of a number of statistical-mechanical theories for the gel electrophoresis of DNA and their application to the analysis of PFGE experiments. We consider first a class of models based on the simple assumption that DNA molecules migrate end-on-through the gel much like snake in thicket of bamboo. This motion is called reputational concept introduced into polymer physics by de Gennes and Doi and Edwards. Repetition model succeed fairly well in describing the behavior of DNA undergoing Electrophoresis in steady fields in dilute gels, such as agarose, and also predict effects of chain reorientation on mobility. Where conventional reputation theories fail dramatically is in their inability to account for the improvement in resolution obtained when the field is inverted. This failure has led to the development of another class of models that allow for other motions of DNA chain in addition to end on-migration. All the later models treat the DNA chain in considerable detail. This

invariably requires that the equations of motion of the chain be solved using computer simulation techniques. The detail of interaction between the chain and the gel are considered in various degrees. Some of the simulations are sophisticated extensions of tube models, whereas others abandon the reputation frame work entirely and consider the interactions of chain with individual obstacles in the gel.

In tube reputation model, DNA chain is considered to move only along its axis. As though confined to a tube in the gel, it has been defined by De Gennes and elaborated by Doi and Edwards. It is referred to as the primitive path. The tube concept permit a simple description curvilinear path of the DNA chain constrained by contacts with the gel fibers and avoid considering interaction of chain with the gel in detail.

-In simulation Model, Due to fluctuation in the contour length of the tube are assumed to be small and un-correlated in second , loop of DNA are assumed not to penetrate the walls of tube, so that branched tube conformations are forbidden.

Operationally, there are at least five parameters that affect resolution of DNA molecules in PFGE: Field strength, pulsed time, re-orientation angle, gel concentration, and temperature. Optimum values of these parameters appear to be interdependent (Durmaz, R).

The strength of the electric field has profound effects on PFGE separations. High field degrade the resolution of very high- mol-wt DNA molecules, possibly by trapping these molecules at the origin of electrophoresis (Durmaz, R).

Separation involving giant molecules, such as, *Neurospora crassa* chromosome believed to be of the order of 5-10 Mbp, requires extremely low fields, of the order of 1V/Cm or less. The requirement for such low fields leads to extremely long electrophoresis times. Often more than 1 week. The optimum pulsed time for separations over a given range of mol wt is likely to be closely coupled to value of the field strength and the reorientation angle (Durmaz, R).

Only obtuse reorientation angles seem to be effective in PFGE. Generally, useful reorientations are obtained when the reorientation angle is between 90-180.The mol wt range resolved depends strongly on reorientation angle. In PFGE experiments in which the reorientation angle is 120, the mobility is generally a monotonically decreasing function of mol wt below some critical value of the mol wt, above which

all DNA s have the same mobility. In FIGE, however, the windows of resolution are relatively narrow and no monotonic (Durmaz, R).

A serious deficiency in all of models reviewed here may be the failure to account adequately for properties of the gel. The models considered here take the gel to be either a featureless tube, possibly allowing some variation in the size of spaces comprising the tube, or a set of point obstacles in two dimensions. Electron microscopic evidence, however, suggests that agarose gels are networks of agarose fibers, these fibers are arranged randomly in space, leading to a complex structure for the gel with a broad distribution of void sizes (Durmaz, R).

It seems DNA molecules are tapped in agarose framework.

We are unaware of any current efforts to understand in detail the dependence of PFGE behavior on temperature. Temperature effects on mobility in steady field electrophoresis arise mainly from changes in the chain fraction coefficient owing to the temperature dependence of the viscosity of water.

It is possible; however, that temperature may exert some nontrivial effect in PFGE through effects on the local tensions in the chain (Durmaz, R).

Field-inversion Gel Electrophoresis (FIGE):

Among the techniques to separate large DNA fragments, field inversion gel electrophoresis (FIGE), is probably the easiest to perform with a minimum of special equipment. Indeed, the only requirement besides a regular gel electrophoresis box and power supply is device enabling the periodic inversion of electric field direction over the course of the experiment. This method has been derived from Orthogonal-field-alteration gel-electrophoresis (OFAGE) apparatus. The widest angle used is 180, the four electrode of PFGE system was reduced to two electrodes.

Contour-clamped Homogenous Electric Fields (CHEF)

CHEF is a particular formulation of pulsed-field gel electrophoresis (PFGE), which uses an array of electrodes positioned around the gel (on a contour) and clamped to specific voltage to produce a nearly homogenous Electric field inside the contour. The direction of the electric field is changed periodically, as with all pulsed-field techniques. In the case of CHEF, field orientation is achieved electronically by

changing the voltage: (potentials) of the various electrodes in the array. Commercial CHEF Devices currently employ a hexagonal electrode array, but other types of contours, such as circles or squares, if properly clamped, can also produce alternating homogenous electric fields (Durmaz, R).

CHEF was developed for two primary reasons. The first pulsed field machines employed in homogenous electric fields that caused the DNA molecules to migrate with curvilinear, arc-like or even wave like trajectories, early workers suggested that voltage gradient produced by these inhomogeneous fields might be required for the resolution of large DNA (Durmaz, R).

The major difficulty in separating molecules in this range has been that, as the size of chromosomal DNA increases, the field strength used to resolve the molecules must be decreased to minimize trapping of the DNA as it moves through the agarose matrix. This necessarily leads to long periods of electrophoresis more than one week for a 10 Mb molecules, making the determination of the optimal condition for resolving a particular sample a time consuming process (Durmaz, R).

The window of resolution in CHEF electrophoresis is the result of interplay of several factors. These include the electric field strength, the temperature, the agarose composition and concentration, the pulsed time, and the angle between alternating electric fields (Durmaz, R).

To successfully resolve multimega base DNA molecules, one must first prepare them intact, it is useful to remember that a 3 Mb DNA molecule is approx. 20 A in diameter, with a CHEF length about 1mm,yielding an axial ratio of 500,000.to prepare such molecules, care must be taken to minimize shearing enzymatic degradation during all manipulations (Durmaz, R).

Bacterial Genome-mapping" by two-Dimensional Pulsed-field Gel Electrophoresis" (2D-PFGE)

Two dimensional pulsed field gel electrophoresis (2D-PFGE) is a powerful PFGE technique for the restriction mapping of bacterial genomic DNA. The method consists of two sequential steps of restriction endonuclease digestion and separation of the fragments by PFGE:

1-Step A, in which partially or completely digested bacterial DNA is separated by PFGE in the first dimension; and

2-Step B, in which a gel slice containing the separated DNA of the first dimension is cut out from the first gel, re-digested with the same or a different restriction enzyme, and separated by PFGE in the second dimension, i.e, perpendicular to the first dimension.

The final gel displays a two dimensional pattern of DNA Spots whose distribution is determined by localization of the restriction sites in the genomic DNA. Hence, it should be possible to deduce from this pattern the position of most, if not all, restriction sites in the original DNA.

Applications of PFGE in molecular biology,

1-Bacterial Genome mapping by two dimential PFGE

2-Protozoan Genome, karyotype analysis, chromosome structure, and chromosome specific Libraries

3-Conformation of yeast artificial chromosome libraries by PFGE

4-Analysis of yeast artificial chromosome clones

5-Strategies for mapping large regions of mammalian Genome

6-Two dimensional DNA electrophoresis, form a mammalians' DNA

7-PFGE &2DE of long arrays of tenderly repeated DNA; Analysis of human centrmeric Alpha satellite

8-Construction of Lambda libraries from large PFGE Fragments

9-In Gel Detection of DNA; application to study of viral DNA Metabolism by use of pulsed field agarose gel electrophoresis

1.30 Aims of the project

P. aeruginosa represents a major cause of morbidity and mortality in both the hospital and community setting. The overall goal of this study was to assemble a database of PFGE profiles and to identify major lineages of *Pseudomonas aeruginosa* circulating in our studied hospital wards.

According to some articles bronchoscopes, endoscope could preserve bacteria in the form of biofilm. So we tried to check this hypothesis and our results confirmed the accuracy of our hypothesis. The purpose of this study was to gain a greater understanding of these processes and the mechanisms by which they are occurring within the epidemiological setting of ANKARA.

-Increasing use of "SpeI" restriction enzyme as an alternative of "XbaI" in pulsed field gel electrophoresis and epidemiological studies obliged us for evaluation its efficiency and definition of its cut off value for coming researches. Is its accuracy as the same of XbaI?

Do our bacterial isolates posses' homogenous or heterogenous dendogram profile?

Do they originate from similar source and reservoir?

Due to difficulty of treatment of MDR *Pseudomonas* in a clinical environment, a number of isolates, presumptive *Pseudomonas aeruginosa* strains, were obtained from an Ankara private hospital, the MESA; it was of interest to study these isolates in order to:

-identify and characterize PFGE profile of the strains

-create an antibiotic resistance profile for each isolate

-investigate the similarity or variety of mechanisms of antibiotic resistance in the multi drug resistant strain, *P. aeruginosa*.

1.30.1 Geographic and demographic setting of study

ANKARA is the capital of Turkey and the country's second largest city after Istanbul. The city has a mean elevation of 938 meters. The city is located at 39°52'30" North, 32°52' East, about 450 km to the southeast of Istanbul, the country's largest city. Although situated in one of the driest places of Turkey and surrounded

mostly by steppe vegetation except for the forested areas on the southern periphery, Ankara can be considered a green city in terms of green areas per inhabitant, which is 72 m^2 per head. According to the Turkish Statistical Institute, as of 2011 the city of Ankara had a population of 4,338,620 and its metropolitan municipality 4,550,662. Ankara provides a unique set of circumstances for the study the epidemiology and pathogenesis of *P. aeruginosa* infection in humans.

CHAPTER 2

MATERIALS AND METHODS

All steps of this research has been summarized in Figure 9.

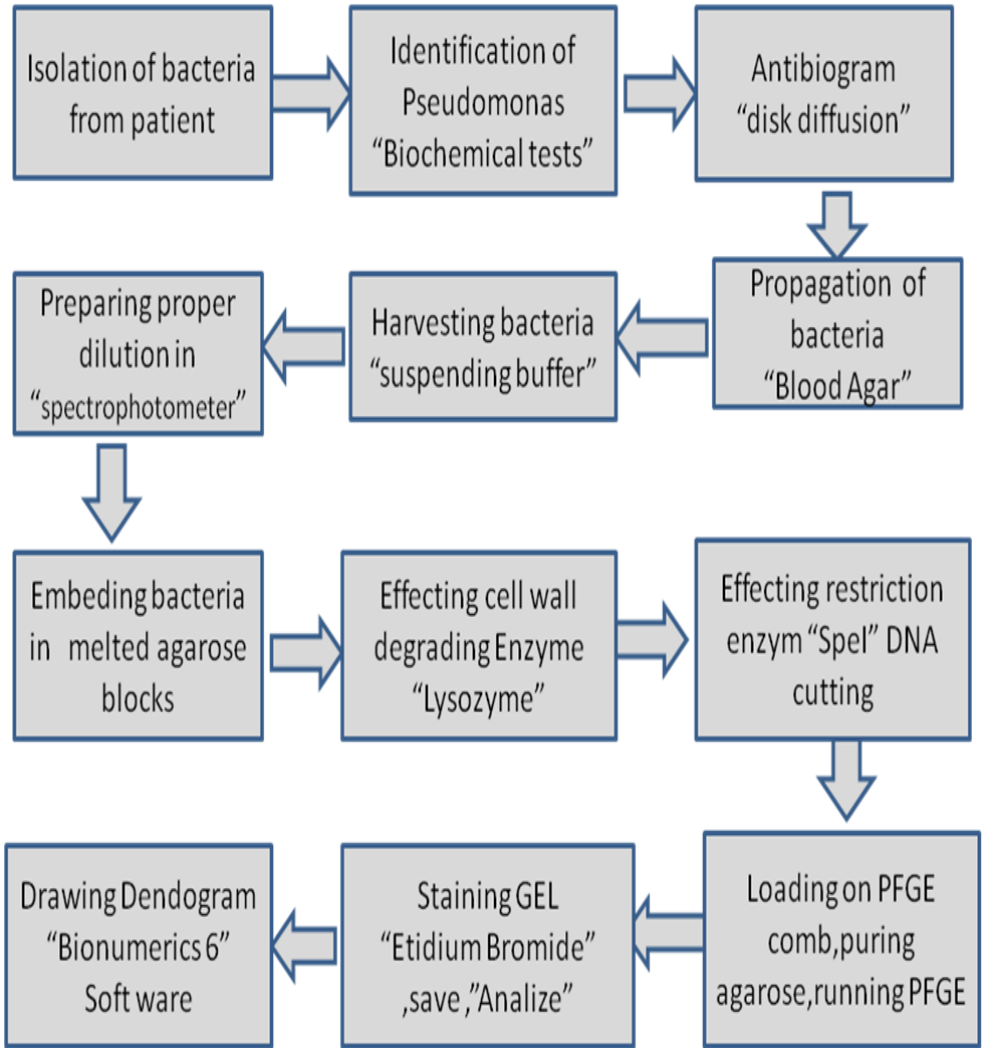


Figure 9. Brief stages carried out in our study

2.1 Clinical isolates

The *Pseudomonas* isolate employed in this study was mainly provided by Dr Abbas Taner , Head of the Microbiology Laboratory of MESA hospital archive strains,
They were 58 isolates from MESA. Isolates were received on blood agar plates and were transported after recovering from frozen condition in blood agar plates (Figure 34 Appendix B).

2.2. Microbiological media

2.2.1Blood agar

Blood agar was made by adding 50 ml of sterile defibrinated human blood to 950 ml of nutrient agar. Nutrient agar was sterilized by autoclaving and allowed cool to 50ºC.
Defibrinated blood was added aseptically. The agar was mixed and distributed into plates. Blood should never be added before autoclaving (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993) (Figure 34 Appendix B).

2.2.2 Cetrimide Agar

Dehydrated cetrimide agar powder (Merck) (4.45g) was dissolved in 100 ml of distilled water. Glycerol (10 ml/L) was added and the agar was autoclaved at 121ºC for 15 minutes. The medium was supplemented with 15 µg/ml naldixic acid to select for *Pseudomonas aeruginosa* (Figure 36 Appendix B).

2.2.3 Hugh and Liefsons' Medium

The solids were dissolved by heating in water and the pH was adjusted to 7.1 with 2 M NaOH. The indicator dye was added and the medium was sterilized. Following sterilization, a sterile solution of glucose was added aseptically to give a final concentration of 1% v/v. The medium was mixed and distributed aseptically in 10 ml

volumes into sterile test tubes (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993) (Table 2, Figure 36 Appendices B).

Table 2: Components of Hugh and Liefsons' medium used for the oxidation Fermentation Test (Todar).

Component	**Amount/L**
Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Agar	3g
Distilled Water	1000 ml
Bromothymol blue (0.2%w/v)	15 ml

2.2.4 Preparation of Mueller-Hinton Agar for disk susceptibility test

Mueller-Hinton agar was prepared as follows. The agar was prepared according to the manufacturer's instructions to a pH of 7.2-7.4. Immediately after autoclaving, the agar was allowed to cool to 45-50ºC. The freshly prepared agar was poured into plastic, flat bottomed Petri dishes on a level surface to give a uniform depth of approximately 4 mm (this corresponded to 25-30 ml of agar in a plate with a diameter of 100 mm).

The plates were allowed to cool to room temperature and, unless the plates were used in the same day, they were stored at 2-8°C in a refrigerator. All plates were used within seven days of preparation. A representative sample of each batch of plates was examined for sterility by incubating at 37°C for 24 hours (Figure 32 Appendix B).

2.2.5 Tests used to identify bacterial isolates

2.2.6 Cell and colony morphology characteristics

The cell and colony morphology of the isolates were observed following growth of the organisms on nutrient agar and incubation for 24 hours at 37°C according to Cown and Steel's Manual for the identification of Medical Bacteria,1993(Figure 34 AppendixB).

2.2.7 Gram Staining

The Gram stain was carried out on 18-24 hour cultures according to the Hucker method (Collins and Lyne, 1985). A loopful of an overnight culture was air-dried and heat fixed on a glass slide. Crystal violet stain (0.3% w/v) was added and allowed to stand for one minute. Excess stain was washed off with a gentle stream of water.
Grams-iodine (0.4% w/v) was added and allowed to stand for 30 seconds before being rinsed off. The stain was washed with ethanol (95% v/v) and then stained with the Secondary stain.Safranin (0.4% v/v) covered slides for one minute. This was then washed with water for 5 seconds. If the bacteria were Gram-negative, it appeared pink under the microscope. If the cell was Gram-positive, it appeared purple under the microscope.

Controls: *Staphylococcus aureus*- positive

Pseudomonas aeruginosa- negative

2.3 Motility test

An overnight culture of the organism was examined in "hanging drop" preparations, using a 100x magnification and reduced illumination. A "hanging drop" slide was prepared by placing a loopful of the bacterial suspension onto the center of a cover slide. A depression slide onto which a ring of Vase line had been spread around the

concavity was lowered onto the cover slip, with the concavity facing down over the drop. When a seal had formed, the hanging drop slide was turned over and examined under a microscope (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: motile- *Pseudomonas aeruginosa*
Non-motile - *Enterococcus facials*

2.4 Catalase activity

A loopful of culture was emulsified with a loopful of 3% (v/v) hydrogen peroxide. Effervescence, caused by the liberation of free oxygen as gas bubbles, indicated a positive result (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: Positive - *Staphylococcus aureus*
Negative - *Streptococcus pyogenes*

2.5 Oxidase activity

(a) Filter paper was impregnated with a 1% (w/v) aqueous solution of tetramethyl para-phenylene-diamine (with 0.1% (v/v) ascorbic acid to prevent auto-oxidation) Bacterial cultures were smeared across the filter paper with a glass rod. The formation of a purple color within 5-10 seconds indicated oxidase positive cultures (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993) (Figure 35 AppendixB).
(b) Oxoid oxidase identification sticks were used to take up some bacterial culture. A positive reaction was recorded when purple coloration formed within 30 seconds (Figure 35 Appendices B).
Controls: Positive – *Pseudomonas aeruginosa*
Negative – *Escherichia coli*

2.6 Oxidation-Fermentation test

Two tubes of Hugh and Liefson's medium were stab inoculated with the test culture. One tube was covered with sterile mineral oil and both tubes were incubated at 37ºC for up to 14 days. Acid production was indicated by a change in the color of the medium from blue-green to yellow. Fermentative organisms produced acid in both tubes and oxidative organisms produced acid in only the open tube and usually only at the surface (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: *Pseudomonas aeruginosa*- oxidation

Serratia marcescens- fermentation

2.7. Hemolysin production

A plate of Blood agar (Section 2.4.3) was inoculated by streaking once across the surface. Plates were incubated at 37ºC for 24 hours. There were three possible outcomes: α-haemolysis (green zones, cell envelopes intact), β-hemolysis (clear, colorless zone, cell envelopes disrupted) or γ-hemolysis (no action on red cells) γ-hemolysis describes a negative result (Cowan and Steel's Manual for the identification of Medical Bacteria, 1993).

Controls: α-hemolysis – *Streptococcus viridians*

β-hemolysis – *Streptococcus pyogenes*

γ-hemolysis – uninoculated medium

2.8. API Tests

The **analytical profile index** or **API** is a classification of bacteria based on experiments, allowing fast identification. This system is developed for quick identification of clinically relevant bacteria. Because of this, only known bacteria can be identified.

The API identification system API20NE (bioMérieux, Marcy-l'Etoile, France) were used for identification of the clinical isolates.

The identification system was used according to the manufacture's instructions. These test reactions were carried out with Phoenix Automatic system.

2.9 Antimicrobial disk susceptibility tests

Preliminary antibiotic susceptibility testing was performed in triplicate with Mastring-S M14 antibiotic multidisks (Mast Diagnostics, Merseyside, U.K.). These disks contained eight antibiotics including ampicillin (10 μg), cephalothin (5 μg), colistinsulphate (25 μg), gentamicin (10 μg), streptomycin (10 μg), sulphatriad 200 μg tetracycline (25 μg) and cotrimoxazole (25 μg). Mueller-Hinton agar was inoculated with overnight culture of the strain to be tested. A multidisc was aseptically laid on the surface of the agar using a sterile tweezers. The plates were incubated for 24 hours. The zones of inhibition were observed.

