Chapter One

Introduction

Cystic fibrosis (CF) is an autosomal recessive inherited disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Normal CFTR gene encodes for a protein that constitutes channels controlling the movement of salt ions. Ion movement across secretory cells is important to maintain normal secretions of these cells. Mutations in the CFTR gene produce dysfunctional CFTR protein. Therefore movement of ions into and out of cells is hindered resulting in the formation of thick mucus in lungs, pancreas and other organs .

The major problem in CF patients is the bronchopulmonary infections. It leads to a decrease of the lung function and it's responsible of a high morbidity and mortality. The primary bacterial pathogens associated with CF pulmonary infections have been identified as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Haemophilus Influenzae*. CF patients must be screened for the presence of these pathogens.

The determination of presence other bacterial species is important because they may also be involved in the progressive loss of lung function. The bacterial pathogens in CF airways are identified through cultivation of expectorated sputum samples on selective media. The culture-based analysis may has problems

since the process is time-consuming, requires species-specific selective media and it may excludes the detection of unculturable bacteria that may predominate in many environments.

Molecuolar biology techniques for correct detection and identification of bacteria is now widely used in clinical microbiology and also developed for identification of isolates obtained from CF patients. Hence, correct identification of bacteria help in the selection of appropriate treatment for CF airway infections

The aim of this study is to determine the bacterial species associate with bacterial infection in CF patients, determine the Susceptibility patterns to the antibiotics, and analyze the efficiency of species-specific primer pair to identify these species by using PCR-RFLP protocol.

Chapter Two

Review of Literature

2.1 Cystic fibrosis

CF is a common recessive genetic disease which affects the entire body, causing progressive disability and often early death. The name cystic fibrosis refers to the characteristic scarring (fibrosis) and cyst formation within the pancreas, first recognized in the 1930s. (Yankas. 2004). It results from mutations of the CF transmembrane conductance regulator (CFTR), which functions as a cyclic adenosine monophosphate (cAMP) chloride channel at the apical epithelial cell surface (Strausbaugh and Davis. 2007). Over 1,500 mutations have been identified and categorized into distinct classes reflecting abnormalities of protein structure or function (Cystic Fibrosis Mutation Database 2007).

The organ's sensitivity to CFTR dysfunction and the amount of functional CFTR are assessing the severity of the disease (Boyle. 2003). The primary organ systems involved are the lungs, Lung disease results from clogging of the airways due to mucus build-up, decreased mucociliary clearance and resulting inflammation. (Flume *et al*. 2010) . The gastrointestinal tract, liver, and exocrine pancreas, are also involved in which inspissation of viscid secretions leads to intestinal obstruction, cholestasis, and malabsorption of fat and protein; and the male reproductive tract, in

which obstructive azoospermia leads to infertility. The hallmark CF lung disease includes airway infection, inflammation, obstruction, and structural lung damage. These abnormalities begin in infancy, often prior to the onset of symptoms, and progress over the first years of life (Davis *et al.* 2010) Thus, early initiation of effective chronic therapies, an opportunity afforded by newborn screening, could potentially delay or prevent progression of CF lung disease. There are no clinical trials of chronic non antibiotic maintenance pulmonary therapies in infants and preschool-aged children with CF, even though this is the population with the greatest potential for long-term benefit (Rosenfeld *et al.* 2012).

CF is most common among Caucasians; one in 25 people of European descent carry one allele for CF. Ireland has both the highest incidence of CF in the world and the highest carrier rate in the world with 1 in 19 individuals classed as carriers (Ratjen and Doring. 2003).CF is found in the Australia, New Zealand, the Middle East, Iran, Pakistan, India and Latin America. If not treated, most CF patients die at a young age.CF had been thought to be rare in Arabs, The incidence of CF in Saudi Arabia was reported to be 1 in 4243 children (Nazer *et al.* 1989). Many genetic and epidemiological data have been described in Gulf countries (Kambouris *et al*. 2000). In 1989, It has been documented CF in 13 children in Saudi Arabia, they were suffering from abdominal distention, failure to thrive and recurrent respiratory infections, often with *P.aeruginosa* (Nazer *et al.* 1989). Nine years later, Banjar and Mogarri reported the demographic and clinical data of 84 Saudi patients (49 males and 35 females) with CF who were admitted to a referral center. Ten of them were died due to respiratory failure and 80-90% of the remaining were presented with cough, wheezing, repeated chest infection and failure to thrive (Banjar and Mogarri. 1998).