2.9.1 Antibiotic susceptibility testing according to Clinical and Laboratory Standard Institute

The tests were conducted according to the approved method of the Clinical and Laboratory Standards Institute (CLSI) [M2-A9] (2006). Mueller-Hinton agar was used as the growth medium in all the antibiotic disk susceptibility tests. All tests were done in triplicate.

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard equivalent to a 0.5 McFarland standard was used. The 0.5 McFarland standards were prepared as follows: a 0.5 ml aliquot of 0.048 mol/L$BaCl_2$ (1.175%

w/v$BaCl_2$. $2H_2O$) was added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1 cm light path and matched cuvette to determine the absorbance. The absorbance at 600 nm was between 0.08 and 0.1 for the 0.5 McFarland standards. The $BaSO_4$ suspension was transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum. These tubes were tightly sealed and stored in the dark at room temperature. The $BaSO_4$ turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appeared, the standard was replaced. The barium sulphate standards were replaced or their densities verified monthly.

The direct colony suspension method is the most convenient method for inoculums preparation. The inoculums were prepared by making a direct saline suspension of isolated colonies selected from an 18- to 24-hour agar plate. This resulted in a suspension containing approximately, 1 to 2 $x10^8$ CFU/ml. To perform this accurately, the inoculums tube and the 0.5 McFarland standards were compared visually. A Unicom 8625 spectrophotometer (Cambridge, U.K.) was then used to confirm that the inoculums tube was at the required turbidity of between 0.08 and 0.1 at OD 600nm.

A sterile cotton swab was dipped into the adjusted suspension, optimally within 15 minutes after adjusting the turbidity of the inoculums suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove the excess inoculums from the swab. The dried surface of the Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated two more times, rotating the plate approximately 60º C each time to ensure an even distribution of inoculums. The rim of the agar was then swabbed as a final step. The lid was left a jar for three to five minutes to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

The pre-determined battery of antimicrobial disks (Oxoid, Hampshire, U.K.) was dispensed onto the surface of the inoculated agar plate using a dispensing apparatus.

They were distributed evenly so that they were no closer than 24 mm from center to center. No more than five disks were placed on a plate at a time to avoid overlapping of zones. Once placed on the plate, the disks were not relocated, because some of the antibiotic diffuses almost instantaneously.

The plates were then inverted and placed in a 37ºC incubator within 15 minutes after the disks were applied. The plates were examined after 16-18 hours of incubation. The zones were measured to the nearest millimeter using a ruler held on the back of the inverted Petri dish. The standard error of the ruler was 0.5 mm. The zone margin was considered to be the area showing no obvious, visible growth that can be detected with the unaided eye. The sizes of the zones of inhibition were interpreted by referring to CLSI standards (Table 8 Appendices B) and were reported as being susceptible, intermediate or resistant to the agents that were tested. The zones of inhibition diameters of each antibiotic were also obtained for the control strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 35218 to ensure the method was being performed correctly. Their zones of inhibition diameters were measured and compared to the expected diameters according to CLSI (Table 3 Appendix B).

2.10 API 20NE Identification of Bacterial Species

The API 20NE (Biomerièux) non fermentative Gram negative bacillus identification system consists of a plastic strip containing 20 micro-wells (consisting of a tube and a capsule), each coated with a separate biochemical substrate. Isolates were grown on MacConkey agar (Taslabs) at 37ºC under aerobic conditions for 24 hours prior to analysis. Oxidase reactions were inputted manually following determination of other testing results. API 20NE testing was performed according to the manufacturer's instructions. The resultant biocode was entered into the Biomerièux on-line database (https://apiweb.biomerieux.com) and used to determine a percentage probability and statistical significance (t) of identification. Quality control was performed upon each new kit lot number by the staff of the Royal Hobart Hospital, according to the manufacturer's instructions and in accordance with the National Australian Testing Authority (NATA) guidelines.

Quality Control: *Sphingobacterium multivoran* ATCC 35656

*Aeromonas hydrophila*ATCC 35654

*Pseudomonas aeruginosa*ATCC 27853

*Achromobacter faecalis*ATCC 35655

2.11 CLSI Antimicrobial Susceptibility Testing

P. aeruginosa isolates were sub cultured from Blood agar onto Mac Conkey agar and incubated at 37ºC in air for 48 hours. A 2.5 mL volume of 0.85% sterile saline in a glass test tube was placed in and used to zero a Vitek nephelometer. Following this, a 0.5 McFarland turbidity colorimeter control (Remel) was tested in the nephelometer to confirm reading accuracy. Sufficient amounts of test organism were emulsified in the saline to reach an absorbance in the red test zone (equivalent to 0. 5 Mc Farland standard) A sterile swab was immersed in the saline suspension, excess saline removed by swirling against the side of the tube. The swab was used to make two lawn plates on Mueller Hinton agar (Oxoid) by crossing the agar in three separate directions using the "cross-hatch method". Inoculated agar plates were allowed to dry in air for 30 minutes. Up to six separate antimicrobial susceptibility discs (Oxoid) were placed on each agar plate using a commercial antimicrobial disc dispenser (Oxoid) Plates were incubated for 18-24 hours in air at 37 ºC and the diameters of zones of inhibition around the antimicrobial discs recorded to the nearest whole millimeter using a graduated ruler. In cases where sparse growth was noted, plates were re-incubated for a further 18-24 hours and zone diameters recorded on the second day of incubation. Results were reported as "sensitive", "intermediate" or "resistant" based upon the cut-off values recommended by CLSI (Table8Appendix B) All tests were performed in duplicate on separate occasions, using new batches of media. In cases where discrepant results were observed, the test was repeated in triplicate and the average result of all three tests recorded.

Quality Control: *Pseudomonas aeruginosa* ATCC 27853

2.12. Laboratory investigation Patient samples

Clinical isolates and reference strains *Pseudomonas aeruginosa* viable clinical isolates identified in MESA laboratory from April 1, 2006 to December 31, 2011(n=58) were studied. They were collected from private hospital of MESA.All clinical isolates had been characterized using classical bacteriological techniques, including growth on Blood agar, EMB, TSI, oxidase, OF (oxidation-fermentation), API tests. All bronchoscopy specimens were plated on 5% sheep blood agar and chocolate agar (Biomerieux, Marcy L'Etoile, and France) and incubated over night at 37 C. Colonies were identified with API system batteries (Biomerieux) following the supplier's recommendations. All samples from the bronchoscopy procedures were reviewed during the study period to identify bacteria potentially related to the outbreaks. In addition to the clinical isolates, P.A 18323 was used as reference strain.

2.13. PFGE

Steps of PFGE have been summarized in (Figure 9, 10). Preparation of chromosomal DNA, restriction enzyme analysis of DNA plugs, PFGE, and analysis of the results were carried out in the Molecular Microbiology Research and Application Laboratory at the Refik Saydam National Hygiene Center (Figure24-30AppendixB).

The preparation of chromosomal DNA was performed as described by Durmaz *et al.* with modification (Durmaz, R)

1-Recovered bacteria were subcultured from frozen clinical isolates of *Pseudomonas aeruginosa* on to blood agar and incubated overnight at 37°C.

2-Bacterial colony were harvested suspended in Tris ethylenediaminetetraacetic acid (EDTA, 100 mM Tris –Hcl, 100 mM EDTA pH =8),

3-Bacterial suspension were centrifuged 2500 g for 15 minutes in 4 ºC (alternative 13000 g for 2 minutes, supernatant were put out.

4-1ml from above buffer was added to sediment of bacteria, vortexed.

5-Bacterial+buffer absorbance adjusted to an optical density of 1(Mc Farland 4) at 590 nm in spectrophotometer (Boeco Germany UV/Vis)

6-0.2% low melting point agarose were prepared with above buffer EDTA-Tris-Hcl.(Gibco BRL,Paisley,UK)it was melted in microwave (45-50ºC)1%SDS was added to agarose. Two hundred µl of each suspension were transferred to 1.5-ml micro centrifuge tubes, and SDS-agarose was used for its solution.

7-After several times mixing with pipetage,

8-it was transferred to special well bearing comb, agarose was blocked in it.

9-then it was placed 10 minutes in ice, then to -4 ºC refrigerator for solidification of blocks of agarose. (Figure10, and Figure 25, 29Appendix B)

10-For degrading embedded bacterias in agaros blocks, 0.5 ml lyzing solution containing 50 mM Tris-Hcl (pH 8.0)50 mM EDTA 2.5 mg/ml Lysozyme,1.5mg/ml proteinase K) were prepared.

11-Then 20 µl of a 10-mg/ml solution of proteinase K (Sigma-Aldrich), Lysozyme (sigma-Aldrich) was added to tubes bearing bacteria embedded agaros.

12-They were placed in 37 ºC stirring water bath for 1 hour for maximum effects of enzymes on bacterial cell wall degradation the tubes were gently inverted and 100 µl of 1.6% low-melting agarose/sodium dodecyl sulfate solution was added.

1- Harvesting Bacterial Colony,washing,suspending in bufer,Turbidity 1 Mc Farlend preparation

2 - Inserting bacteria into agarose and preparing Bloks

Bacterial Solution based on Mc Farlen Standard(Spectro)

50°C Low Melting point agarose

100 µl

Cold agarose Frame

3 - Bacterial Cell wall degredation (Enzymaticly),DNA isolation

Lysing Solution

Cold agarose Frame

Incubation in proper temperature and time

Washing in TE Buffer

4- Cutting DNA with SpeI Restriction Enzyme Digestion

Incubation of TE Buffer +Enzyme in proper temperature and time

5 - Electrophoresis

Cutting Agarose Block with Bistury

Agarose

High Melting point agarose

6 - Result

Reading with Etidium Bromide,Photo taking,Analyzing with soft ware and drawing Dendogram
Similarity of strains are detected

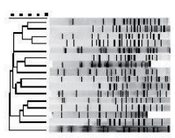


Figure 10 Pulsed Field Gel Electrophoresis different Stages

13-The cell and agarose suspension was mixed and immediately dispensed into a 100-µl disposable plug mold (Bio-Rad Laboratories, Hercules,CA) Solidified plugs were transferred to 10-ml round-bottom glass tubes containing 1.5 ml of EDTA/sarcosine buffer and 80 µl of a 10-mg/ml solution of proteinase K. The tubes were horizontally incubated in a reciprocal shaking water bath (60 strokes per min) at 55°C for 2 h before washing plugs were kept on ice 15 minutes,

14-supernatant were removed with pipet aspiration.

15-The plugs were washed sterile in distilled water (three times) and Tris EDTA buffer (six times) in shaking water bath 15 minutes.

16-Then, the plugs were digested with 50 U of SpeI (Fermentas Life Sciences) by incubation at 37°C for 1.5 h. Plugs firstly were cut to 4 parts with bistouries blades, 1/4 were transferred to Spel restriction enzyme containing buffer, then placed on comb (Figure 25, 29 AppendixB)

17-Band electrophoresis was conducted in 0.5× Tris borate EDTA(TBE) buffer (44.5 mM Trisma base , 44.5 mM boric acid 1m M EDTA pH=8.0)1gram PFGE agarose with 100 ml 0.5 X TBE are mixed, heated in heater for 1 minute, then is transferred to water bath. Plugs are placed on comb of electrophoresis, gel is pured, comb is converted and plugs are inserted into gel. In a contour clamped homogenous electric field apparatus (CHEF-DR II; Bio-Rad) Electrophoresis was performed at 6 V/cm for 24 h at 14°C with a ramped switched time of 5 s to 45 s. The gels were stained for 20 min in 250 ml of deionized water containing 25 µl of a 10-mg/ml solution of ethidium bromide and then washed in deionized water for 20 min. DNA fragments were visualized on a gel imaging System (Figure 11, Figure 28, 30,31 Appendix B)

18-The band patterns were analyzed by Bionumerics (Applied Maths, Inc., Belgium) (version 6.01) software by using 1.5% band tolerance and 1% optimization settings. *P.A* 18323 reference strain was used for external quality control. Clinical isolates were classified in two ways. Firstly, clinical isolates were classified as clustering or non clustering. Secondly; the clinical isolates were grouped according to the

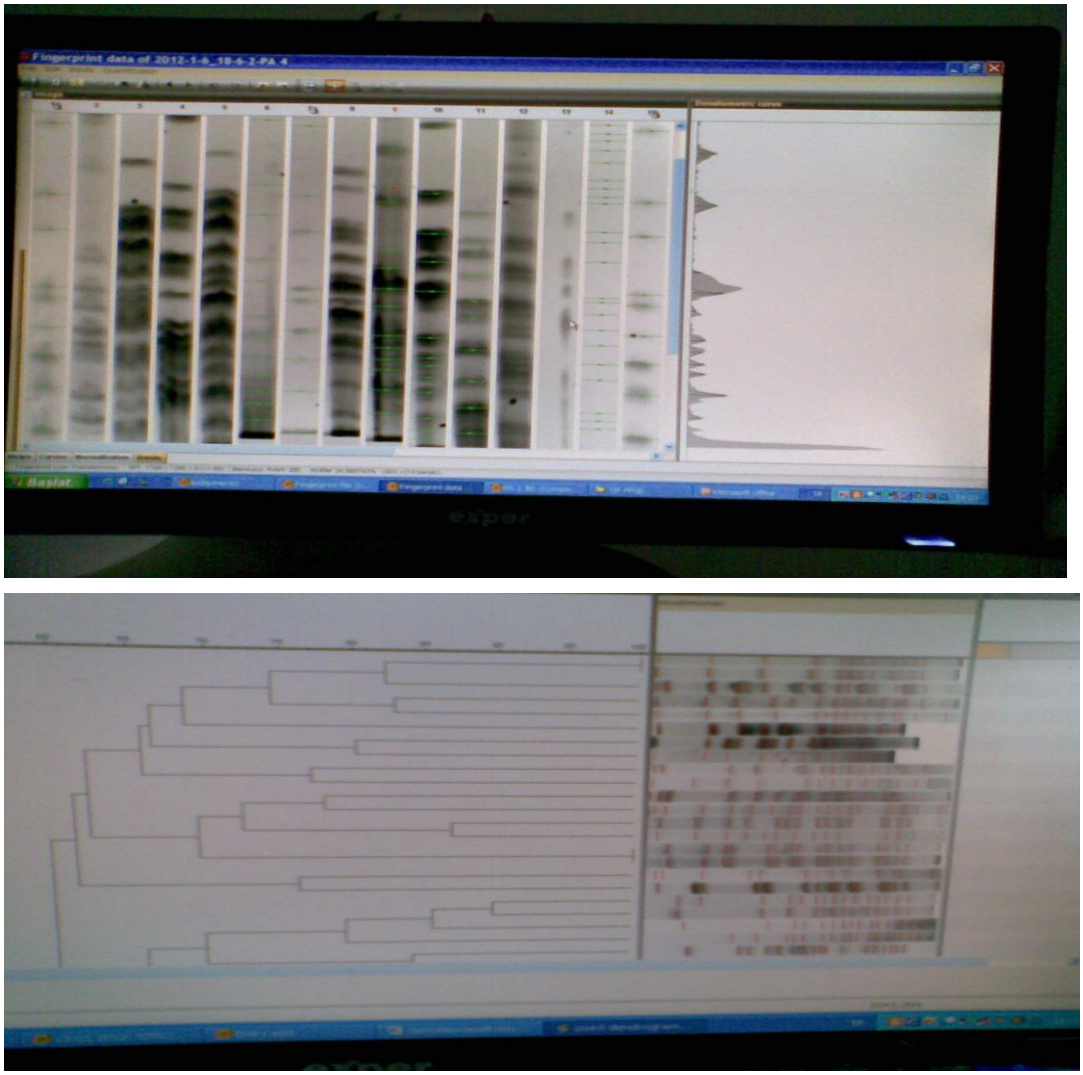


Figure11.PFGE gel of 15 sample genome *Pseudomonas aeruginosa* (above PFGE analyzing, below dendogram drawing with software)

similarity coefficient higher than 80%, to show clonal relationships (Figure30, 31AppendixB)

Interpretation of the DNA banding patterns was according to the criteria of Tenover *et al*. (1995). Dice coefficient of similarity was calculated to compare the macro restriction patterns (Thong *et al*. 1994) This coefficient expresses the proportion of shared DNA fragments in two isolates and was calculated by the following formula:

F = 2nXY/ (nX + nY), where nX is the total number of DNA fragments from isolate X, nY is the total number of DNA fragments from isolate Y, and nXY is the number of DNA fragments that were identical in the two isolates.

PFGE was performed on all isolates grown for 20 hours on Mueller Hinton agar (Excel laboratory products), suspended in 3 mL cold CSB buffer (0,1 mol Tris (Merck), pH 8.0, 0.1 mol EDTA (Invitrogen, pH 8.0) to a 0. 5 McFarland standard. 200 µL of cell suspension was added to 10 µL of 20 mg/mL proteinase K (Promega) 200 µL of this suspension was added to 200 µL of 1,1% molten agarose with 1% SDS and allowed to set. Plugs were placed in 2.5 mL lysis buffer (0.05 mol Tris, pH 8.0, 0.05 mol EDTA, pH 8.0, 1% Sarcosyl (Sigma)), 0.1 mg proteinase K and incubated at 54˚C for 4 hours. Plugs were washed twice in 4 mL Hi pure water at 54˚C for 15 min with shaking, then washed four times in 2.5 mL 1x Tris EDTA buffer for 15 min with shaking at 54˚C. Plugs were then washed twice in 300 µL buffer A (Fermantes) with 1% BSA, followed by restriction in 300 µL buffer A (fermantes) with 1% BSA and 30 U of *Spe*1 restriction enzyme (Promega) at 37˚C overnight. Following restriction, plugs were placed in a 1% SKG agarose gel in 0.5x Tris Borate EDTA buffer and electrophoresed on a Chef-DRIII (Biorad) apparatus at 14˚C. Pulsed field parameters were; two linear ramps 0.5 to 2.5 seconds for twenty hours, followed by 30 to 60 second ramps for four hours at 6V/cm. The international standard ACTC BAA-664 (P.*aeruginosa* ser Braenderup H9812) was included as a marker in all gels. Isolates were considered to be genotypically indistinguishable when their PFGE macrorestriction patterns did not differ by more than one to three bands, representing minor genetic events such as DNA insertions, deletions or point mutations (Armstrong, Nixon *et al*. 2002, Bradbury, Richard).

Quality Control: Positive: International standard ACTC BAA-664
(P.*aeruginosa* Braenderup H9812)

2.14 Dendrogram of Isolate Relatedness by PFGE

Gel analysis of PFGE patterns was carried out with Bio-Rad Diversity Database software (Bio-Rad) and a dendrogram of isolate relatedness was produced from PFGE results using un-weighted pair group matched analysis with arithmetic averages and a dice coefficient (Figure,31AppendixB).

Quality Control: Genotype: *Pseudomonas aeruginosa* PAO1

Outlier: *Pseudomonas fluorescence* ATCC 13525 bands, representing minor genetic events such as DNA insertions, deletions or point mutations (Armstrong, Nixon *et al.* 2002).

Quality Control: Positive: International standard ATCC BAA-664 (*Pseudomonas aeruginosa* ser Braenderup H9812)

2.15 Statistical Methods

Χ2 test, κ tests, student's t- tests, correlation co-efficient tests and determination of mean averages were all performed in Microsoft Office Excel 2003. Statistical analysis of clinical data was carried out by using Fisher's exact test (Freeman-Halton test) in the SPSS statistical package was used for all analyses (SPSS 12.0.1 for Windows, 2003) p-value of ≤ 0.05 was considered to be significant.

CHAPTER 3

RESULTS AND DISCUSSION

We studied a collection of 58 clinical isolates by PFGE. All of them were viable isolates recovered from suspected *Pseudomonas aeruginosa* cases during the period 2006–2011 from one private hospital laboratory archive.