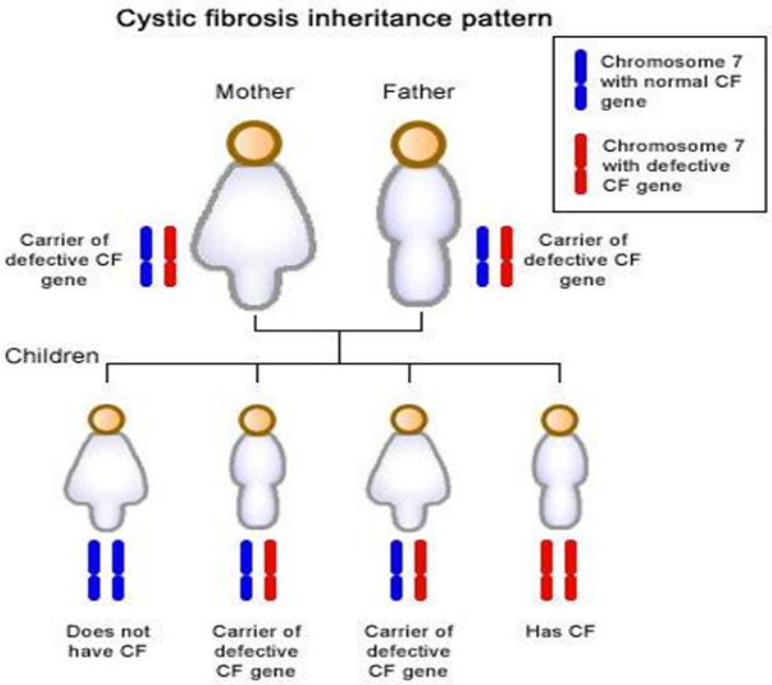


Figure 2.1 Cystic fibrosis inheritance patterns.

Source: http://www.virtualmedicalcentre.com/health-investigation/genetic-testing-for-hereditary-diseases/62

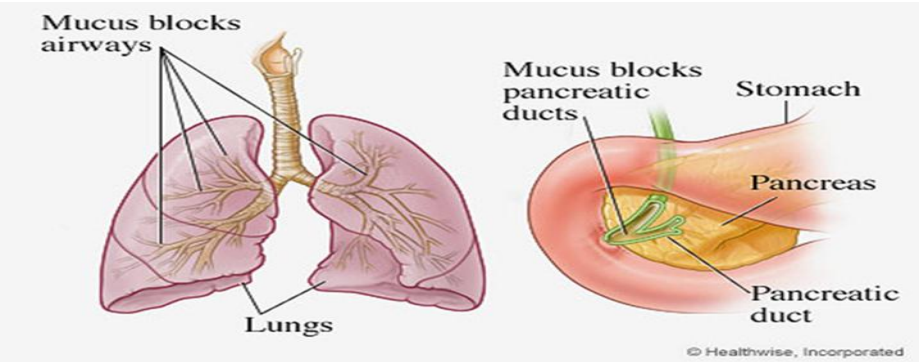


Figure 2.2 Shown the mucus blocks the airways and pancreatic ducts in cystic fibrosis patients.