The epidemiological characteristics of the 58 cases isolated from MESA hospital (presently TOBB) based on time of *Pseudomonas aeruginosa* isolation are shown in (Table3 and Table 10 Appendix B).

Table 3. Bacterial isolation based on year and section**.**

Year of isolation	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**
	3	**6**	**8**	**14**	**22**	**5**
Operation room	**ENT**		**ICU**	**EYE**		**Other-sections(Internal, Emergence, Orthopedic)**
26	**5**		**12**	**1**		**14**

The majority of cases n=22; (40%) were seen in 2010 and n=14 (27%) in 2009, indicating an outbreak of infection in these two years (Table 3). Most of *Pseudomonas aeruginosa* were isolated from Operating room, Pulmonary (Broncoscpy, Tracheal) and ICU sections ($p<0.05$).

Twenty six of the 58 cases (45%) were isolated from female patients and thirty one of 58 cases (53%) were isolated from male patients .Most of patients were belonged to over 81 years age group (Table4, Figure12) it was expectable because in higher ages due to immune-compromises risk of infection with *Pseudomonas aeruginosa*

increases. This increase start from 60 years and reach its peak over 80 years old groups, mean age 62.4±25SD, these are shown in (Table 4) (Figure 12) ($p<0.05$).

Figure 12 shows studied age groups percentage in the form of diagram.

Table.4. Number of isolated bacteria in different age group

Age groups	Number of isolation
0-10	6
11-20	1
21-30	2
31-40	6
41-50	1
51-60	1
61-70	9
71-80	13
Over 81	19
Total	58
Mean age : 62.40±25SD	

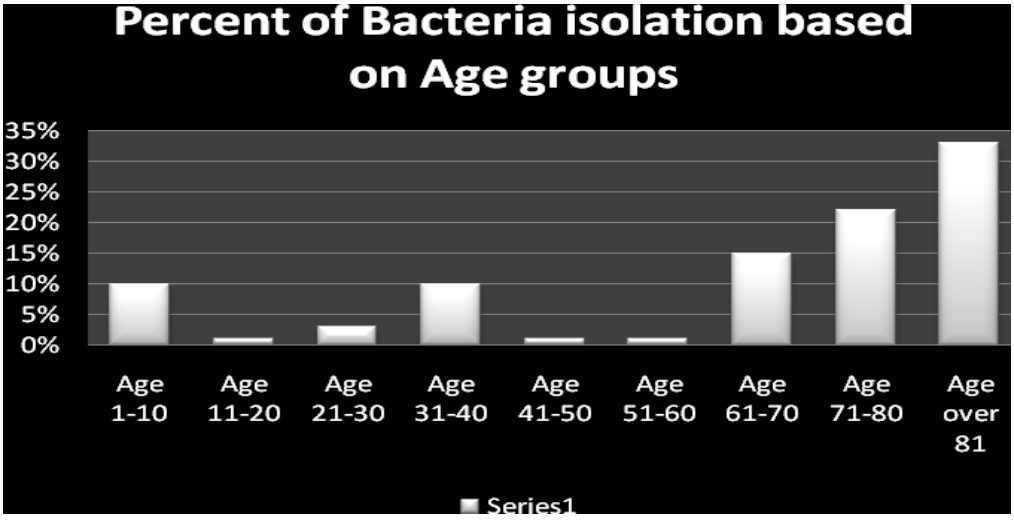


Figure12.Percent of bacterial isolation based on age groups. Mean 62.40±25 SD ($p<0.05$)

In a glance, diagram shows patients age proportional distribution (maximum, minimum),It acts as an internal control,because *Pseudomonas* infection occure mostly in top ages. X age groups,Y percent of age groups.

Most of samples have been isolated from operating rooms, ICU, the least amount from ENT (Ear, Nose, Throat) and Ophthalmology(eye) sections.(Table 3,5, Figure13,14) Wound and blood samples have moderate frequency (Table5).

Table 5. Number of bacteria isolated from different organs

Sample	number
Ear	5
Eye	1
Bronch,Trachea	29
Wound	16
Blood	7
Total	58

Figure 13 shows above results in the template of columns.Operating room was the most riskful place for infection, Bronchoscopy ICU ENT was in the subsequent proportions. ($p<0.05$)

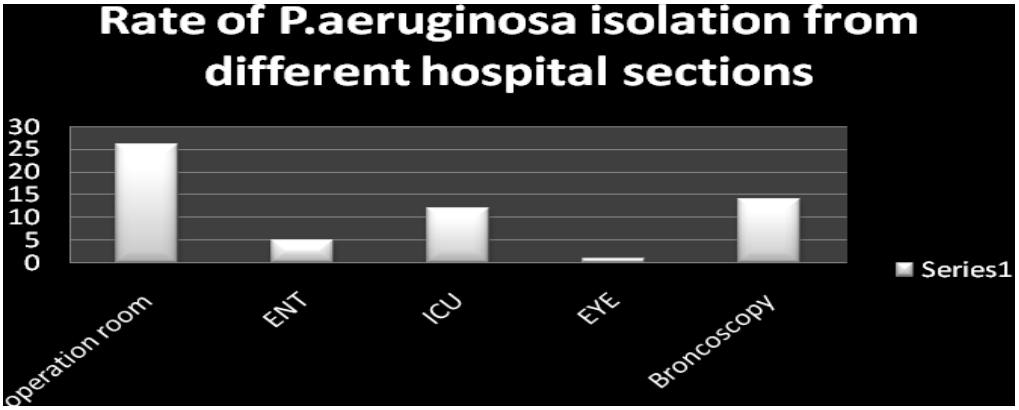


Figure 13. Rate of isolation from different sections ($p<0.05$)

In a glance, diagram shows samples proportional distribution.(For Fast investigators) ENT (Ear,Nose throat)ICU(intensive care unites).

From aspect of isolating organ, most of specimens were isolated through bronchial lavage and bronchoscopy (Table 3, 5, Figure13, 14) Wound, blood, Ear and eye samples were of lesser proportional frequency.

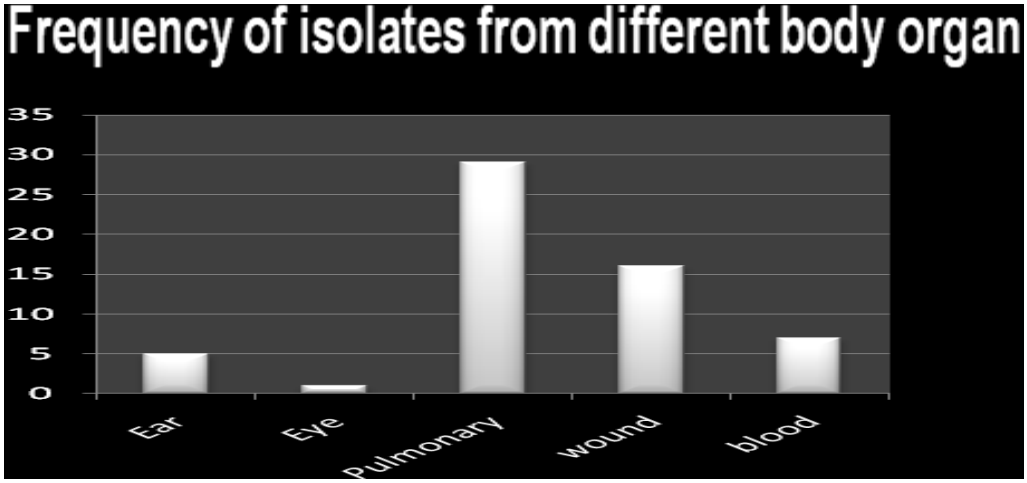


Figure 14 Percent of isolate from different body organs

In a glance, diagram above shows isolated organs samples proprtional distribution.

All "*Pseudomonas aeruginosa*" isolates, their anti-biogram, MIC, MBC etc specifications have been determined by above mentioned hospital's microbiology department relevant automated machine (Phonixe) in special style based on mentioned methods (Table 10, Appendix B).

From 61 P.*aeruginosa*, 3 of them have been diagnosed wrongly, they were *Pseudomonas pseudomalei* while that machine has detected *Pseudomonas aeruginosa*, and we excluded these three samples from our study.

A total of 58 *P. aeruginosa* strains isolated from various clinical specimen in MESA hospital."In the North West region of Ankara-Turkey" during 2006–2011 strains were isolated from 56 patients admitted to different wards of this hospital. *P. aeruginosa* had been isolated from one, two organs or sites of body of patients during staying days of their hospitalization.

Phenotypic study; *P. aeruginosa* isolates were identified by the biochemical profile index procedure (Automated hospital lab phoenix machine). Pyocin production was tested on selective Cetrimide Agar (Merck, Germany).

Susceptibility to antibacterial drugs was studied by the disk diffusion method according to CLSI (Clinical and Laboratory Standards Institute, formerly

NCCLS- National Committee for Clinical Laboratory Standards) for following agents: Carbenicillin (Cb), piperacillin-tazobactam (Tzp), ceftazidime(Caz), imipenem(Imp), meropenem(Mem) , gentamicin(Gn) , tobramycin(Tb), netilmicin(Net), amikacin(Ak), ciprofloxacin(Cip), colistin (Ks).

Performance standards for antimicrobial susceptibility testing (CLSI-2006-2010) (Table6, Figure 15) .

Table 6 Antibiotic resistant rate between isolated bacteria ($p<0.05$)

Antibiotic	Resistance %	Sensitive%	Antibiotic	Resistance %	Sensitive%
Gentamycin	21(44%)	27(56%)	Ceftazidim	10(23%)	33(76%)
İmipenem	4(11%)	34(89%)	Cefepim	2(6%)	32(94%)
Meropenem	9(39%)	14(60)	Cefoxitin	3(16%)	15(83%)
Ertapenem	14(77%)	5(22%)	Ampicilin sulbactam	15(28%)	40(72%)
pipraciline	15(31%)	34(69%)	Tobramycin	14(25%)	40(75%)
Ciprofloxacil ine	5(10%)	45(90%)	Aztronam	5(10%)	51(90%)
SXT	18(50%)	18(50%)	MDR	19(33%)	36(67%)
Colistin	0(0%)	58(100%)	Tazobactam	5(10%)	51(90%)

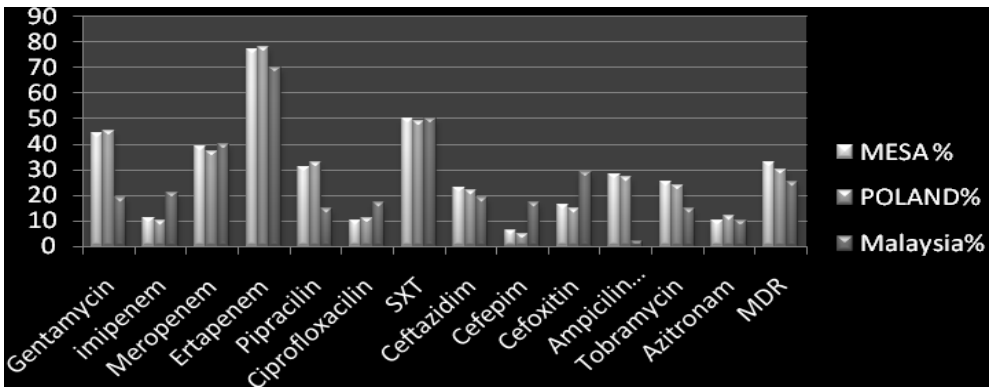


Figure 15 Antibiotic resistant rate diagram.Comparison of its rate between our results in MESSA hospital Ankara,Poland (Urszula)Malaysia(king ting lim) based on CLSI 2010 guideline,Ertapenem ,positive control,Colistin negative control disk.

Drug resistance of isolated bacteria to existing antibiotics has been shown in (Table 6) and (Figure 15). It is considered a way of phenotypic typing method.
Resistances to carbapenems (Ertapenem, imipenem, meropenem) were high, equal to that of Eastern European countries (Urszula 2006).

However results of these hospitals cannot be considered as representative of Turkey or Ankara, but it offer good information about quantity of drug resistance in part of Ankara.
Cefepim, cefoxitin, ceftazidim, azetronam are the most effective drugs against *Pseudomonas aeruginosa*. Our results confirmed it (Table 6, Figure 15).

Multi-drug resistance and pan-Drug resistance were observed in 19 isolates of MESA hospital. Results from the characterization of the 58 clinical isolates of *P. aeruginosa*, obtained from various wards of MESA hospital indicated that the isolates were mostly susceptible to aztreonam, colistin and, to a lesser extent, to amikacin and piperacillin-tazobactam.

All ICU 14 *P.aeruginosa* isolates were resistant to amoxicillin-clavulanic acid, imipenem, meropenem. This is in concordance with the results of Poirel *et al*, which showed that the minimal inhibitory concentration rate is more than 512 mg/mL, indicating that their *P. aeruginosa* isolates were resistant to amoxicillin-clavulanic acid (Poirel L,200). High resistance rates were also found for tetracycline (73%) and chloramphenicol (60%).These maybe due to its natural (non-acquired) resistance. The only other published data on antimicrobial resistance rates among *P. aeruginosa* in Turkey, did not cover some of the antibiotics used in this study, such as tetracycline, chloramphenicol, and aztreonam (Bayram A, Balci I 2006). However, a similar trend was observed for the other antibiotics that were characterized in other studies. Disturbingly, the resistance rates for these antibiotics were higher when compared with a study performed in 2006 by Turkish group in Istanbul, with the exception of cefepime for which a resistance rate of 38.9% was reported (Bayram A, Balci I 2006); The resistance rate of *P. aeruginosa* isolates to ciprofloxacin in MESA hospital is still relatively low when compared

with other cities or countries. For example, in a survey carried out at a teaching hospital in Istanbul, Turkey, it was reported that ciprofloxacin was the most active agent against *P.aeruginosa* despite a resistance rate of 25% (Gençer S, Ak Ö, Benzonana 2002). A more recent survey carried out in a surgical intensive care unit of a hospital in Turkey showed a 59.2% rate of resistance against ciprofloxacin (Bayram A, Balci I 2006), which is higher than the usual reported range of 30% to 40% (Jones RN, 2002, Van Eldere J2003). Although the ciprofloxacin resistance rate reported in this study was relatively low (17%). Similarly, the resistance rate for gentamicin is reasonably low (19%) when compared with the 54.9% rate reported from Spain (Sevillano E,2006), 48.4% rate from Istanbul ,Turkey (Bayram A, Balci I 2006), and 75% rate from Russia(Stratchounski LS1998).

MDR isolates of *P. aeruginosa* have been reported in numerous countries with increasing frequency (Jiang X, Zhang Z 2006.Pagani L, 2004, Pellegrino FL2002). In this study, 33% of the 58 *P. aeruginosa* isolates that were investigated were MDR. The high incidence rate of multidrug resistance, due perhaps to prolonged use of antibiotics, poses serious therapeutic problems in Turkey and should be further investigated. Both ESBL producing isolates were detected by the double-disk synergy test, Gençer *et al* (Gençer S, Ak Ö, Benzonana 2002) reported that the phenotypic laboratory detection of ESBL producers might be difficult due to the combined presence of an AmpC system and class D ESBLs as they confer resistance to β-lactam inhibitors. In this study, all the isolates were resistant to amoxicillin-clavulanic acid and this may be the reason why synergistic effects were not present in the majority of the isolates, although many ESBL-encoding genes have been described.

Oxacillin-hydrolyzing enzymes are frequently found in *P. aeruginosa* and OXA-10 derivatives are one of the most common OXA enzymes reported in *Pseudomonas aeruginosa* The presence of the OXA-10 derivative has been reported in *Pseudomonas aeruginosa* isolates from Turkey, Taiwan, France, and China (Aktas Z, Poirel 2003, Mugnier P, 1998).

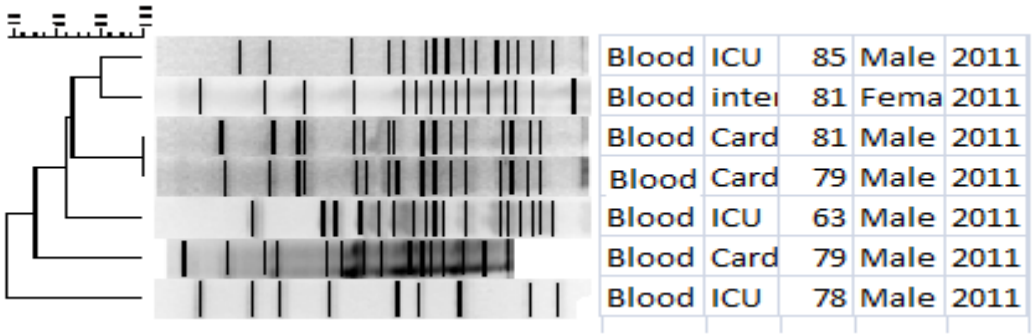
From international prospective, the prevalence of infections in ICUs are higher than other wards, and nosocomial outbreaks are more frequent in ICUs.

Difference of drug resistance in ICU with other wards were expected. Our study showed meaningful difference between drug resistance incidence with other wards. Antibiotic consumption consequently relatively high in ICU. Because of these factors, selection of resistant strains is expected, and hence many antibiotic resistance surveillance studies have been carried out in ICUs only a few studies have compared antibiotic resistance in ICUs and that in other wards (Monnet DL, Archibald L1997). Therefore, longitudinal studies, comparing ICUs with the whole hospital, are of value. For some antibiotic and bacterial species, the resistance levels were high in the ICU s, but even higher in other parts of the hospital. Antibiotic resistance surveillance in the hospital should therefore cover all wards. There were too few isolates from the ICU s to permit analysis.

Pseudomonas aeruginosa is a leading cause of nosocomial infections (National Nosocomial Infections Surveillance2004). Nearly half of the imipenem-resistant *P.aeruginosa* strains were isolated from the ICU s, which contained only 1-5% of the hospital beds. The high prevalence of imipenem-resistant *Pseudomonas aeruginosa* among isolates from the ICUs has been reported by other investigators (National Nosocomial Infections Surveillance 2004). The high ICU resistance rates of 68% were found to be due to an accumulation of isolates from nosocomial outbreaks in the medical/surgical ICU s.

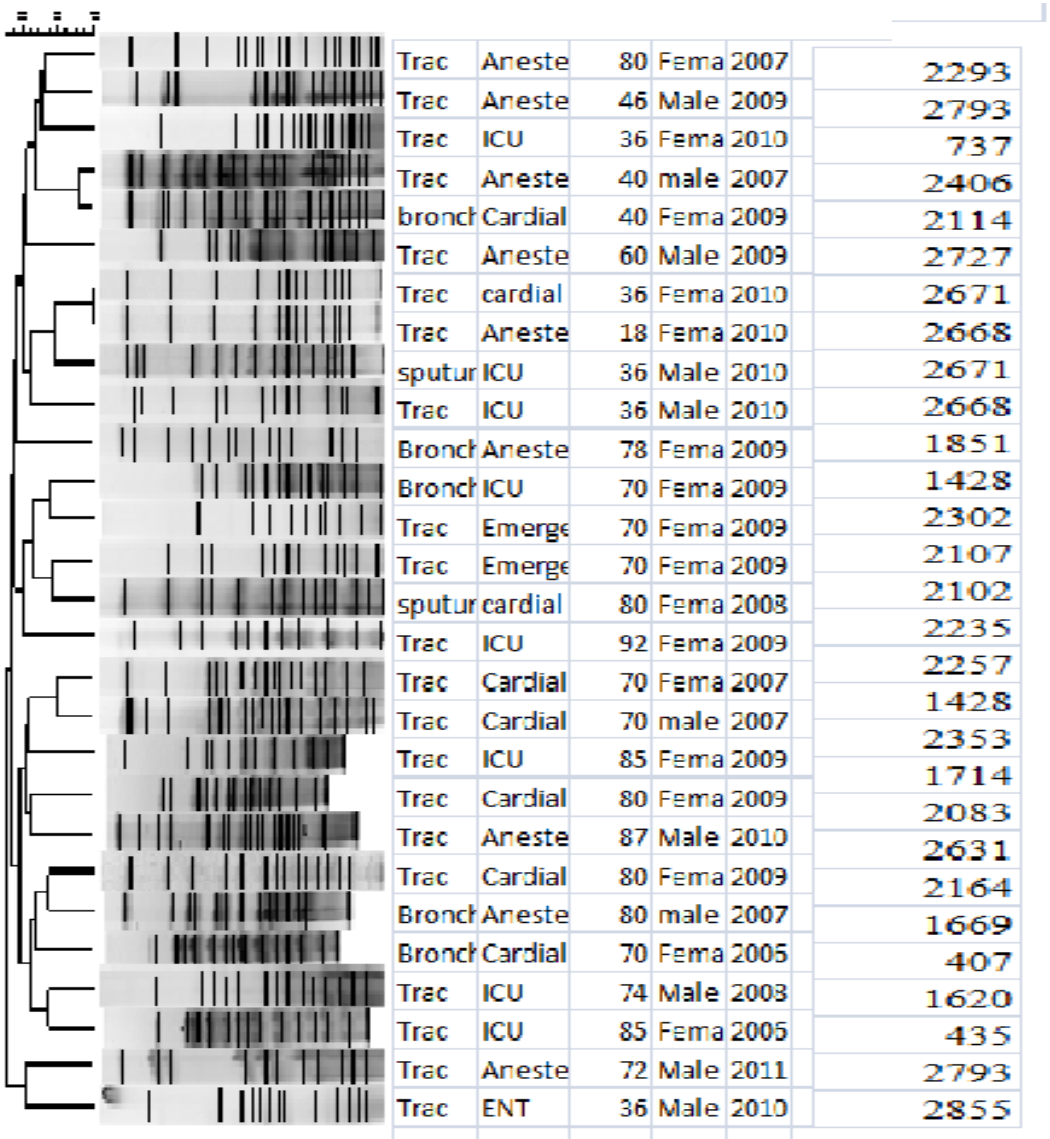
MDR rate from units outside ICUs have been found at rates as high as ICUs.

One interesting thing is that most of MDR (18 of 19) were isolated from patients who had undergone bronchoscopy with the same bronchoscope of the hospital (Figure17).



Blood	ICU	85	Male	2011
Blood	inter	81	Fema	2011
Blood	Card	81	Male	2011
Blood	Card	79	Male	2011
Blood	ICU	63	Male	2011
Blood	Card	79	Male	2011
Blood	ICU	78	Male	2011

Figure 16.PFGE dendogram of 7 P aeruginosa isolated from blood samples



Trac	Aneste	80	Fema	2007	2293
Trac	Aneste	46	Male	2009	2793
Trac	ICU	36	Fema	2010	737
Trac	Aneste	40	male	2007	2406
bronch	Cardial	40	Fema	2009	2114
Trac	Aneste	60	Male	2009	2727
Trac	cardial	36	Fema	2010	2671
Trac	Aneste	18	Fema	2010	2668
sputur	ICU	36	Male	2010	2671
Trac	ICU	36	Male	2010	2668
Bronch	Aneste	78	Fema	2009	1851
Bronch	ICU	70	Fema	2009	1428
Trac	Emerge	70	Fema	2009	2302
Trac	Emerge	70	Fema	2009	2107
sputur	cardial	80	Fema	2008	2102
Trac	ICU	92	Fema	2009	2235
Trac	Cardial	70	Fema	2007	2257
Trac	Cardial	70	male	2007	1428
Trac	ICU	85	Fema	2009	2353
Trac	Cardial	80	Fema	2009	1714
Trac	Aneste	87	Male	2010	2083
Trac	Cardial	80	Fema	2009	2631
Bronch	Aneste	80	male	2007	2164
Bronch	Cardial	70	Fema	2005	1669
Trac	ICU	74	Male	2008	407
Trac	ICU	85	Fema	2005	1620
Trac	Aneste	72	Male	2011	435
Trac	ENT	36	Male	2010	2793
					2855

Figure 17. Dendogram of PFGE of *P aeruginosa* samples isolated from bronchial lavage, sputum, Tracheal aspirate

Generally in most cases *in vitro* results do not overlap with in *vivo*. Isolates may show high sensitivity *in vitro* and resistance to antibiotics *in vivo* due to formation of biofilm and non-penetration of drug from biofilm barrier.

Such controversial cases cannot be investigated in this research due to lack of access to clinic results. Physicians prescribe the dose of drugs they prefer, taking into consideration of the patients' situation and bacterial colonization organ.

Generally forth generations of cephalosporins are the most effective choice drug against *P.aeruginosa.*

Largest outbreaks of P.aeroginosa were observed in 2010 and the smallest in 2011(Table3).
The high dice similarity between isolates of 2006 and those of 2010 was surprising.

The decrease in *Pseudomonas aeruginosa* samples in 2011 must be due to application of modified sterilization methods in the medical equipments as consequence of the transformation of the hospital from private "MESA" to academic "TOBB".

Isolation of similar strains of *Pseudomonas aeruginosa* from pulmonary section were unusual. There must be a common "lost ring" between different patients.

According to some articles bronchoscopes, endoscope could preserve bacteria in the form of biofilm. So we tried to check this hypothesis and our results confirmed the accuracy of our hypothesis. "Phenotypic similarity" of drug resistance to MDR and "Genotypic similarity" pulsed-field gel electrophoresis profile showed us that bacteria can be hidden in bronchoscope, endoscopes in the form of biofilm (Figure 17, Table 10 Appendix B).

Bronchoscopes must be sterilized in a proper way, because bacteria can become resistant to different kinds of antibiotics in biofilms and be detached from bio films and transfer to another persons' body and colonize there. Especially when bacteria become MDR or pan-drug resistant, they may lead to an outbreak in hospital due to

using such equipments (S. Schelenz and G.French 2000, P.Corne2005, R. Bou2006). Similar cases have been reported from other researchers in the world (S. Schelenz and G. French 2000, J.-C.Cetre2005, P. Corne2005, R. Bou2006, Hans-Jürgen Woske2001). Consequently the Olympus Company admitted a deficiency in their products and informed that they have corrected that deficiency in their new models. Their verification need to extra research on them.

We followed "Tenover criteria" for band interpretation. Tenover criteria are globally well-known and applied. It define that difference of 2-3 bound in PFGE define close relatedness with outbreak strain (Table 7).

Table 7.Tenover Criteria for interpreting PFGE results.

Category	No. of genetic differences compared with outbreak strain	Typical no. of fragment difference compared with out break	Epidemiologic pattern interpretation
Indistinquishable	**0**	**0**	**Isolate is part of outbreak**
Closely related	**1**	**2-3**	**Isolate is probably part of the outbreak**
Possibly related	**2**	**4-6**	**Isolate is possibly part of the outbreak**
Different	**>3**	**>7**	**Isolate isnot part of the outbreak**

Similarly 3-4 band difference show possible relatedness, whereas more than 7 band difference show isolate completely different from outbreak strain.(Table 7) special soft-ware has been planned for interpretation of PFGE bands based on Tenover criteria.

This software has become standard for western countries and European countries, where level of hygiene is extremely high and incidence of infection is very low.

We used SpeI restriction enzyme for our PFGE research which breaks *P.aerugiosa* DNA to 20 fragments. it is used routinely in Europe PFGE research, while in former researches XbaI restriction enzyme were used which cut P.aeruginosa DNA to 50 fragments.(Table 9,Table 11 Appenix B).

For differentiation two strains of *Pseudomonas aeruginosa* based on their genome, using Xbal can be more advantageous than SpeI in the same way. We can differentiate them with more resolution.

So generalization of differentiation based on Tenover criteria with less DNA fragments can not have same resolution that it could have with more fragments. So Tenover rules need for modification and updating to new restriction enzymes with less cutting fragments (Table9, 11 Appendix B).

SpeI may be applied in European countries that rate of nosocomial infection; severity of present bacteria is limited; while in Middle East countries using less fragment cutting restriction enzymes cannot offer the same results. Such mistakes have been seen in Malaysia, in Poland and some Arabian countries (King-Ting Lim2009, URSZULA2006) it could have affect our research result,but it didnot.

Observation of same bacteria from different organs of same patient in the same date of sampling alarmed and awoke us from falling in such an error.

Moreover, in USA on other bacteria e.g. *staphylococcus* with similar restriction enzyme they have used above 80% of dice similarity instead of 95% similarity.We did similar action in our research (Linda K. McDougal2010).

According to Tenover criteria above 70 percent similarity must be used for dendogram interpretation. European countries use above 95 percent of similarity for their interpretation. We noticed special patients with two different samples from different site of body (e.g. blood and bronchi) in the same time, despite having same anti-biogram pattern their band were differed from each other. Dice coefficient similarity was less than 95%!

According to commonly used Tenover criteria we must define them two different strains, while it could not be possible. So we standardized it with 80 percent of dice similarity. Moreover we found double samples of a same patient in the same time, according to 95% of similarity these two samples could be determined different genotype, while it could not be correct. Same bacteria isolation from two locations only can be interpreted with decreasing dice similarity and standardization of Tenover to a local cut off value. So we concluded that 80 % of dice similarity is the most proper criteria for defining of Turkey hospitals infections interpretation. These results strongly take interpretation of other researchers under question.

Perhaps they had not faced with two samples of same patient. So they have not noticed to the matter precisely. I think they have investigated only e.g.urine samples and based on European reference articles, they have reported them based on 95% of similarity (King-Ting Lim2009).

Samples of eye, ear section were unique; it was overlapping with other researchers results. However they had investigated "las" gene involvement in their research (Lomholt, Poulson *et al*. 2001)(Figure 18). Wound samples had semi-homogenic dendogram (Figure 19).Three ICU sections patients strains had different pulsed types, while bronchoscopy strains were homogen (Figure 20).

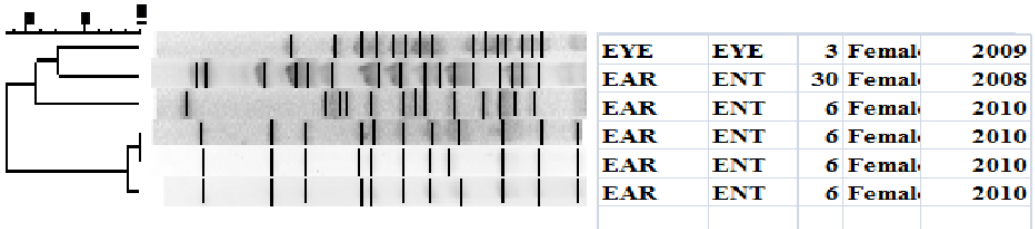
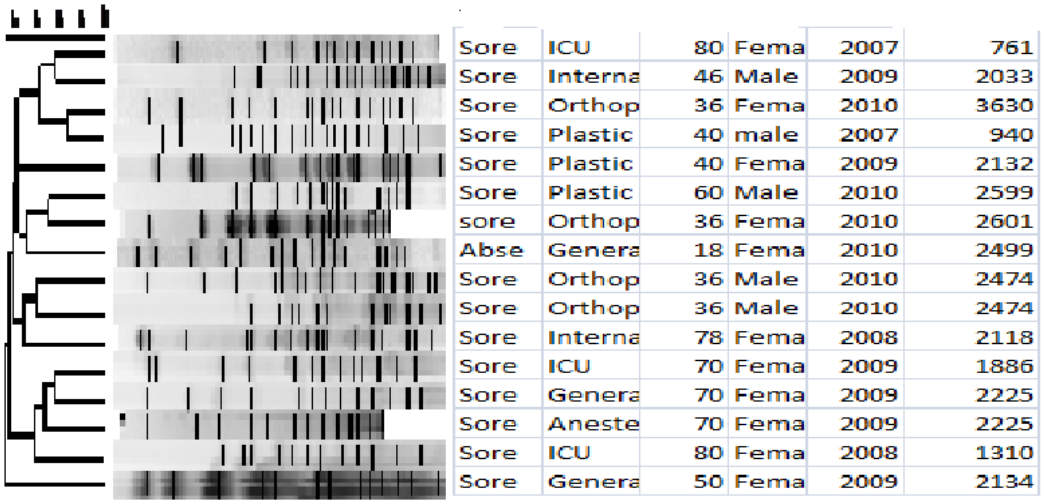
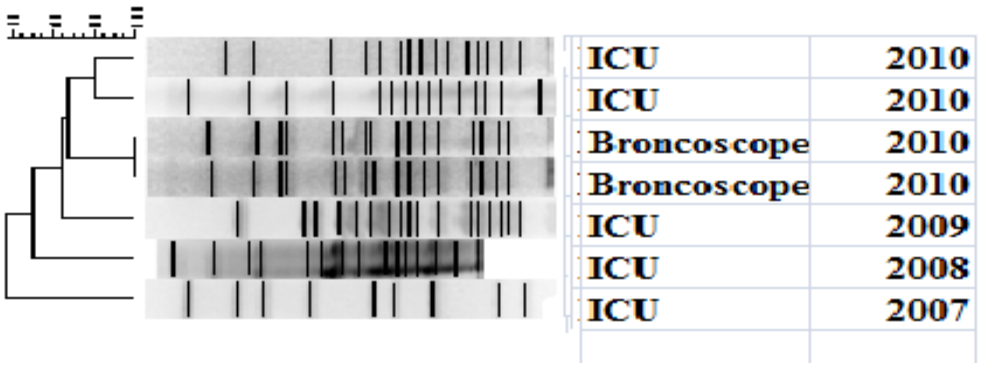


Figure 18, PFGE dendogram of *P.aeroginosa* isolated from Eye Ear section patients



Sore	ICU	80	Fema	2007	761
Sore	Interna	46	Male	2009	2033
Sore	Orthop	36	Fema	2010	3630
Sore	Plastic	40	male	2007	940
Sore	Plastic	40	Fema	2009	2132
Sore	Plastic	60	Male	2010	2599
sore	Orthop	36	Fema	2010	2601
Abse	Genera	18	Fema	2010	2499
Sore	Orthop	36	Male	2010	2474
Sore	Orthop	36	Male	2010	2474
Sore	Interna	78	Fema	2008	2118
Sore	ICU	70	Fema	2009	1886
Sore	Genera	70	Fema	2009	2225
Sore	Aneste	70	Fema	2009	2225
Sore	ICU	80	Fema	2008	1310
Sore	Genera	50	Fema	2009	2134

Figure 19, PFGE dendogram of *P.aeroginosa* isolated from wounds



ICU	2010
ICU	2010
Broncoscope	2010
Broncoscope	2010
ICU	2009
ICU	2008
ICU	2007

Figure 20.PFGE dendogram of *P aeruginosa* isolated from ICU, Broncoscopy

Genetic types were determined based on cluster analysis comparing values of Dice coefficient for PFGE patterns. Two main groups (I–II) at the level of 70% of Dice coefficient were observed (Figure 20).

PFGE typing revealed 25 groups of genotypes (A-Z) containing from 2 to20 isolates

of high similarity according to Dice coefficient values(80–100%) and 7 unique isolates (A1,A2,H,T,,Z,,**β**) (Fig.15,20,21). Among them, genotypes persistently present in a particular ward for some years were detected,e.g. PFGE types A,B,C,D isolated from Cardial Surgery patients undergone broncoscopy, in the hospital from 2006 to 2011 ,as well as strains typical of different wards of the same hospital, e.g. PFGE type U,V,X,Y,μ_1,$\mu_2\mu_3$,β (ICU-Bronchoscopy)and type F,O(Anesthesia-bronchoscopy). It was observed that some PFGE type so occurred simultaneously for a year or longer in same ward of hospital,e.g. PFGE types K and J detected in plastic surgery,2008 in bronchoscopy. Details are presented in (Table10 AppendixB) (Figure 21). Most often the same clones were isolated from two to ten patients, but the different clones in sequential samples from respiratory tract or from two sites of the same patient were found also Strains classified to the same genotypic type were phenotypically similar, i.e. they displayed the same susceptibility to antimicrobial agents and the same type of growth on selective medium as well as they belonged to the same PFGE type. Within the same type up to several resistance profiles, types of growth on selective medium were presented. Detailed data on comparison of phenotypic and genotypic strain features are presented in (Table 10 Appendix B).

Irrespectively of the hospital and/or ward, most of *Pseudomonas aeruginosa* isolates showed much differentiated resistance to antimicrobial agents tested. Different resistance patterns in various arrangements were observed, from sensitivity to all tested antibiotics, through single resistance to carbenicillin to multidrug resistance for almost all tested drugs. All strains were susceptible to colistin. Strains isolated in 2006 and 2007 and 4 from 5 unique types (L, M, N, P) were generally less resistant to chemotherapeutic agents than isolated since the end of 2008 (Figure 21).

Moreover, correlation was found between susceptibility to antibiotics and the type of growth on selective medium.

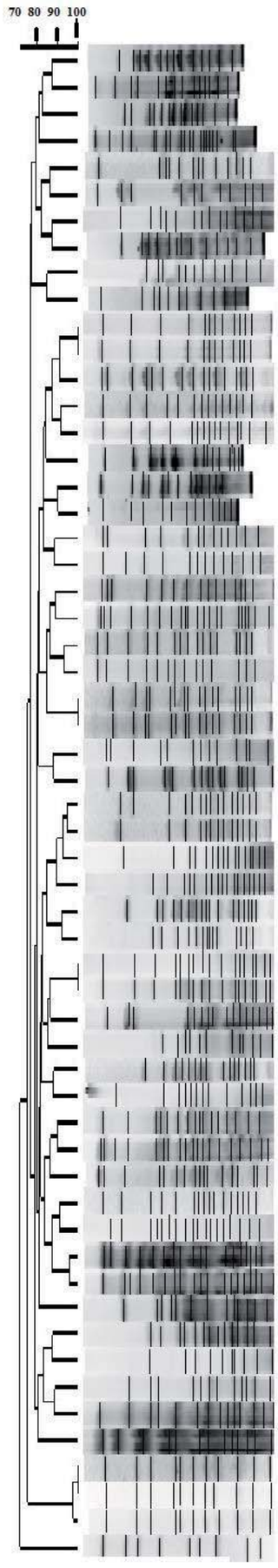
The totals of 58 *Pseudomonas-aeruginosa* strains were tested on selective

cetrimide agar. A green-yellow type of growth appeared most frequently (56 strains98%) where as a blue type was the most rarely found (2%) Details are presented in (Table 10) (Figure21).

We were looking to research on cystic fibrosis patients and investigate existing or non-existing special multidrug resistant equal to LES, MES, AES.....equal involvement in Turkey.

Plenty of multidrug resistance and pan drug resistance were observed.

Normally drug resistance are categorized in 4 level of resistance, sensitive, intermediate While intermediate is considered resistant in our research. Rate of drug resistance of our research overlap with Eastern Europe results (Urszula 2006).