Source:http://www.docstoc.com/docs/107227457/Effective-treatment-for-cystic-fibrosis--in-Mindheal-Homeopathy-clinic

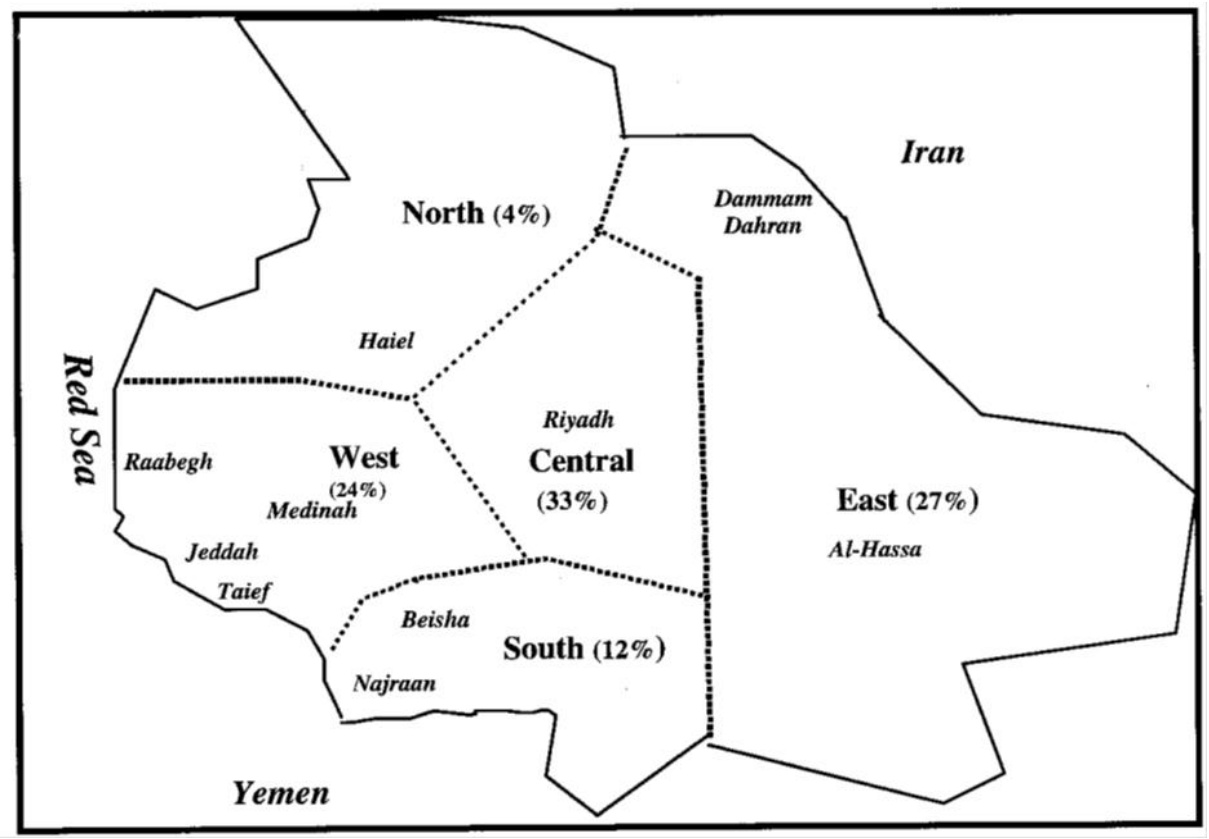


Figure 2.3 Provinces of Saudi Arabia and percentage of families with identified CFTR mutations (Banjar and Mogarri. 1998).

2.2 Bacterial pathogens in cystic fibrosis patients

This disease affects persons without distinction of age or sex but can be asymptomatic in a great number of cases (Chaparro *et al*. 2001). Failure of innate defense mechanisms and the lack of mucocilliary clearance in the airways stimulate primary and recurrent bacterial infections, blockage of airways, inflammation and chronic bacterial infections (Boucher. 2004 ; Accurso. 2005). During the first decade of life of CF patients, *Staphylococcus aureus* and *Haemophilus influenzae* are the most common bacteria isolated from the sputum, but in the second and third decade of life, *Pseudomonas aeruginosa* is the prevalent bacteria (Coutinho *et al*. 2008) Beside the main agents (*P.aeruginosa* and *S.aureus*), Gram-negative non fermentative microorganisms (*Burkholderia cepacia*, *Stenotrophomonas maltophilia, Acinetobacter baumannii*) have been identified in sputum of CF patients (Shaginian *et al.* 2010)

2.2.1 *Pseudomonas aeruginosa*

P.aeruginosa is a gram negative bacilli which have very simple growth requirements. It colonizes the respiratory and gastrointestinal tract. (Fujitani *et al,* 2011), and causes life-threatening infections to the patients with immunodeficiency such as cystic fibrosis and cancer (Chang *et al*. 2005a; Small *et al*. 2007). *P.aeruginosa* is a leading cause of nosocomial infection primarily because it is resistant to many antibiotics and antimicrobials, in part because of its effective efflux systems (Chang *et al*. 2005b) .