NO	Source Of Sample	Hospital Ward	Age	Sex	Date of Sampling	Hospital	Pigment	Pulse Type	
1	Broncial Lavage	Cardial Surgery	70	Female	2006	MESSA	GREEN	PA1	A
2	Blood	Cardial Surgery	80	Male	2006	MESSA	GREEN	PA2	A
3	Blood	Cardial Surgery	80	Male	2007	MESSA	GREEN	PA3	A1
4	Broncial Lavage	cardial surgery	70	Male	2007	MESSA	GREEN	PA4	A2
5	Broncial Aspirate	Cardial Surgery	80	Female	2010	MESSA	GREEN	PA5	B
6	Broncial Aspirate	Cardial Surgery	87	Male	2010	MESSA	GREEN	PA6	B
7	Thracial Aspirate	Cardial Surgery	78	Male	2010	MESSA	GREEN	PA7	C
8	Thracial Aspirate	Cardial Surgery,Anestezia	91	Male	2010	MESSA	GREEN	PA8	C
9	Thracial Aspirate	Cardial Surgery	80	Female	2009	MESSA	GREEN	PA9	D
10	Blood	Cardial Surgery	79	Male	2009	MESSA	GREEN	PA10	D
11	Thracial Aspirate	Anestezia	63	Male	2010	MESSA	GREEN	PA11	E
12	Thracial Aspirate	Anestezia	84	Male	2010	MESSA	GREEN	PA12	E
13	Bronchial Lavage	Anestezia	80	Male	2010	MESSA	GREEN	PA13	F
14	Bronchial Lavage	Anestezia	84	Male	2010	MESSA	GREEN	PA14	G
15	Thracial Aspirate	Anestezia	90	Male	2010	MESSA	GREEN	PA15	G
16	Blood	Internal	81	Female	2009	MESSA	GREEN	PA16	H
17	SORE	Orthopedia	36	Female	2010	MESSA	GREEN	PA17	I
18	ABSE	GENERAL SURGERY	18	Female	2010	MESSA	GREEN	PA18	I
19	SORE	GENERAL SURGERY	70	Female	2009	MESSA	GREEN	PA19	J
20	SORE	PLASTIC SURGERY	40	Female	2009	MESSA	GREEN	PA20	J
21	SORE	PLASTIC SURGERY	60	Male	2010	MESSA	GREEN	PA21	K
22	SORE	PLASTIC SURGERY	60	Male	2010	MESSA	GREEN	PA22	K
23	SORE	Orthopedia	36	Female	2010	MESSA	GREEN	PA23	L
24	SORE	Orthopedia	36	Male	2010	MESSA	GREEN	PA24	L
25	Thracial Aspirate	ICU	92	Male	2008	MESSA	GREEN	PA25	M
26	Blood	ICU	83	Male	2008	MESSA	GREEN	PA26	N
27	Thracial Aspirate	Cardial surgery	70	Male	2010	MESSA	GREEN	PA27	N
28	Thracial Aspirate	Cardial Surgery	70	male	2010	MESSA	GREEN	PA28	O
29	SORE	Anestezia	70	Female	2009	MESSA	GREEN	PA29	O
30	Broncial Lavage	Anestezia	84	Male	2009	MESSA	GREEN	PA30	O
31	Thracial Aspirate	Anestezia	72	Male	2011	MESSA	GREEN	PA31	O
32	Broncial Aspirate	Anestezia	72	Male	2011	MESSA	GREEN	PA32	O
33	SORE	Orthopedia	36	Male	2010	MESSA	GREEN	PA33	O
34	SORE	Orthopedia	36	Male	2010	MESSA	GREEN	PA34	O
35	Blood	Cardial Surgery	79	Male	2010	MESSA	GREEN	PA35	P
36	SPUTUM	Cardial surgery	80	Male	2010	MESSA	GREEN	PA36	P
37	SORE	GENERAL SURGERY	50	Female	2009	MESSA	GREEN	PA37	Q
38	Thracial Aspirate	Anestezia	72	Male	2011	MESSA	GREEN	PA38	Q
39	SORE	Internal	46	Male	2008	MESSA	GREEN	PA39	R
40	SORE	Internal	78	Female	2008	MESSA	GREEN	PA40	R
41	Thracial Aspirate	EMERGENCY	22	Male	2009	MESSA	GREEN	PA41	S
42	Thracial Aspirate	EMERGENCY	57	MALE	2009	MESSA	GREEN	PA42	S
43	EAR	ENT	30	Female	2008	MESSA	GREEN	PA43	T
44	Thracial Aspirate	ICU	74	Male	2008	MESSA	GREEN	PA44	U
45	SORE	ICU	80	Female	2008	MESSA	GREEN	PA45	U
46	SORE	ICU	70	Female	2009	MESSA	GREEN	PA46	V
47	Sputum	ICU	94	Female	2009	MESSA	GREEN	PA47	V
48	Blood	Cardial Surgery	80	Male	2007	MESSA	GREEN	PA3	W
49	Thracial Aspirate	ICU	85	Female	2006	MESSA	GREEN	PA49	X
50	Thracial Aspirate	ICU	85	Female	2009	MESSA	GREEN	PA50	X
51	SORE	ICU	80	Female	2007	MESSA	GREEN	PA51	Y
52	Thracial Aspirate	ICU	74	Male	2007	MESSA	GREEN	PA52	Y
53	EAR	ENT	6	Female	2010	MESSA	GREEN	PA53	Z
54	Blood	ICU	63	MALE	2011	MESSA	GREEN	PA54	μ_1
55	Broncial Lavage	ICU	73	Female	2009	MESSA	GREEN	PA55	μ_2
56	Broncial Lavage	ICU	73	Female	2009	MESSA	GREEN	PA56	μ_3
57	Blood	ICU	78	Female	2007	MESSA	BLUE	PA57	β

Figure 21. Dendogram of Bacteria genome of full samples

For knowing reason of drug resistance, and location of mutation in strains, it is required for extra research, on mutations on bacteria genome with molecular methods e.g. PCR, DNA sequencing, they were out of our project aim.

All of *Pseudomonas aeruginosa* samples have been confirmed through biochemical methods. Automated equipment has confirmed all 58 samples and their MIC, MBC to antibiotics. Three of samples results which was reported *Pseudomonas aeruginosa*, due to offering confusing response in PFGE, they were re-investigated, We noticed they were *non-aeruginosa pseudomonas* that automated phoenix has failed in correct detection of it. We excluded them from our research. It shows reliance to automatic equipment must not be considered 100%. They recognize samples based on their biochemical tests in shortest time that can be possible.

Automatic machines can be useful for hospitals with plenty of samples, while their sensitivity must be increased with application of professional laboratory specialist.

In Turkey graduates of high schools works as lab technicians that may not be able for differentiate near strains from each other. So using such automatic equipments can be useful, nothing can be alternative for professional expert specialist using in labs.

Despite normalization of bands with standard strain bands, consideration of band up curve or down curve can change results of borderline (80% Dice similarity) so it seems we need for clearer criteria and higher precision software for analyzing of bands. According to these criteria in some cases same patients" multiple samples (*P.aeruginosa* strains) differ from each other despite having similar source and anti-biogram pattern.

DNA band movements in different gel running do not overlap absolutely with other running. However its isolation pattern "style" similarity. Due to such kind of similarity in most cases researchers manually cluster or differentiate them from each other.

Only on clusters with 100% similarity no doubt is existed, in other boarder line similarity cases confusing doubtless govern on analyzing.

However this hospital is not represented of Ankara city, Or Turkey. But results of it show hidden rings in hygiene chain of health centers and hospitals. We must not ignore from such facts and their proper sterilization for preventing subsequent events.

The hospital environment remarkably promotes selection and quick distribution of resistant strains (Dzier¿anowska, 1997; Giedrys-Kalemba, 2000; Hanberger *et al*., 2004; Jaworski *et al*, 1993; Eopaciuk, 1996; Sader *et al*., 2004) one of the essential steps leading to a reduction of nosocomial infections is a constant monitoring of etiological agents and resistance of intra-hospital strains. It is of crucial importance to carry out epidemiological surveys including a detailed characteristic and relationship among strains isolated in particular environment and time, as well as to become aware of risk factors, sources and ways of infection distribution (Czekajo-Koodziej, 2001; Eopaciuk, 1996). To obtain reliable results, especially in case of isolates without characteristic phenotypic markers, application of molecular methods seems to be non-inevitably.

To differentiate precisely among particular *P aeruginosa* strains isolated from different sections of hospitals in the north-west region of ANKARA, PFGE typing was carried out. However, due to the large number of strains and their different origin, the classification of strains/genetic patterns was conducted at the level of 80% and more of Dice coefficient (Struelens *et al*, 1993). Dendrogram analysis enabled to divide strains into main groups (70%of similarity), then subgroups (80%), genotypes (70–80 %) and subtypes (81–100%).PFGE typing revealed, presence of 25 PFGE types of *P.aeruginosa*.

A high number of PFGE types pointed to mark intra-hospital differentiation of *Paeruginosa* strains that are widely distributed in nature, especially in humid environments. It indicated various sources of strains and their constant exchange, also with the same patients. Such strains were generally highly resistant to

antibiotics what confirmed the development of secondary resistance and their intra-hospital selection. At the same times strains of unique fingerprints, frequently expressing higher susceptibility to chemotherapeutic agents, were isolated. It gave evidence of the temporary incidence of new endogenous strains entering the hospital environment.

Some of the genetic types expressing the same/similar of PFGE pattern were numerically dominant within the ward(s) for some months/years. It might prove horizontal transmission of clones or clonal-related groups and epidemic/endemic character of registered infections (Fiett *et al* 1998). The incidence of the same genetic types of *Pseudomonas-.aeruginosa* in different hospital wards drew attention to a possibility of a long-distance strain transmission. It might be linked to the movement of patients; visitors, medical and paramedical staff, or commonly use of similar equipment in related hospital sections.

Based on dates of strains isolation and their resistance to antibiotics, it is highly probable that selection of highly resistant isolates takes place in ICU s, where *Pseudomonas–aeruginosa* is one of the most frequent and severe cause of infections, especially in patients with mechanical ventilator. In the absence of epidemic clones, secondary resistance development during combined antibacterial therapy appeared to be the main factor contributing to the prevalence of resistance in ICU, what was observed in sequential samples from the same patient preliminary criterion indicating the incidence of a potential intra-hospital strain and signaling the necessity of conducting further investigations.

Results of PFGE typing revealed, between strain fingerprints and their phenotypic features overlap existence. A majority of *Pseudomonas-aeruginosa* strains presented a high differentiation of phenotypic patterns within genotype. It confirmed existence of correlation between molecular and conventional typing, e.g. types of growth on Cetrimide Agar, susceptibility/resistance pattern to antimicrobial agents. Similar results also with the other genera of microorganisms were proved (Bouza *et al*., 1999; Fierobe *et al*, 2001; Dinesh *et al*.2003; Giedrys-Kalemba *et al*., 2001). The lack of strong correlation between *Pseudomonas-aeruginosa* PFGE types and their phenotypic features indicates that phenotypic analysis should not be exclusive method of evaluating of strain relationship and

conducting epidemiological investigations of nosocomial infections. It is necessary to carry out analysis at the molecular level (Cavallo *et al*. 2000; Gomez *et al* 2000).

However, phenotypic studies are a valuable tool supplementing genotyping as they enable tracing of phenotypic feature expression influenced by different environmental conditions (Biendo *et al*. 1999).

This thesis describes outbreaks of BAL, Ear, and Eye specimen contamination, occurring among 58 patients, due to Possibility of infective bronchoscopes, ottoscopes, and other hospital equipments. Due to non-existing former report from such infection, we consider this as outbreak of nosocomial infection through bronchoscope. In this report we discuss in detail the strengths and weaknesses of our study followed by some recommendations. "The large number of patients involved in the outbreaks gave us an opportunity to describe, with enough power and detail, individuals for whom a clear association was established between bacteria and bronchoscopes, ottoscopes present. To the best of our knowledge, a similar sample size has been reported only for outbreaks that occurred in Baltimore (Johns Hopkins University) (Srinivasan A 2003) also France (Kirschke DL2003).

Ccntamination of BAL due to bacteria trapped in the bronchoscope may occur by two mechanisms. On the one hand, the liquid may be contaminated as it is injected into the biopsy channel and can, thus, contaminate the lungs (true contamination) alternatively, the liquid may be contaminated only after re-aspiration through the suction channel.

In the latter case, the BAL findings are considered to be false positives. In a study of France researchers (cetre *et al*) two bronchoscopes were available but one was contaminated bronchoscopes were used more often as they were video endoscopes. It seems unlikely that a deficiency in the disinfection procedure was responsible for the contamination. However, the specific characteristics of these two bronchoscopes prevented the disinfectant from reaching the contaminated area. The plastic cap of the biopsy channel port housing became loose and was probably trapping bacteria in an area in-accessible to the normal disinfection process. This plastic cap was

subsequently removed and replaced with a more secure, stainless steel cap. Delays in identification and notification of such problems may also be prevented by more frequent dialogue between manufacturers and professionals.

Some lessons can be learned from these outbreaks. The endoscopy personnel adhered closely to published guidelines, except that the bronchoscopes were stored flat rather than hanging, (Mehta A1999, Honeybourne D 1997) and this was confirmed by audits. (Honeybourne D 1997).

However, we observed material deficiencies which highlight the need to test bronchoscopic samples regularly. Leak testing systematically performed before each dis-infection and may have allowed much earlier detection of the problem.

We underscore the importance of vigilance and follow-up of deficiencies at international level as discussed by Culver *et al* (Culver DA 2003).The publication of the alert in the USA shows the need for communication between professionals. We emphasize the necessity of installing software for the detection of outbreaks at laboratory level. At Johns Hopkins University (Srinivasan A 2003) as in one institution, dozens of cases and several weeks were needed before the outbreak could be detected. Manufacturers and healthcare professionals should collaborate and communicate not only regarding equipment maintenance, but also to improve the design and structure of bronchoscopes.

This will prevent the existence of sites, not accessible to cleaning or disinfection which facilitates bacterial growth"(Ahmad M, 1999).

Because of its high discriminatory power, "PFGE is a valuable tool for investigating outbreaks of *Pseudomonas aeruginosa* infections, particularly in hospital settings PFGE has also been used to discriminate among community and health care-acquired *Pseudomonas* strains.

In the past, the sharing of PFGE data among laboratories was difficult, and typing results for the same strains performed in different laboratories often lacked

concordance. However, recent advances in gel analysis software programs allow the creation and storage of large databases of normalized fragment patterns in which similarity calculations and cluster analyses can be performed with relative ease. Normalization of the fragment patterns using established standards (such as *Pseudomonas aeruginosa*) and the advent of new database sharing tools both serve to facilitate the exchange of PFGE strain typing data and epidemiological information among reference laboratories, even in different countries. Thus, the traditional barriers to the sharing of PFGE patterns, even those run using different switching parameters, have to a large extent been overcome with powerful new software programs. The development of a surveillance system that can integrate these databases and make the information available to other reference centers is critical.

Some studies have shown that PFGE can identify stable lineages of *Pseudomonas aeruginosa* PDR and can be used to track the spread of these lineages from continent to continent over extended periods of time".

From international prospectives, "the prevalence of infections in ICUs are higher than other wards, and nosocomial outbreaks are more frequent in ICU(Figure22).
Difference of drug resistance in ICU with other wards were expected. our study showed meaningful difference between drug resistance incidence with other wards ($p<0.05$)".

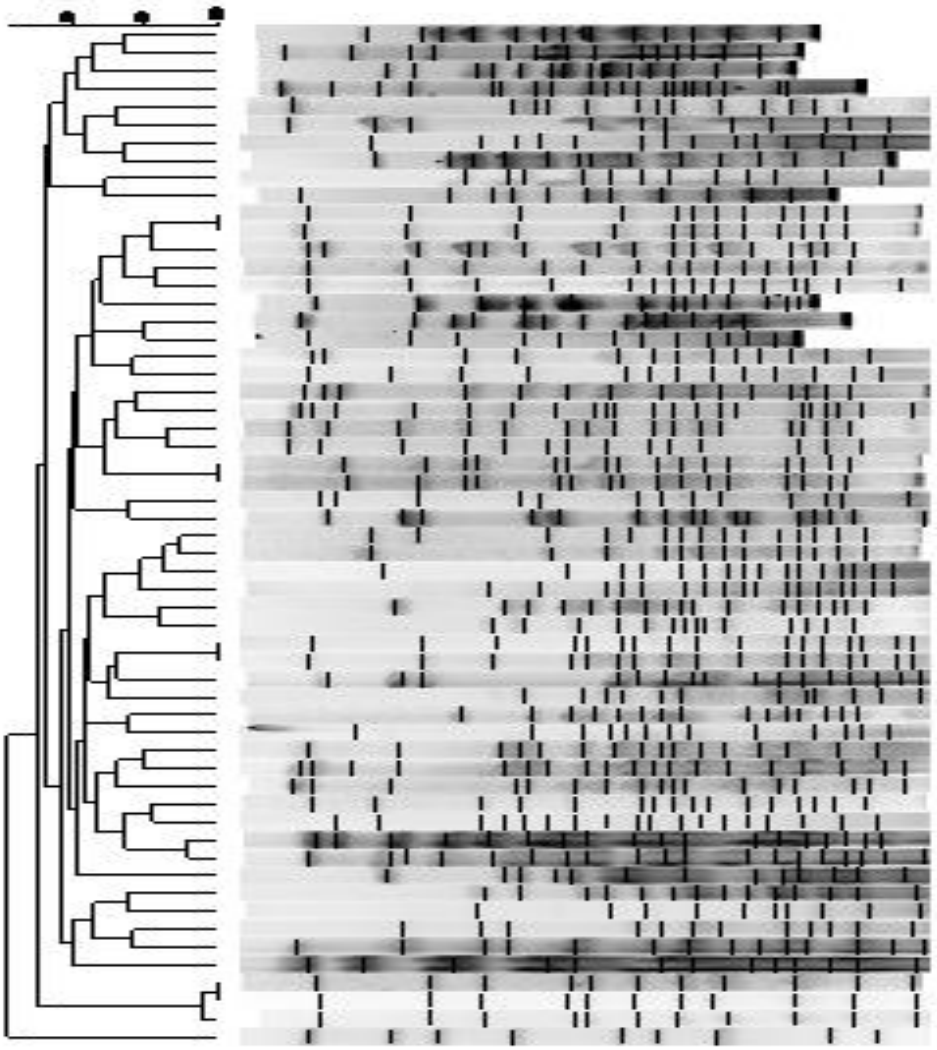


Figure22. PFGE dendogram based on genotype of *Pseudomonas aeruginosa*

Antibiotic consumption consequently relatively high in ICU. Because of these factors, selection of resistant strains is expected, and hence many antibiotic resistance surveillance studies have been carried out in ICUs only (Fraenkel CJ).Only a few studies have compared antibiotic resistance in ICUs and that in other wards (Monnet DL, Archibald L).

Therefore, longitudinal studies, comparing ICUs with the whole hospital, are of value. For some antibiotic and bacterial species, the resistance levels were high in the ICUs, but even higher in other parts of the hospital. Antibiotic resistance surveillance in the hospital should therefore cover all wards. there were too few isolates from the ICUs to permit analysis. The number of cultures taken was too low to provide data on species distribution and susceptibility (Erlandsson M). The findings of less antimicrobial resistance for some antibiotic and bacterial combinations in the ICUs compared to the whole hospital might have several explanations. some ICUs are characterized by short stays, and a majority of the cases are hospitalized directly after emergency admittance.

Isolation care in general is essential to prevent nosocomial transmission of infection and colonization with resistant bacteria, and detailed guidelines are available. For the nursing of potentially contagious cases, the importance of good-quality barrier nursing (Hartstein AI) preferably in single rooms (Jernigan JA) has been well documented as a means to avoid the spread of resistant organisms. Cross-transmission of multi-resistant micro organisms is common, particularly in ICUs (Chetchotisakd P) If transmission occurs, this is an indicator of poor quality of nursing care, nursing overload and crowding being most important (Kibbler CC).

The ICU patients also seem to be well protected from transmission of infection/colonization by good barrier nursing, except during periods of over crowding and under staffing. Many published studies on antibiotic resistance cover only shorter time periods (Fraenkel CJ, Archibald L, Burwen DR Sörensen TL). We found large fluctuations over time in our study, indicating that it is important to perform antibiotic resistance surveillance studies over longer time periods. This is especially important for ICUs, where the fluctuations seem to be more pronounced. These fluctuations might be explained partly as a result of outbreaks of nosocomial infections. The two outbreaks might be explained partly as a result of outbreaks of nosocomial infections.

The two outbreaks overcrowding and under staffing at the Hospital. Moreover, previous publications have given the results of multi center studies, with the data

pooled from different centers (Fraenkel CJ, Livermore DM, Burwen DR, Sörensen TL) Other studies have indicated that a detailed analysis of the source of the data might provide a more differentiated view of resistance emergence (Archibald L): registered data of MDR from units outside ICUs have been found at rates as high as ICUs (Trick WE, Loeb MB). Our data concerning ICU in relation to the whole hospital support this view. Further analysis of antibiotic resistance in ICUs should be performed at the MESA Hospital in order to answer these questions.

Pseudomonas aeruginosa is a leading cause of nosocomial infections (National Nosocomial Infections Surveillance (NNIS) System Report).Nearly half of the imipenem - resistant *P.aeruginosa* strains were isolated from the ICUs, which contained only 5% of the hospital beds.

The high prevalence of imipenem - resistant *P. aeruginosa* among isolates from the ICUs has been reported by other investigators ([National Nosocomial Infections Surveillance (NNIS) System Report). The high ICU resistance rates of 68% were found to be due to an accumulation of isolates from nosocomial outbreaks in the medical/surgical ICUs.

CHAPTER 4

CONCLUSION AND SUGESSTIONS

This thesis describes outbreaks of BAL, ICU, Blood, wound, Ear, and Eye specimen contamination with *P. aeruginosa*, occurring among 58 patients, due to Possibility of infective bronchoscopes, ottoscopes, and other hospital equipments.

In this report we discuss in detail the strengths and weaknesses of our study followed by some recommendations.

Nosocomial infections are the most common hospital problems around the world. The most developed countries pay great amount of their budgets for minimizing its harmful effects to society. *Pseudomonas aeruginosa* is a common cause of nosocomial infections, particularly in intensive care units (ICUs), bronchoscope, oncology, and urology.

"P. aeruginosa represents a major cause of morbidity and mortality in both the hospital and community settings.

The importance of the organism is increasing due to increasing immunocompermise patients and resistance to full antimicrobials due to bearing efflux pumps genome by this bacteria.

The hospital environment remarkably promotes selection and quick distribution of resistant strains of *Pseudomonas aeruginosa*.

The study presented in this thesis represent a thorough genomic investigation of hospital acquired *P. aeruginosa* strains associated with human infection in the most developed, hygiene, high quality modern, MESA private hospital of Ankara Turkey.

-So these results reflect minimal percent of nosocomial infection in Ankara. It represents the first work comparing multiple drug resistant and multiple isolates of *P. aeruginosa* from diverse clinical sources in a private hospital through molecular "PFGE" method.

For determination the sources of *Pseudomonas aeruginosa* infection in this hospital, We did retrospective study and analyzed genotypically a collection of 58 clinical isolates recovered during the period 2006–2011 from MESA private hospital microbiology department's bacterial archive, PFGE was carried out at the Rafik Saydam National Hygiene Center.

The aim of this study was to characterize *P. aeruginosa* clinical isolates clonally relatedness by pulsed-field gel electrophoresis (PFGE) typing and it's cut off value determination.

-We profited from isolates' drug resistance pattern as control phenotypic character , "similar to puzzle particles assortment", consequently determined correct cut off value, interpreted observed "A-Z" PFGE pulsed types and introduced true relationships between strains based on drawn dendograms.

-According to similar published articles, we firstly carried out PFGE with SpeI restriction enzyme and considered above 95% of dice similarity "cut off value". We got contradict results in interpreting our dendogram and strains relationship.

-According to Tenover criteria, above 70 percent similarity must be used for dendogram interpretation. European countries use above 95 percent of similarity for their interpretation. We noticed special patients with two different samples from different site of body (e.g. blood and bronchi) in the same time, despite having same anti-biogram pattern their Pulsed types were differed from each other!!! Dice coefficient similarity must be considered less than 95%!

-According to commonly used Tenover criteria we must define them two different strains, while it could not be correct. So we standardized it with 80 percent of dice similarity.

-Observation of same bacteria from different organs of same patients in the same date of sampling alarmed and awoke us from falling in such an error.
-So we concluded that 80 % of dice similarity is the most proper cut off value for defining of Turkey hospitals infections interpretation. These innovative results strongly take interpretation of other researchers under question.

-We used Spe1 restriction enzyme for our PFGE research which breaks DNA to 20 fragments. It is widely used in nowadays *P-aeruginosa* PFGE researches in the world, while in former researches XbaI restriction enzyme were used, which cut DNA to 50 fragments.

-PFGE cut off value with SpeI restriction enzyme must be different from XbaI due to changing of scale.
-Bionumerics (version 6) which has been set up to Xbal restriction enzyme offer contradict results to SpeI. It should be up to dated and corrected in version 7.

-Pulsed-field gel electrophoresis with SpeI-digested genomic DNA resulted in 53 different genomic profiles. "With upper 95 % dice similarity cut off", while with decreasing cut off value to upper 80 % dice similarity strains accumulated in 25 clusters, "A-Z", each one representing different wards of hospital and nine individuals.
-This cut off value fully described same patients' different samples "*Pseudomonas* placement in a same cluster correctly, while former cut off value interpret them with contradiction in separate genotypes.
-Multi drug resistant strains showed to be genetically identical origin.
-PFGE demonstrated the existence of a common two clones in a (ICU) critical care area and bronchoscope unites, we defined it bronchoscope related infection.

-The large number of patients involved in the outbreaks gave us an opportunity to describe, with enough power and detail, individuals for whom a clear association was established between bacteria and bronchoscopes, ottoscopes present. To the best of our knowledge, a similar sample size has been reported only for outbreaks that

occurred in Baltimore (Johns Hopkins University) (Srinivasan A 2003) also France (Kirschke DL 2003) contamination of BAL due to bacteria trapped in the bronchoscope may occur by two mechanisms. On the one hand, Bacteria can be colonized in bronchoscope tube and secrete biofilm around itself for protection from environmental effects, preserve for long time there and detach and distribute to other immunocompermise patients with subsequent bronchoscopy alternatively, biofilm particles containing bacteria can be transferred by forceps and bronchoscope.

-According to some articles bronchoscopes, endoscope could preserve bacteria in the form of biofilm. So we tried to check this hypothesis and our results confirmed the accuracy of our hypothesis. "Phenotypic similarity" of drug resistance to MDR and "Genotypic similarity" pulsed-field gel electrophoresis profile showed us that bacteria can be hidden in bronchoscope, endoscopes in the form of biofilm.

-Bronchoscopes must be sterilized in a proper way, because bacteria can become resistant to different kinds of antibiotics in biofilms and be detached from biofilms and transfer to another persons' body and colonize there with subsequent broncos copying. Especially when bacteria become MDR or pan-drug resistant, they may lead to a deadly outbreak in hospital due to using such equipments (S. Schelenz and G.French 2000, P.Corne2005, R. Bou2006). Similar cases have been reported from other researchers in the world (S. Schelenz and G. French 2000, J.C.Cetre2005, P. Corne2005, R. Bou2006, Hans-Jürgen Woske2001). Consequently the Olympus Company admitted a deficiency in their products and informed that they have corrected that deficiency in their new models. Their verification need to extra research on them.I recommend health Ministry for withdrawing, replacing olds models with new models in full Turkey hospitals and training hospitals with correct ways for sterilizing of biofilms.

-Due to non-existing former report from such infection, we consider this as outbreak of nosocomial infection through bronchoscope.

-Despite not existing horizontal transferring from patient to patient due to high level of hygiene plans of this hospital, isolating similar strain (*Pseudomonas aeruginosa*) between years 2006-2010 maybe due to biofilm formation and non-correct sterilization of medical equipments, e.g. bronchoscope, Oxygen mask, laryngoscope.

-These means may be sources of infection in this hospital. Reinforcement of infection control measurement is needed to avoid iatrogenic and horizontal transmission and severe infections.

-This is the first study on the molecular characterization of *Pseudomonas aeruginosa* isolates in such a wide level in a private hospital in Turkey. Morphological typing method based on anti-biotic Resistance method fully overlapped with PFGE method and confirmed our results in higher level of sensitivity and reliance.

-The majority of laboratory-confirmed cases n=22; (40%) were seen in 2010 and n=14 (27%) in 2009, they showed outbreak of infection in these two years and subsequently decreased to its minimum amount in 2011 due to application correct sterilization method on bronchoscope.

-Most of *Pseudomonas aeruginosa* have been isolated from operating rooms, Pulmonary (Bronchoscopy, Tracheal), ICU, the least amount from ENT (Ear, Nose, Throat) and Ophthalmology (eye) sections ($p<0.05$).

-Operating room was the most riskful place for infection; Bronchoscopy, ICU, ENT was in the subsequent proportions.

-Resistances to carbapenems (Ertapenem, imipenem, meropenem) were high, equal to that of Eastern European countries (Urszula 2006).
-Colistin, azetronam Cefepim, cefoxitin, ceftazidim were the most effective drugs against *Pseudomonas aeruginosa*, resistance rate to chloramphenicol (60 %), gentamycine (55 %), imipenem (11 %), meropenem (39 %), pipracilin (31 %), ciprofloxacilin (10 %), ceftazidim (23 %), cefepim (6 %), cefoxitin (16 %), tobramycin (25 %), and aztreonam (10 %) was observed.
-There are some reports from involvement of genetically indistinquishable *Pseudomonas* (non- *aeruginosa*) involvement in hospital infections, we found three of such cases, that were belonged to P*seudomonas pseudomalei*, they had been

recognized *Pseudomonas aeruginosa,*secluded it from our study. So it is recommended for precision investigation of *Pseudomonas* named isolates in hospitals.

-There is five globally known efflux pumps phenotypes and genotypes that cause (MDR,PDR) pan drug resistance in hospital settings and cause high mortality and morbidity rate in hospitals,however such phenotype circulation was not observed in MESA hospital but we recommend for searching existance of such genotypes in all turkey hospitals and their DNA sequencing ,using Efflux pump disruports drug for them, our studied cases resistance were due to biofilm formation and other resistance mechanisms (MexXY-OprM, MexAB-OprM, MexCD-OprJ, Mex E,F).

-The hospital environment remarkably promotes selection and quick distribution of resistant strains (Dzier¿anowska, 1997; Giedrys-Kalemba, 2000; Hanberger *et al*., 2004; Jaworski *et al*, 1993; Eopaciuk, 1996; Sader *et al*., 2004).One of the essential steps leading to a reduction of nosocomial infections is a constant monitoring of etiological agents and resistance of intra-hospital strains. It is of crucial importance to carry out epidemiological surveys including a detailed characteristic and relationship among strains isolated in particular environment and time, as well as to become aware of risk factors, sources and ways of infection distribution (Czekajo-Koodziej, 2001; Eopaciuk, 1996). To obtain reliable results, especially in case of isolates without characteristic phenotypic markers, application of molecular methods seems to be inevitable.

- MDR rate from units outside ICUs have been found at lower rates than ICUs.
One interesting thing is that most of MDR (18 of 19) were isolated from patients who had undergone bronchoscopy with the same bronchoscope of the hospital.

-From international prospectives, the prevalence of infections in ICUs are higher than other wards, and nosocomial outbreaks are more frequent in ICU .While in our study broncoscopy and operating room had higher prevalence to infection to this bacteria than ICU.

-Based on Nosocomial Infections Surveillance (NNIS) System Reports, Nearly half

of the imipenem - resistant *P.aeruginosa* strains were isolated from the ICUs, which contained only 5% of the hospital beds. Our results fully confirmed above report.

-The high prevalence of imipenem - resistant *P. aeruginosa* among isolates from the ICUs has been reported by other investigators ([National Nosocomial Infections Surveillance (NNIS) System Report). The high ICU resistance rates of 68% were found to be due to an accumulation of isolates from nosocomial outbreaks in the medical/surgical ICUs.

- The ICU patients also seem to be well protected from transmission of infection/colonization by good barrier nursing, except during periods of over crowding and under staffing.

- The decrease in *Pseudomonas aeruginosa* samples in 2011 must be due to application of modified sterilization methods in the medical equipments as consequence of the transformation of the hospital from private "MESA" to academic "TOBB".
- Samples of eye, ear section were unique; it was overlapping with other researchers results. However they had investigated "las" gene involvement in their research.

It seems special strains of *P.aeruginosa* has tropism to special organ, we could not research on it, due to financial limitations.

- We were looking to research on cystic fibrosis patients and investigate existing or non-existing special multidrug resistant equal to LES, MES, AES.....equal involvement in Turkey, due to non-access to cystic fibrosis patient samples, non-cooperating hacettepe university cystic fibrosis and microbiology section with us we changed our research route to nosocomial infection.
- For knowing origin of drug resistance, and location of mutation, it is required for extra research, on mutations on bacteria genome with molecular methods e.g. PCR, DNA sequencing, they were out of our project aims, we may carry out it in post doc program.

Moreover,Standardization of PFGE cut off value with SpeI restriction Enzyme to above 80% of dice coefficient similarity and substituting this cut off to formerly believed upper 95% of dice coefficient similarity, correcting of Tenover criteria to cases that use similar restriction enzymes with lesser cutting fragments was our innovation of this thesis

We suggest other researchers attention to this matter and re-consideration of their results with lower cut off value.Also doing complementary researches with using XbaI, SpeI on same strains with same soft ware for determination our observation in a scientific way.

Due to spread of MDR, PDR strains through bronchoscopes and otoscopes, We recommend for their proper sterilizations. Also for trapping pan drug resistance *Pseudomonas aerognosa* in hospitals, close cooperation of all health and hygiene system is required.

Due to non-access to patient information of GAZI hospital and pan-drug resistance in our received samples, I suggest some student carry out similar research in their similar sections with Precision information and sampling in future.

Most of similar global research are focused on cystic fibrosis patients, and gene expression, mutation, molecular mechanisms of antibiotic resistant, gene discovery of such phenomenons. They have reported associations between disease type and T3SS virulence gene prevalence. or increased prevalence of *exoU gene* from intensive therapy units.

Our results confirmed that similar genotype, phenotype of *Pseudomonas aeruginosa* involved in ear and ocular infections.

Drawn dendogram, proved similar strains of *Pseudomonas aeruginosa* cause infection in skin wounds and ocular infection. our results overlap with other researcher results.(Lomholt, Poulson *et al*. 2001) however they have reported role of genes lasA, *lasB*, *apr*, *exoS*, *exoT*, *exoU*, but not *exoY*. In their report. This study

could not investigate such details due to above mentioned reason, so it is recommended investigation of such genes involvement or non-involvement in future researches.

Also some strains involvement in bronchial aspirate, bronchial lavage, that were used with same bronchoscope in the same field of time prove bronchoscope mediated nosocomial infection.

With consideration role of biofilm formation in such equipments, it warn alarm for sterilization of such equipments with biofilm removing detergent solutions.

Old model of bronchoscope of olympus company has been reported for their eligibility for forming bacterial biofilm. It seems they have removed or decreased this deficiency in their new models. So it is recommended for using new model of bronchoscope for hospital relevant sections or proper sterilization solutions or methods with anti-biofilm effects on them.

Nowadays, Quorum sensing has attracted interest of plenty of researchers for discovering its role in drug resistant genes "horizontal gene transferring phenomenon"it seems it trigger some genes, switch on and make some bacteria eligible "Competent" for getting foreign genes. their investigation need for development of experimental biofilm models using and development. some of Eukaryota models (eg Amoeba), researchers have developed Eukaryote Amoeba, derosophila, *D. Discoideum, E. elegans* models for such kind of investigations. using of such kind of models and such phenomens investigation is strongly recommended for future researches.

Due to effective and preventive role of "Quorum sensing disruptor","Biofilm disruptor"materials in development of new generation of drugs, cosmetic, hygiene solution, it is strongly recommended for using such compounds in such drugs in future and fulfilling relevant research and development on them.

DNA finger printing of P*seudomonas aeruginosa* isolated from hospital sections and biofilm embded multidrug resistant,"pan drug resistant"*Pseudomonas aeruginosa* clone epidemy has been presented in 3rd Iranian graduate congress, it will be published as article in "Hospital infection journal" in near future. They are first reports from Ankara hospital by this sensitive molecular method.

A number of conclusions have been drawn from other studies regarding the prevalence of specific virulence factor genes and the type of infection that may be caused by *Pseudomona aeruginosa*, as well as the degree of global conservation of virulence genes in the *P. aeruginosa* genome. A number of these studies have investigated potential associations between the presences of known virulence Factor genes and differing types of infection. Other studies have identified a specific link between possession of *exoU* and resistance to the fluoroquinolone antimicrobial agent, ciprofloxacin" (Wong-Beringer, Wiener-Kronish *et al*. 2007).

Some study have found *exoT* to be universally present, with variable prevalence of other T3SS exo-enzyme genes. It also identified a decreased prevalence of *exoY* in urinary isolates and *exoU* in CF isolates. CF isolates of *P. aeruginosa* were found to have a higher prevalence of *exoS* in comparison to isolates from other sources. No other variations in T3SS exo-enzyme gene prevalence were noted (Feltman, Schulert *et al*. 2001) difference between level of research of nosocomial infections between developed countries and developing countries can be shown in such kind of results.

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APPENDIX A

SOLUTIONS, CHEMICALS, MATERIALS, CULTUR MEDIA

A1- Solutions

General solutions

EDTA (0,5 M Stock, pH 8,0)

EDTA 186,1 g

Make up to 1 L with distilled H2O

Correct to pH 8,0 with Sodium hydroxide pellets

Autoclave at 121ºC for 20 minutes

Ethidium Bromide

1 tablet ethidium bromide

Sterile distilled H2O 10 mL

Wrap container in aluminium foil to protect from light.

Phosphate Buffered Saline

Phosphate buffered saline (Dulbecco A) tablets 1 tablet

Make up to 100 mL with distilled H2O

Autoclave at 121ºC for 20 minutes

Proteinase K solution

Proteinase K powder 0.200 g

Make up to 10 mL with distilled H2O

Ensure powder has completely dissolved in distilled H2O. Dispense 160 µL aliquots in 0,6 mL microfuge tubes and store at -20ºC.

0.85% Saline Solution

Sodium Chloride 8.5 g

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

20 % Sodium Dodecyl Sulfate

SDS 100 g

Make up to 500 mL with distilled H2O

Autoclave at 121ºC for 20 minutes

10% Sarcosyl

N-lauryl sarcosine 100 g

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

50x Tris Acetate EDTA (stock solution)

Tris 242 g

0.5 M EDTA (pH 8,0) 100 mL

Glacial acetic acid 57,1 mL

Make up to 1 L with Distilled H2O

Autoclave at 121ºC for 20 minutes

1 x Tris Acetate EDTA (working solution)

50x Tris acetate EDTA stock solution 20 mL

Make up to 1 L with Distilled H2O

Tris Chloride (2,0 M Stock, pH 8.0)

Tris 242,2 g

Conc. Hydrochloric acid 100 mL

Correct to pH 8,0 with Conc. Hydrochloric acid

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

Cell Suspension Buffer

Tris chloride (2,0 M Stock, pH 8,0) 50 mL

0.5M EDTA (pH 8,0) 200 mL

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

5 x Tris Borate EDTA (stock solution)

Tris 54 g

Boric acid 27,5 g

EDTA (0,5 M Stock, pH 8,0) 20 mL

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

0.5 x Tris Borate EDTA (working solution)

5 x Tris borate EDTA (stock solution) 250 mL

Make up to 2,5 L with distilled H2O

Tris-EDTA Buffer

Tris chloride (2,0 M Stock, pH 8.0) 100 mL

EDTA (0,5 M Stock, pH 8.0) 100 mL

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

Cell Lysis Buffer

Tris chloride (2,0 M Stock, pH 8,0) 25 mL

EDTA (0,5 M Stock, pH 8,0) 100 mL

10% Sarcosyl 100 mL

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

Proteinase K solution 2,5 mL

Add proteinase K solution immediately prior to use

1x Blocking Solution

10 x Easy-hyb blocking solution 10 mL

Maleic acid buffer 90 mL

Colour Substrate Solution (per 100 cm2 of nylon membrane used)

Easy-hyb NBT/BCIP stock solution 200 μL

Detection buffer 10 mL

Detection Buffer

Tris 6.057 g

Sodium chloride 2.922 g

Make up to 400 mL with distilled H2O

Correct to pH 9,5 with 1 M Hydrochloric acid

Autoclave at 121ºC for 20 minutes

Maleic Acid Buffer

Maleic acid 11.610 g

Sodium chloride 8.767 g

Make up to 1 L with distilled H2O

Correct to pH 7.5 with Sodium hydroxide pellets

Autoclave at 121ºC for 20 minutes

A-2 Culture Media

Unless otherwise stated, all culture media was allowed to cool to 50ºC in a water bath prior to pouring 30 mL aliquots into sterile plastic petri dishes, the media was set at room temperature. One plate from each batch was incubated at 37ºC overnight and examined for growth the following day as quality control. All media was stored at 4ºC, and allowed to equilibrate to room temperature prior to use.