P. aeruginosa is a widespread organism that is capable of adapting to the unique environment of the CF lung. It is able to create biofilm by assume a

mucoid phenotype. The biofilm enhances bacterial resistance to phagocytosis and antipseudomonal antibiotics. The first descriptions of an association between mucoid *P.aeruginosa* strains and chronic pulmonary colonization in patients with CF appeared in the 1960s (Doggett. 1969). Once established in the CF lung, *P. aeruginosa* is actually impossible to eradicate. Although the age of *P. aeruginosa* acquisition is an important determinant of prognosis (Nixon *et al*. 2001). Numerous studies have demonstrated that people with CF typically harbor their own unique strain of *P. aeruginosa*, which is presumably acquired from the environment during childhood (Romling *et al.* 1994; Mahenthiralingam *et al.* 1996). The sharing of strains is considered unusual, with the exception of siblings where this occurrence is well-documented (Grothues *et al*. 1988 ; Wolz *et al*. 1989). Cross-infection with *P. aeruginosa* has been reported in some Specialist CF Centers and guidelines are required to reduce the spread of transmissible strains (Govan. 2000; Jones and Webb. 2003; Saiman *et al*. 2003). Genome sequence analysis of *P. aeruginosa* PAO1 has provided several insights as to why the bacterium is able to adapt to chronic lung infection (Stover *et al.* 2000).Dependent on this we can say that the molecular techniques is the disentangle method for understand how to treat this organism.

2.2.1.1 Characterization

P.aeruginosa is a gram negative rod that belongs to the family Pseudomonadaceae. It is motile by means of a single polar flagellum (Ryan and Ray. 2004), and it is a facultative anaerobic. The thick layers of lung mucus and alginate surrounding mucoid bacterial cells can limit the diffusion of oxygen so it can adapt to microaerobic or anaerobic environments (Cooper *et al*. 2003; Williams *et al*. 2007). It has very simple nutritional requirements. It can grow in

distilled water, which is evidence of its minimal nutritional needs. The optimum temperature for growth is 37 degrees, and it is also can grow at temperatures as high as 42 degrees. Its tolerance to a wide variety of physical conditions, including temperature, contributes to its ecological success as an opportunistic pathogen in the laboratory. The simplest medium for growth of *P. aeruginosa* consists of acetate as a source of carbon and ammonium sulfate as a source of nitrogen (Todor. 2011). It produces two types of soluble pigments, Pyocyanin and (fluorescent) pyoverdin. The latter is produced abundantly in media of low-iron content, and could function in iron metabolism in the bacterium. Pyocyanin (from "pyocyaneus") refers to "blue pus" which is a characteristic of supportive infections caused by this organism.it has large and irregular colonies with a characteristic fruity odor. The colonial morphology, staining, pigment production and biochemical tests are the conventional methods for identify *P.aeruginosa*.

2.2.1.2 Virulence factors

P. aeruginosa has a uniquely large genome containing genes for many different virulence factors and regulatory mechanisms allowing it to adapt to hostile environments (Kipnis *et al.* 2006). This organism able to produce a large number of toxins and surface components . These include pili, flagella, membrane bound lipopolysaccharide, and secreted products such as exotoxins A, S and U, elastase, alkaline protease and cytotoxins (Vallés and Mariscal. 2005).

Figure 2.4 *Pseudomonas aeruginosa*

Source: http://www.buzzle.com/articles/pseudomonas-aeruginosa-infection-symptoms-and-treatment.html

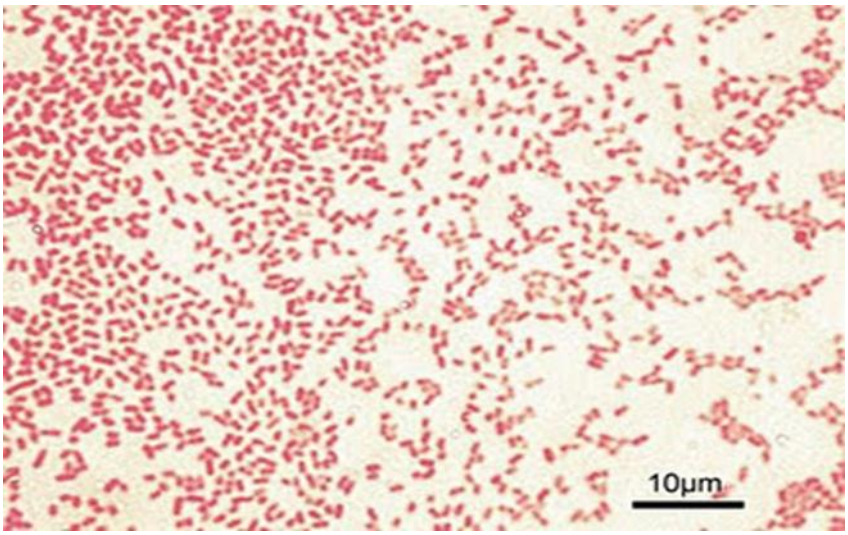


Figure 2.5 Gram stain of *P.aeruginosa*

Source: http://en.academic.ru/dic.nsf/enwiki/1168819

2.2.1.2.1 Flagella

Flagella are highly complex bacterial organelles which are unusually well conserved among diverse bacterial species. The synthesis and function of flagella are depending on over 50 genes. Different studies suggest that the flagella preservation and role in chemotaxis and motility are important in the survival of many organisms (Shapiro. 1995). The flagellum of *P.aeruginosa* is required for swimming motility but also plays crucial roles in biofilm dispersal and adhesion to the surface of host cells (Arora *et al.* 1998). During infection flagellin (the primary structural component of the flagellum) is recognized by Toll-like receptor 5 (TLR5) on the surface of host cells. Therefore, toll-like receptor 5 is used by the host as a surveillance mechanism to detect invading *P.aeruginosa* bacteria and in turn trigger the immune response by inducing the synthesis of cytokines such as Tumor necrosis factors (TNF), Interleukin (IL-6) and IL-8 (Zhang *et al*. 2003; Zhang *et al*. 2005). Throughout the course of chronic infection in the CF lung *P.aeruginosa* down regulates expression of flagellin, perhaps to evade the host immune response (Wolfgang *et al*. 2004; Palmer *et al,* 2005). Elastase released by neutrophils in respiratory mucus degrades the flagellar hook protein FlgE at the bacterial surface (Sonawane *et al.* 2006), in the absence of FlgE, the flagellar apparatus is no longer competent for export, and the otherwise secreted anti-sigma factor FliM accumulates within the bacterium and binds to FliA. Sequestration of FliA prevents expression of flagellar genes normally targeted by this transcriptional activator, resulting in the absence of flagella (Jyot *et al.* 2007). Animal models have demonstrated that antibodies against the flagellum, induced by either active or passive immunization, are protective (Landsperger *et al*. 1994 ; Holder and Naglich, 1986). Prevention of

P.aeruginosa lung infection by immunization against flagellar antigens might therefore be a suitable adjuvant therapy in individuals with CF.