General culture and storage media

Glycerol Freezing Medium

Glycerol 25 mL
L37 Bacteriological peptone 1,0 g
Sodium Chloride 0,5 g
Make up to 100 mL with distilled H2O

Autoclave at 121ºC for 20 minutes
Dispense into 1,5 mL cryogenic vials

Minimal Maintenance Media

Agar bacteriological (No.1) LP0011 5,0 g
Sodium chloride 5,0 g
Bacteriological peptone L37 3,5 g
Neutralised bacteriological peptone L34 2,5 g
Sodium Phosphate dibasic 2,9 g
Potassium phosphate monobasic 1,3 g
Make up to 1 L with distilled H2O
Heat until dissolved,
correct to pH 6,7 with glacial acetic acid,
distribute into 3 mL
aliquots in sterile bijoux bottles.
Autoclave at 121ºC for 20 minutes.

MacConkey Agar

MacConkey agar CM7 53 g

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

***Pseudomonas* Selective Agar**

Pseudomonas agar CM0559 48,4 g

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

Tryptone Soya Agar with Yeast Extract

Tryptone soya broth CM0129 60 g

Yeast extract LP0021 6 g

Agar bacteriological (No.1) LP0011 24 g

Make up to 1 L with distilled H2O

Autoclave at 121ºC for 20 minutes

Tryptone Soya Broth

Tryptone soya broth CM0129 30 g

Make up to 1 L with distilled H2O

Ensure powder has completely dissolved into distilled H2O

Dispense 10 mL aliquots into 50 mL glass universal screw cap containers

APPENDIX B

FIGURES, RAW DATA, TABLES

Table8. Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for *Pseudomonas aeruginosa* (Clinical and Laboratory Standards Institute M100-S16, 2006 above, 2010 down,)

Antibiotic (amount)	Diameter (mm) of inhibition zone	Interpretation
amikacin (30 µg)	17	susceptible
ampicillin (10 µg)	23	susceptible
benzylpenicillin (10 IU)	22	susceptible
cefuroxime (30 µg)	28	susceptible
cephalothin (30 µg)	18	susceptible
chloramphenicol (30 µg)	20	susceptible
colistin (10 µg)	11	susceptible
gentamicin (10 µg)	17	susceptible

Drug name (Dose strength)	Zone Diameter (mm)		
	Resistant (mm or less)	Intermediate (mm or less)	Susceptible (mm or more)
Tetracycline(30 µg)	≤14	15-18	≥19
Gentamicin (10 µg)	≤12	13-14	≥15
Cefotaxime (30 µg)	≤14	15-22	≥23
Amikacin (30µg)	≤14	15-16	≥17
Piperacillin(100µg)	≤17	---	≥18

Table 9. Restriction Enzymes are used for PFGE of different bacteria (Xbal, SpeI specific PA), (Wikipedia, Todar)

Enzyme	Source	Recognition Sequence	Cut
EcoRI	*Escherichia coli*	5'GAATTC 3'CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'
EcoRII	*Escherichia coli*	5'CCWGG 3'GGWCC	5'--- CCWGG---3' 3'---GGWCC ---5'
BamHI	*Bacillus amyloliquefaciens*	5'GGATCC 3'CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'
HindIII	*Haemophilus influenzae*	5'AAGCTT 3'TTCGAA	5'---A AGCTT---3' 3'---TTCGA A---5'
TaqI	*Thermus aquaticus*	5'TCGA 3'AGCT	5'---T CGA---3' 3'---AGC T---5'
NotI	*Nocardia otitidis*	5'GCGGCCGC 3'CGCCGGCG	5'---GC GGCCGC---3' 3'---CGCCGG CG---5'
HinfI	*Haemophilus influenzae*	5'GANTCA 3'CTNAGT	5'---G ANTC---3' 3'---CTNA G---5'
Sau3A	*Staphylococcus aureus*	5'GATC 3'CTAG	5'--- GATC---3' 3'---CTAG ---5'
PvuII*	*Proteus vulgaris*	5'CAGCTG 3'GTCGAC	5'---CAG CTG---3' 3'---GTC GAC---5'
SmaI*	*Serratia marcescens*	5'CCCGGG 3'GGGCCC	5'---CCC GGG---3' 3'---GGG CCC---5'
HaeIII*	*Haemophilus aegyptius*	5'GGCC 3'CCGG	5'---GG CC---3' 3'---CC GG---5'
HgaI[58]	*Haemophilus gallinarum*	5'GACGC 3'CTGCG	5'---NN NN---3' 3'---NN NN---5'
AluI*	*Arthrobacter luteus*	5'AGCT 3'TCGA	5'---AG CT---3' 3'---TC GA---5'
EcoRV*	*Escherichia coli*	5'GATATC 3'CTATAG	5'---GAT ATC---3' 3'---CTA TAG---5'
EcoP15I	*Escherichia coli*	5'CAGCAGN_{25}NN 3'GTCGTCN_{25}NN	5'---CAGCAGN_{25} NN---3' 3'---GTCGTCN_{25}NN ---5'
KpnI[59]	*Klebsiella pneumoniae*	5'GGTACC 3'CCATGG	5'---GGTAC C---3' 3'---C CATGG---5'
PstI[59]	*Providencia stuartii*	5'CTGCAG 3'GACGTC	5'---CTGCA G---3' 3'---G ACGTC---5'
SacI[59]	*Streptomyces achromogenes*	5'GAGCTC 3'CTCGAG	5'---GAGCT C---3' 3'---C TCGAG---5'
SalI[59]	*Streptomyces albus*	5'GTCGAC 3'CAGCTG	5'---G TCGAC---3' 3'---CAGCT G---5'
ScaI*[59]	*Streptomyces caespitosus*	5'AGTACT 3'TCATGA	5'---AGT ACT---3' 3'---TCA TGA---5'
SpeI	*Sphaerotilus natans*	5'ACTAGT 3'TGATCA	5'---A CTAGT---3' 3'---TGATC A---5'
SphI[59]	*Streptomyces phaeochromogenes*	5'GCATGC 3'CGTACG	5'---GCATG C---3' 3'---C GTACG---5'
StuI*[60][61]	*Streptomyces tubercidicus*	5'AGGCCT 3'TCCGGA	5'---AGG CCT---3' 3'---TCC GGA---5'
XbaI[59]	*Xanthomonas badrii*	5'TCTAGA 3'AGATCT	5'---T CTAGA---3' 3'---AGATC T---5'

Key:*=blunt-ends N=C or G or T or A W = A or T

Table 10-Detailed information of studied patients

No	**Hospital name**	patient file number	origin of sample	Section of hospital	age	sex
1	**MESA**	2838*	Blood	internal	47	M
2	**MESA**	2763*	Blood	ICU-General	63	M
3	**MESA**	2162*	Blood	First floor internal	81	F
4	**MESA**	2612-2628	Blood	Cardial surgery	79	M
5	**MESA**	1357*	Blood	ICU	85	M
6	**MESA**	960*	Blood	Cardial surgery	80	M
7	**MESA**	525-459	Blood	Cardial surgery	80	M
8	**MESA**	822*-761*	Blood	ICU-General	78	F
9	**MESA**	1834*	Eye	Eye	3	F
10	**MESA**	1917	Eye Contact LENZ	Eye	3	M
11	**MESA**	1621	Eye	General Surgery	3	M
12	**MESA**	2596*	Ear Marker	ENT	6	F
13	**MESA**	1864*	Ear	ENT	6	F
14	**MESA**	822	Blood	ICU	78	F
15	**MESA**	2530,2601*	Sore	Orthopedic	36	F
16	**MESA**	761*	Sore	ICU	80	F
17	**MESA**	MESA -459*	Sore		76	M
18	**MESA**	MESA -2599*	Sore	Plastic Surgery	73	F
19	**MESA**	MESA -1508*	Ear-	ENT	70	F
20	**MESA**	MESA -1463*	Sore	ICU GENERAL	91	M
21	**MESA**	MESA -2118*	Sore	1.Floor	78	F
22	**MESA**	2225*,2161,2162,2163	Sore	Anesthesia	70	F
23	**MESA**	MESA1310*,1281	Sore	ICU	80	F
24	**MESA**	MESA -1886*	SORE	ICU	70	F
25	**MESA**	MESA -2474*	Sore	orthopedic	36	M
26	**MESA**	-2271*,2293	Tracheal	Anesthesia	72	M
27	**MESA**	MESA -2134*	Sore	General Surgery	50	F
28	**MESA**	MESA2033,1963*	Sore	1.floor Internal	46	M
29	**MESA**	MESA -2132	Sore	Plastic Surgery	40	F
30	**MESA**	MESA -640*	Sore	Plastic Surgery	40	M
31	**MESA**	MESA -2447*	Sore	ORTHOPEDİ	36	M
32	**MESA**	MESA1963*,2033	Sore	First floor Internal	44	M
33	**MESA**	2164,2162,2163	Tracheal lavage			F
34	**MESA**	MESA -2727*	Tracheal lavage	Anesthesia 72	90	M
35	**MESA**	MESA -671*	Tracheal lavage	Heart Surgery	78	M
36	**MESA**	MESA -2668	Tracheal lavage	ANESTEZİA91		M
37	**MESA**	MESA -435*	Tracheal lavage	ICU-General	85	F
38	**MESA**	2093*	Tracheal lavage			F
39	**MESA**	2612*,2628	Tracheal lavage	Cardiac Surgery	68	M
40	**MESA**	1714*,1732,1697	Tracheal lavage	ICU General	85	F
41	**MESA**	MESA -2631*	Tracheal lavage	Anastasia	87	M
42	**MESA**	2235*	Tracheal lavage	B1	57	M
43	**MESA**	MESA -1428*	Tracheal lavage	ICU	92	M
44	**MESA**	MESA -2293*	Tracheal lavage	Anesthesia	63	M
45	**MESA**	MESA -1010*	sore	ICU	65	F
46	**MESA**	MESA -2114*	Tracheal lavage	ICU	91	M
47	**MESA**	MESA -2102*	Tracheal lavage	Emergence	22	M
48	**MESA**	MESA -2793*	Tracheal lavage	ANESTEZİA 72	78	M
49	**MESA**	MESA -2406*	Tracheal lavage	Anesthesia	84	M
50	**MESA**	MESA -2855*	Tracheal lavage	ENT	36	M
51	**MESA**	MESA -2629*	Tracheal lavage	Cardial surgery	72	F
52	**MESA**	MESA-2353*,2378*	Tracheal lavage	Cardial surgery	70	M
53	**MESA**	MESA -737*,753	Tracheal lavage	ICU-General	74	M
54	**MESA**	MESA -1620*	Tracheal lavage	ICU	74	M
55	**MESA**	MESA -669*	Bronchial	ICU	74	M
56	**MESA**	MESA -407*	Bronchial	ICU	70	F

Table 10 (continiued)

57	**MESA**	MESA -2107*,2093	Bronchial	ICU	73	F
58	**MESA**	MESA -2114	Bronchial	ICU	70	M
59	**MESA**	MESA -2302*,2347	Bronchial	ANESTEZİ	84	M
60	**MESA**	MESA -1851	Sputum	ICU	94	F
61	**MESA**	MESA -2257*	Sputum	Cardiology	80	M
62	**MESA**	MESA -2499*	ABSE	Surgery	18	F
63-1	**GAZİ**	701708-6370307	Priton	Surgery	62	F
64-2	**GAZİ**	1793013-6373549	ETA	PED-ICU	1	F
65-3	**GAZİ**	1799126-6374308	Blood	Oncology	50	F
66-4	**GAZİ**	1112767-6382535	Sore	Dermatology	32	M
67-5	**GAZİ**	1797947-6382007	Catheter	Hematology	51	F
68-6	**GAZİ**	1802149-6384152	Sputum	cardial Surgery	75	F
69-7	**GAZİ**	1805080-6389082	Priton	PED-surgery	2	M
70-8	**GAZİ**	1806433-6387296	Sore	İnfection	55	M
71-9	**GAZİ**	822351-6390276	Sore	İnfection	47	M
72	**GAZİ**	1362738-6901083	Priton	Pediatric-Surgery	2	M
73	**GAZİ**	120045-6399594	Sore	cardiology	80	M
74	**GAZİ**	1581004-6406704	puse	cardiology	80	M
75	**GAZİ**	1760262-6404474	Endocrinology	Endocrinology	72	M
76	**GAZİ**	1789641-6309564	Blood	Pediatric-ICU	2	F
77	**GAZİ**	1793753-6317661	Puse	Orthopedic	70	F
78	**GAZİ**	1707700-6302994	Blood	Pediatric	4	F
79	**GAZİ**	594082-6244927	Sputum	Hematology	21	M
80	**GAZİ**	1744146-6067454	Sputum	ICUPULMONARY	94	M
81	**GAZİ**	1732911	Pleura	Tracheal surgery	50	M

Table 11 Restrictions Enzymes used for PFGE (Xbal, SpeI specific to P.A)

(Tenover)

Organism	*Restriction enzyme*	Approximate no of	Fragment size range (kb)
Gram positive organisms			
Entrococcus	*SmaI*	15-20	5-400
Clostridium dificile	*SmaI*	10--15	10-900
Clostridium dificile	*SacII*	10--15	10-900
Clostridium perfrigensis	*SmaI*	12	45-1460
Clostridium perfrigensis	*SacII*	10	45-1640
Staphylococcus aurus	*SmaI*	15-20	10-700
Saphylococcus aurus/	*CspI*	10--15	30-500
Staphylococcus spp,coagulase Negative	*SmaI*	15--20	5-400
Streptococcus spp(A&B)	*SmaI*	15-20	5-500
Streptococcus penumoniae	*SmaI*	10--19	20-300
Streptococcus penumoniae	*ApaI*	10--19	20-250
Gram Negative Organisms			
Acintobacter culcaceticus	*SmaI*	20-25	5-300
Acintobacter baumani	*SmaI*	20-40	5-300
Acintobacter baumani	*ApaI*	20-30	10-300
Bacterioides spp	*NotI*	8--10	200-1200
Bordettela pertusis	*XbaI*	20--30	20-700
Borrellia burgdoferri	*SmaI*	10--20	10-300
Pseudomonas cepacia	*SpeI*	20--25	40-700
Campylobacter jejuni	*SmaI*	8--10	40-400
Campylobacter fetus	*SmaI*	10--15	40-400
Campylobacter fetus	*SarI*	10--15	40-300
Chlamydia trachomatis	*Sse83871*	17	9-220
Coxiella burnetii	*NotI*	19	10-293
Entrobacter spp	*XbaI*	ca20	10-700
Echerichia coli	*XbaI*	ca20	10-500
Echerichia coli	*NotI*	12--15	10--1000
Echerichia coli	*SfiI*	15--20	10--700
Haemophilus influenza	*SmaI*	10--12	10-500
Haemophilus influenza	*RsrII*	10--12	10-500
Klebsiella spp	*XbaI*	Ca 20	10--700
Legionella Pnumophila	*SfiI*	10--15	50-700
Legionella penumophila	*NotI*	5--10	50--2000
Mycobacterium spp	*AseI*	12--20	10--700
Neisseria gonerrhoeae	*SpeI*	12--17	10-500
Neisseria meningitidis	*NotI*	20--30	5-200
Neisseria meningitidis	*Bg II*	20--30	5-200
Proteus mirabilis	*SfiI*	7--10	50-700
proteus mirabilis	*NotI*	6--10	75-700
Pseudomonas aeruginosa	*SpeI*	20--25	10-700
Pseudomonas aeruginosa	*XbaI*	40--50	10-300
Salmonella spp	*NotI*	40-50	5-400
Shigella spp	*XbaI*	15-23	10-700
Shigella spp	*SfiI*	15-20	10-700
vibrio cholerae	*NotI*	20-30	10-400
Xantamonas maltophila	*XbaI*	ca 15	10-700
Yersinia pestis	*XbaI*	ca 20	10-700

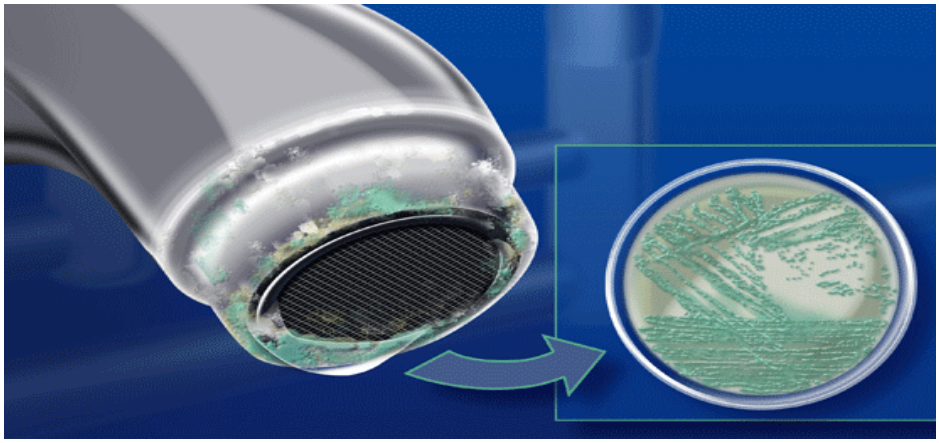

Figure 23. Biofilm formation around water pipe (bronchoscope)

Figure 24.PFGE Apparatus (Bio-Rad)

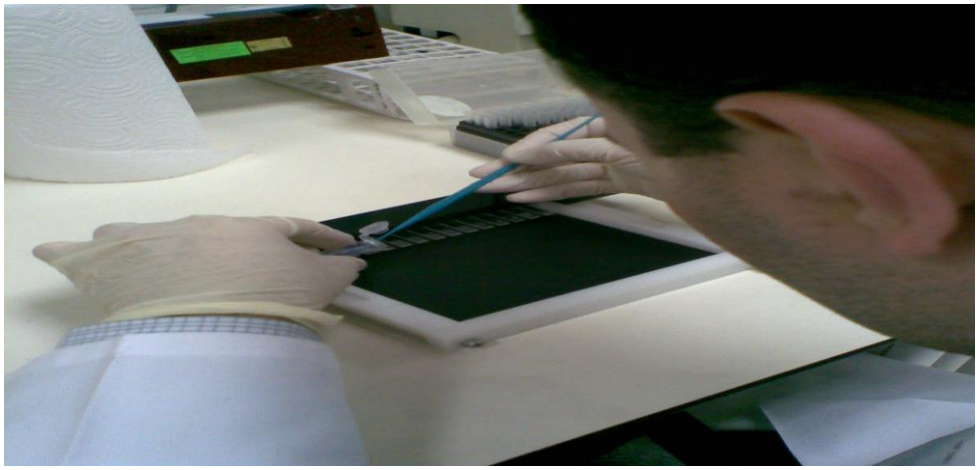

Figure 25.comb for placing plugs samples on it. (Before purring gels plugs are deposited on comb)

Figure26. PFGE Apparatus Surface of Electrophoresis area

Figure27. Purring Agarose on PFGE frame,

(Comb containing samples in Blocks are implanted in gel)