2.2.1.2.2 Pili

Pili or fimbriae are smaller filamentous surface appendages of *P. aeruginosa*. Multiple pili are usually present on the surface. *P. aeruginosa* pili are among the rare prokaryotic pili involved in bacterial motility (Kipnis *et al.* 2006). Some studies have shown that both pili-mediated adherence and twitching motility are critical to *P. aeruginosa* virulence (Farinha *et al*. 1994; Comolli *et al*. 1999). In an infant mouse model of lung infection, piliated strains of *P. aeruginosa* caused more severe and diffuse pneumonia than corresponding non-piliated mutants (Tang *et al*. 1995)

2.2.1.2.3 Lipopolysaccharides (LPS)

LPS of *P. aeruginosa* is composed of three distinct regions: lipid A, core oligosaccharide (OS), and the long-chain O antigen. The core OS of *P. aeruginosa* is composed of L-glycerol-D-manno-heptose, 3-deoxy-D-manno-oct-2-ulosonic acid, D-galactosamine, D-glucose, and L-rhamnose. Non-carbohydrate substituents are also found in the core OS including phosphate, 2-aminoethyl (di) phosphate, acetyl, alanyl and carbonyl groups. (Kocíncová and Lam. 2011). The variable O-specific polysaccharide chains are the basis of antigenic identification of *P.aeruginosa* serotypes. This immunogenicity makes them obvious targets for immunotherapy .However, the active immunization elicited by O-antigen based vaccines is lacking in protectiveness even when multiple O antigens from different serotypes are conjugated. (Lang *et al*. 2004; Pier. 2003) Thus, various O-antigen based vaccines have been tested over

decades with limited success (Pier. 2003); to circumvent this problem several strategies have been developed. Multiple serotype conjugates can be further conjugated with another target such as exotoxin A (Lang *et al*.2004).

The adaptation of *P.aeruginosa* during chronic infection rely of different factors, the LPS is one of them. It is already known that *P. aeruginosa* acute infection also implies a consistent change in LPS lipid A structure (Ernst *et al.* 2007). Indeed, the Lipid A component of *P. aeruginosa* LPS activates multiple pro-inflammatory pathways (Wieland *et al.* 2002 ; Hajjar *et al.* 2002). In cystic fibrosis patients, *P. aeruginosa* adapts by selecting mutants with specific Lipid A modifications that allow resistance to host antimicrobial peptides and increase TLR4 activation (Hajjar *et al*. 2002 ; Ernst *et al*. 2003).

2.2.1.2.4 Alginate

The mucoid phenotype of *P. aeruginosa* is one of the most extensively researched characteristics of this species (Ramsey and Wozniak. 2005). Mucoid strains overproduce the exopolysaccharide called alginate.

Alginate is an unbranched linear polymer of partially acetylated β-d-mannuronic acid and its C5 epimer α-l-guluronic acid. Alginate synthesis begins in the cytoplasm and ends with secretion to the extracellular milieu (Rao *et al*. 2011) . This mucoid alginate-producing phenotype is commonly found in cystic fibrosis airways over the course of *P. aeruginosa* infections. Overexpressed alginate protects *P. aeruginosa* from phagocytosis, antibiotics and even attenuates the host response (Cobb *et al*. 2004). However, because of the excessive production of alginate in cystic fibrosis, alginate has been target in immunotherapy (Theilacker *et al*. 2003; Pier *et al*. 2004) .

2.2.1.2.5 Pyocyanin

Pyocyanin is a blue pigment metabolite of *P. aeruginosa* that has been shown to have numerous pathogenic effects such as depressing host-response apoptosis in neutrophils (Allen *et al*. 2005). In animal models of acute and chronic lung infection, Pyocyanin was shown to be essential to *P. aeruginosa* virulence (Lau *et al*. 2004). Although no therapeutic strategies directly target Pyocyanin, several antioxidant therapies have proved useful in cystic fibrosis. Glutathione delivery through aerosolization improved Forced Expiratory Volume (FEV) (lung capacity test) in cystic fibrosis patients (Wood *et al*. 2003; Griese *et al*. 2004). Lung function was improved in cystic fibrosis patients after an 8-week antioxidant supplemented regimen (Wood *et al*. 2003).

2.2.1.2.6 Pyoverdine

Pyoverdine is a siderophore, a small molecule chelating iron from the environment for use in *P. aeruginosa* metabolism. Pyoverdine has also been shown to play a role in *P. aeruginosa* virulence (Takase *et al*. 2000). In *P. aeruginosa*, Pyoverdine is necessary for infection in several different disease models. The occurrence of Pyoverdine-defective strains in chronic infections of patients with cystic fibrosis and the extremely high sequence diversity of genes involved in Pyoverdine synthesis and uptake indicate that Pyoverdine production is subject to high evolutionary pressure (Visca *et al*. 2007) and its regulate the secretion of other *P. aeruginosa* virulence factors, exotoxin A and an endoprotease and its own secretion (Lamont *et al*. 2002)

2.2.1.2.7 Alkaline protease

It is a fibrin lysing protease secreted by *P. aeruginosa*. It may participate in the pathogenesis of acute lung injury. It has been shown that there is an early massive intra-alveolar formation of fibrin in *P. aeruginosa* acute lung injury and that inhibition of this initial fibrin formation is deleterious in an animal model (Kipnis *et al*. 2004).

2.2.1.2.8 Exotoxin A

It inhibits elongation factor-2 (Yates and Merrill. 2004). This then causes the elongation of polypeptides to cease. ExoA has also been shown to depress host response to infection (Schultz *et al.* 2000).

2.2.1.2.9 Type III secretion system (T3SS)

T3SS is a clinically important virulence mechanism in *P. aeruginosa* that secretes and translocates up to four protein toxin effectors into human cells, facilitating the establishment and dissemination of infections (Aiello *et al*. 2010).

Four T3SS effectors have been identified in *P. aeruginosa* strains: ExoS, ExoT, ExoY, and ExoU. ExoS and ExoT are bifunctional proteins consisting of an N-terminal small G-protein-activating protein (GAP) domain and a C-terminal ADP ribosylation domain, ExoY is an adenylate cyclase, and ExoU is a phospholipase (Engel and Balachandran. 2009). In studies with strains producing each effector separately, ExoU and ExoS contributed significantly to persistence, dissemination, and mortality, while ExoT produced minor effects on virulence in a mouse lung infection model, and ExoY did not appear to play a major role in the pathogenesis of *P. aeruginosa* (Shaver and Hauser. 2004). While not a prototypical effector toxin, flagellin (FliC) also may be injected into the

cytoplasm of host cells from *P. aeruginosa* via the T3SS machinery. (Franchi *et al*. 2009; Miao *et al*. 2008)

2.2.1.2.10 Quorum-sensing

Production of several *P. aeruginosa* virulence factors is coordinated by a cell density monitoring mechanism termed Quorum Sensing (QS) (Rumbaugh, Grisworld and Hamood. 2000).

This ability of *P. aeruginosa* to coordinate the upregulation of virulence genes in a whole population translates into increased pathogenicity of QS capable strains compared to QS deficient mutants throughout a variety of animal models (Lesprit *et al*. 2003). And it use quorum sensing to coordinate the formation of biofilms, swarming motility, exopolysaccharide production, and cell aggregation (Lewis *et al.* 2002). These bacteria can grow within a host without harming it, until they reach a certain concentration. Then they become aggressive; develop to the point at which their numbers become sufficient to overcome the host's immune system, and form a biofilm, leading to disease within the host. Another form of gene regulation that allows the bacteria to rapidly adapt to surrounding changes is through environmental signaling. There are studies have discovered that anaerobiosis can significantly impact the major regulatory circuit of QS. This important link between QS and anaerobiosis has a significant impact on production of virulence factors of this organism (Cornelis. 2008).

2.2.1.3 Epidemiology of *P. aeruginosa*

P.aeruginosa is a usually noncapsulate, nonsporing, and nonfermenting Gram-negative bacillus that is common in the environment, especially in water.

The ability of *P. aeruginosa* to persist and multiply in moist environments (soil detritus and equipment such as humidifiers in hospital wards, urinary catheters, bathroom sinks, and kitchens) is of particular importance in cross infection (Döring *et al*. 1996). *P. aeruginosa* is a pathogen of great relevance in infectious disease for different reasons :

a) Reservoirs for infection can develop, especially in intensive care units, often associated with water in sinks or respiratory equipment .

b) The microorganism displays a predilection for infecting immunocompromised hosts (including burn patients) whose proportion is increasing in hospitals and society .

c) It is the most serious pathogen in ventilator-associated pneumonia and one of the most common in other nosocomial infections.

d) There is an increase in occurrence of *P. aeruginosa* strains with resistance to multiple antibiotics. (National Nosocomial Infections Surveillance System report. 2004).