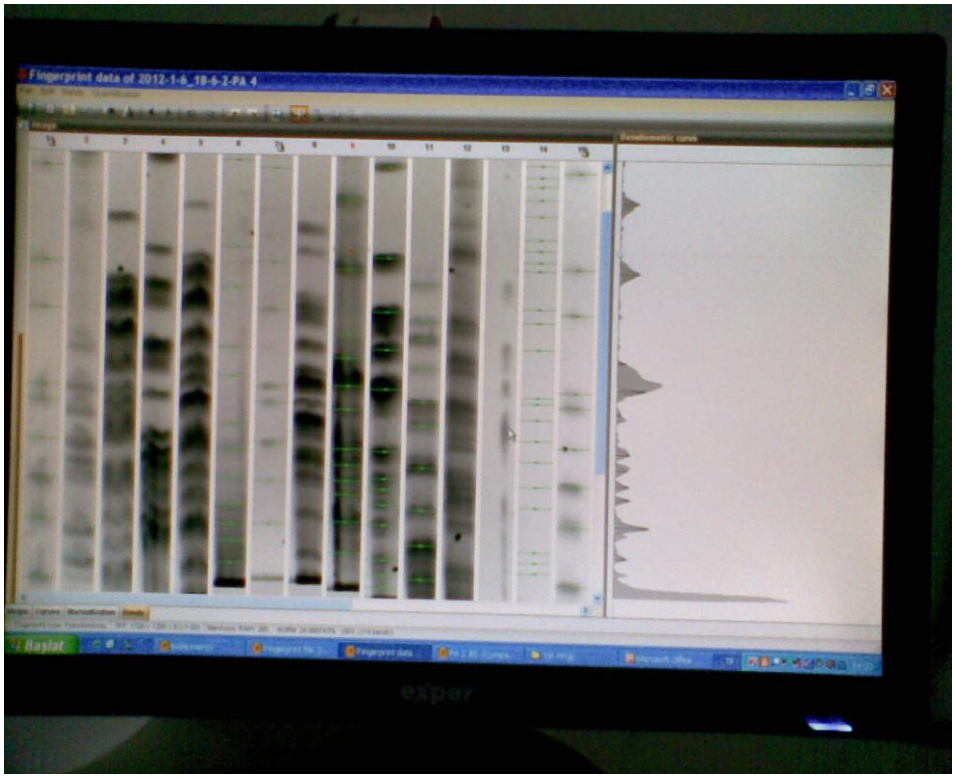

Figure 28. Analyzing PFGE gel with software Dendogram preparing

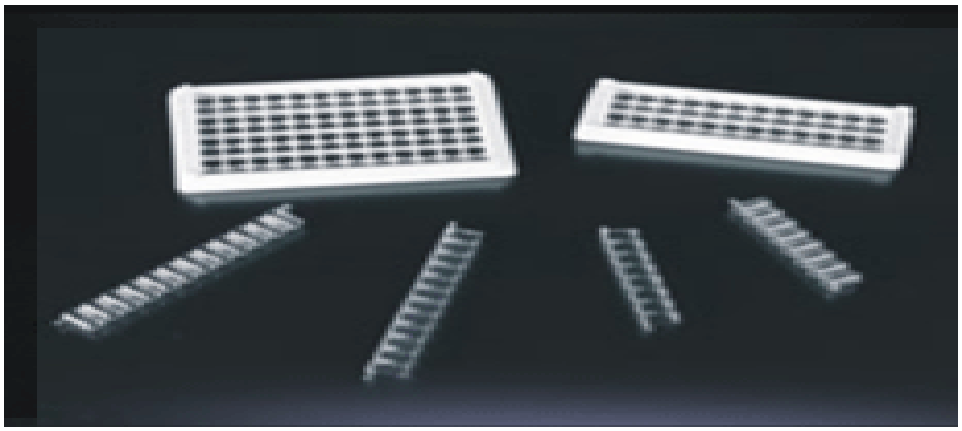

Figure 29. Well bearing comb for agarose frame

(Bacteria containing agarose become blocked in its wells)

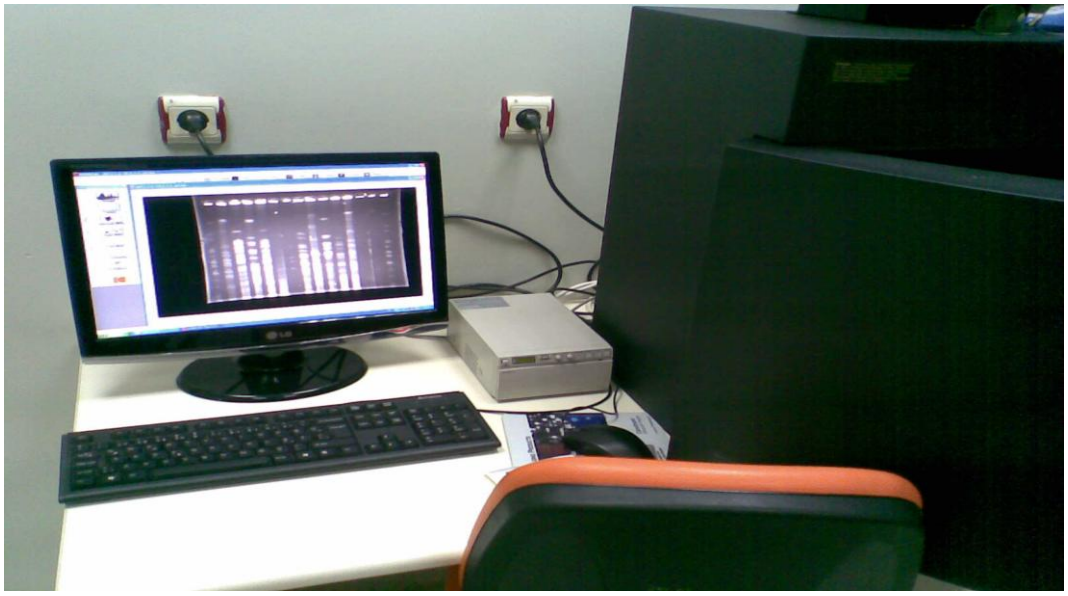

Figure 30. Etidium bromide stained gel on Monitor

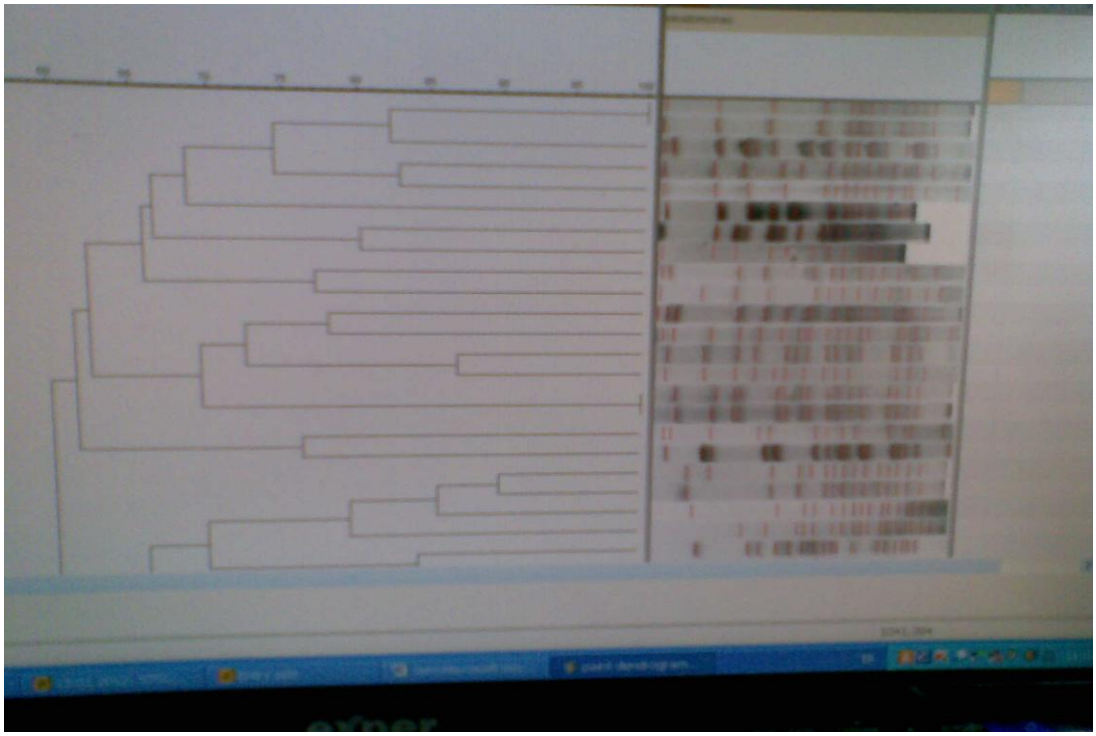

Figure 31. Dendogram drawn based on gel. (Clusters shows relevance of strains)

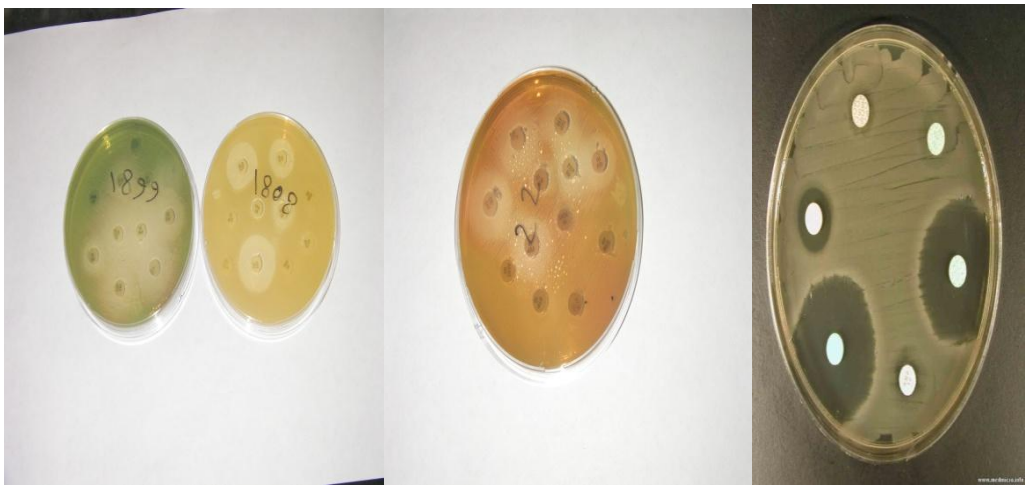

Figure 32. Plate of Anti-bio gram of P.A (Disk diffusion Muller Hinton)

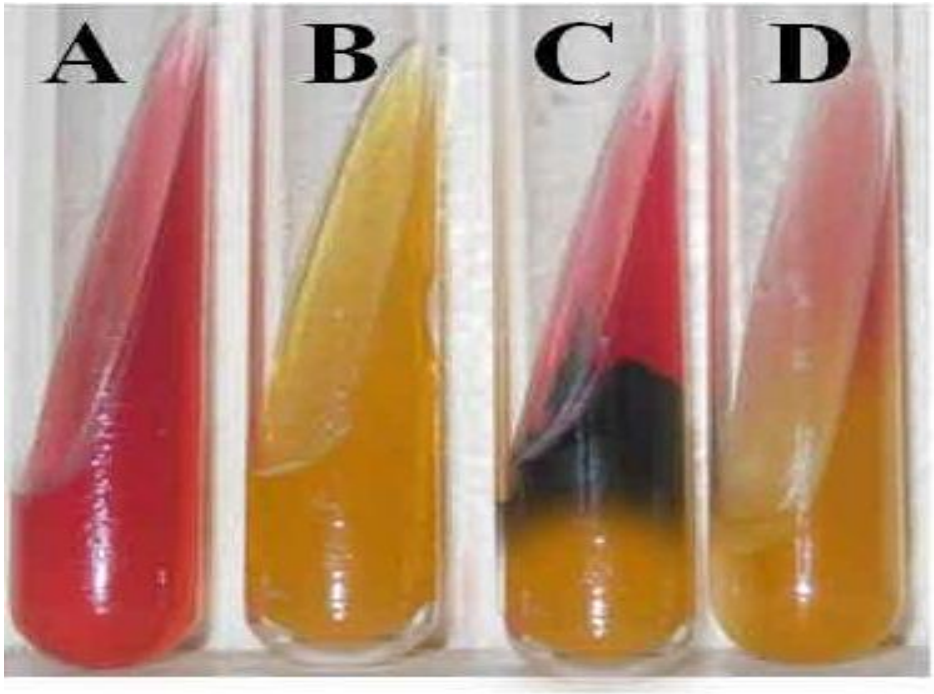

Figure 33.TSI Alk/Alk,

Acid/Acid, Alk/Acid+H_2S, Alk/Acid

Red“Represent non-fomenters” Yellow”Represent fermenter”

A)*Psuedomonas aeruginosa*:Gluc(-),Lac/Suc(-),H_2S(-),

B)*Escherichia coli*:Gluc(+)Lac/Suc(+),H_2S(-)

C)*Salmonella typhimurium*:Gluc(+),Lac/Suc(-),H2S(+)

D)*Shigella boydili:Gluc*(+),Lac/Suc(-),H_2S(-)

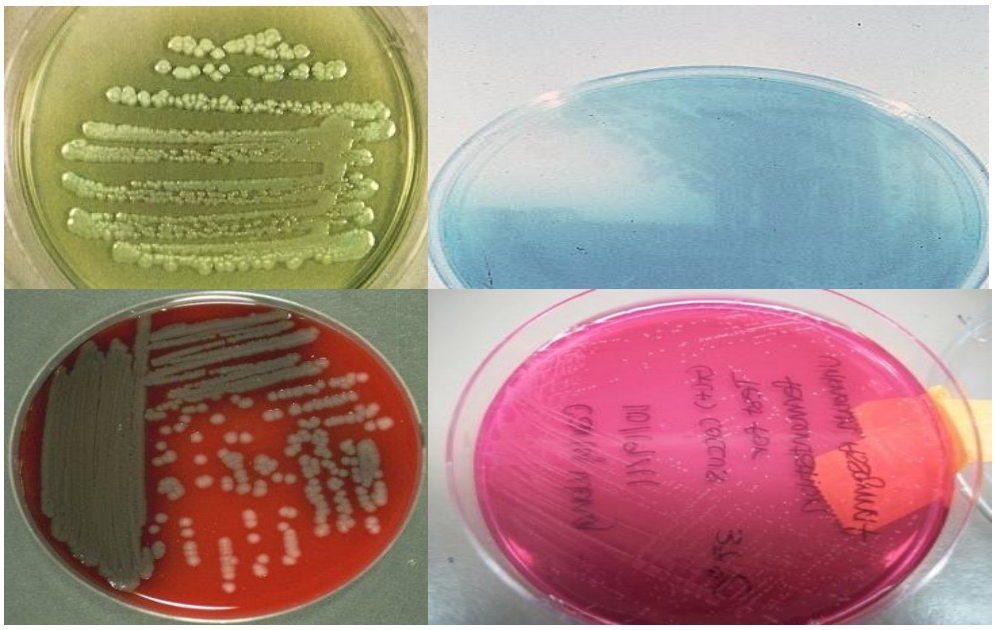

Figure 34. Colony of P.A in plates Up (Hugh leifson pigmentation) Down Blood agar and Mckoncy.

Figure 35. Oxidase test

(Blue positive, white negative)

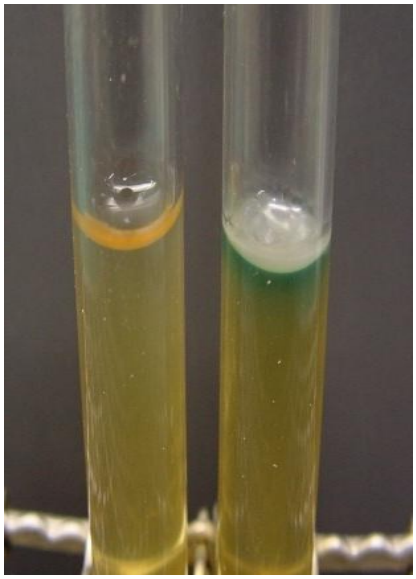

Figure 36. Hugh Leif son test

(Green positive)

CURRICULUM VITAE

PERSONAL INFORMATION

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Education:

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PhD	METU, Biotechnology, 2013
MSc	Tarbiat Modares Medical Microbiology, 1996
BSC	Tabriz University, Biology, 1986
High school	Ferdowsi high school 1983

WORK EXPERIENCE:

Year	Place	Enrolment
2007-2013	Metu, Biotechnology	PhD thesis works
1996-2006	Iran Health Ministry universities	Teacher or Researcher
1996-1998	Iran pasteur institute, Tehran	Researcher
1998-2004	Tabriz Zakaria Pharmaceuticals	Quality control
1998-2006	Toos diagnostic kit producing company	Manager
1998-2005	Tabriz Azad university	Teacher
1990-1993	Tabriz Medical university	Hospital, Lab Management

FOREIGN LANGUAGE

English

PUBLICATION

1-DNA Finger printing of *Pseudomonas aeruginosa* isolated from hospital sections. (Under publication)Iranian 2.conferance2012, Turkish Microbiology association conference, Ankara, (poster 2012)

2-Molecular characteristics of Pan drug resistant, Biofilm embedded *Pseudomonas aeruginosa*, isolated from hospital environment. (Under publication)

3-Evaluation of IFA in diagnosis *H.pylori* infection in children and adults journal of Faculty of Medicine 1999, 57th year, NO2 29-36

4 - Evaluation of rapid &ultra-rapid Urease test, the journal of Faculty of Medicine 1999, NO.3 P.30-35

5-Limitation of Rapid urease test in detection of H.pylori eradication (under publishing)

6-Evaluation of Elisa kit for detection of *H.pylori* infection in different age and sex groups, Iranian graduates 2.conferance, 2011

VII... LIST OF PAPERS PRESENTED IN CONGRESSES AND SEMINARS

3lectures: 2nd congress of Blood and Related, disease 1996 Dec 21-23 pasture Institute, IRAN.

1. Correlation between blood group Lewis (Le+, Le-) and *H-pylori* infection
2. DNA finger printing of *Pseudomonas aeruginosa* isolated from hospital sections, 2012 agri-food congress, Antalya, Turkey
3. DNA finger printing of *pseudomonas aeruginosa* isolated from hospital sections, 2012, Molecular-Microbiology conf.Ankara, TURKEY
4-DNA Finger printing of *Pseudomonas aeruginosa* isolated from hospital sections, Iranian 2.conferance2012
5-Molecular characteristic of Pan drug resistant, Biofilm embedded *Pseudomonas aeruginosa*, isolated from hospital environment. Iranian graduates 2.conferance, 2013
6-Evaluation of Elisa kit for detection of H.pylori infection in different age and sex groups, Iranian graduates 2.conferance, 2011

7-Failure of Bionumerics 6soft ware in interpretation of SpeI mediated pulsed field gel electrophoresis.4th International scientific conference of Iranian Academics2014

8-Outbreak of pseudomonas aeruginosa nosocomial infection through biofilm "Olympus Broncoscope 4th International scientific conference of Iranian AcademicsAnkara Turkey 2014

HOBBIES: internet, Football, Racing, Walking, swimming, Chess, Music.

TEZ FOTOKOPİ İZİN FORMU

ENSTİTÜ

Fen Bilimleri Enstitüsü ☒

Sosyal Bilimler Enstitüsü ☐

Uygulamalı Matematik Enstitüsü ☐

Enformatik Enstitüsü ☐

Deniz Bilimleri Enstitüsü ☐

YAZARIN

Soyadı: AlipourGhorbani.....
Adı: Nader.....
Bölümü: Biotechnology

TEZİN ADI (İngilizce) : GENOMIC CHARACTERIZATION OF PSEUDOMONAS AERUGINOSA CLINICAL ISOLATES BY PULSE FIELD GEL ELECTROPHORESIS METHOD

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.....

TEZİNTÜRÜ : YüksekLisans ☐ Doktora ☒

1. Tezimin tamamı dünya çapında erişime açılsın ve kaynak gösterilmek şartıyla tezimin bir kısmı veya tamamının fotokopisi alınsın. ☒
2. Tezimin tamamı yalnızca Orta Doğu Teknik Üniversitesi kullancılarının erişimine açılsın. (Bu seçenekle tezinizin fotokopisi ya da elektronik kopyası Kütüphane aracılığı ile ODTÜ dışına dağıtılmayacaktır.) ☒
3. Tezim bir (1) yıl süreyle erişime kapalı olsun. (Bu seçenekle tezinizin fotokopisi ya da elektronik kopyası Kütüphane aracılığı ile ODTÜ dışına dağıtılmayacaktır.) ☒

Yazarın imzası ..... Tarih ..25-04-2014.....

N-Alipourse