Acquisition of *P. aeruginosa* begins early in childhood. (Li Z *et al.* 2005). It is believed that the bacterium is initially acquired from environmental sources, but patient-to-patient spread has also been described. (Renders *et al*. 1997; Kosorok *et al.* 1998). In patients with CF, prevalence of Pseudomonal pneumonia ranges from 21% in those younger than 1 year to >80% in those older than 19 years. The increasing longevity of patients with CF has created a significant shift in the proportion of adult patients with CF; their proportion has increased fourfold, from 8% in 1969 to 33% in 1990 (Chen and Rudoy. 2010).

Approximately 70% of patients with CF in the US are chronically infected with *P.aeruginosa* often with multiple strains, and it is generally accepted that chronic bacterial lung infections accelerate the deterioration of lung function that ultimately accounts for approximately 85% of all CF-related deaths (Cystic Fibrosis Foundation).

2.2.1.4 Treatment of *P.aeruginosa*

The acquisition of *P. aeruginosa* as a chronic airway pathogen is associated with a decline in lung function, and increased morbidity and mortality (Emerson *et al.* 2002). Once patients become colonized by *P.aeruginosa* it is imperative to utilize appropriate antipseudomonal therapy for exacerbations of lung disease.

Many practitioners recommend initial outpatient treatment with oral and inhaled therapies, (Tobramycin) and use intravenous therapies for patients with severe exacerbations or those failing outpatient treatments. Standard treatment courses for exacerbations generally last for 14–21 days (Flume *et al*. 2009)

Chronic *P. aeruginosa* infection in CF is often characterized by multidrug resistance. The selection of antibiotic agents is generally based on finding two agents with differing mechanisms of action which demonstrate in vitro efficacy on conventional drug susceptibility testing. Another strategy involves utilizing multiple combination bactericidal antibiotic or 'synergy' testing. This technique offers the potential benefit of identifying agents that may have additive or synergistic effects, even in those bacterial strains that demonstrate resistance by conventional methods (Saiman *et al*. 1990). However, a randomized, clinical trial evaluating the efficacy of combination antibiotic susceptibility testing in CF patients with multidrug-resistant Gram-negative organisms failed to improve

clinical outcomes (Aaron *et al.* 2005). The fluoroquinolones (ciprofloxacin or levofloxacin) are oral agents most commonly used for outpatient treatment of exacerbations associated with *P. aeruginosa* (Remmington *et al*. 2007). Common intravenous regimens include the use of an antipseudomonal β-lactams (pipercillin or ticarcillin), third-generation cephalosporins (ceftazidime), fourth-generation cephalosporins (cefepime), carbapenems (meropenem or imipenem) or monobactams (aztreonam), combined with an aminoglycoside (amikacin, gentamicin or tobramycin) can be administered in an inpatient or ambulatory setting via chronic intravenous access (Bosworth and Nielson. 1997). There are research indicates that azithromycin may enhance chloride efflux from airway epithelial cells, alter biofilm formation, protect airway epithelial cells from damage and have direct antioxidant properties (Bergamini *et al*. 2009).

2.2.2 Staphylococcus aureus

S.aureus is one of the most commonly isolated pathogens from the respiratory tract of patients with cystic fibrosis and one of the first microbes to infect the lungs of patients with cystic fibrosis (Saiman and Siegel. 2004). The prevalence of respiratory tract colonization/ infection with both methicillin-susceptible and methicillin-resistant *S.aureus* has increased over the past decade. Affinity of staphylococcus for cystic fibrosis mucus, mucociliary abnormalities and unknown factors contribute to persistent colonization with this organism causing progressive pulmonary damage and possibly influencing *Pseudomonas* infection (Razvi *et al*. 2009).

The clinical significance of colonization/infection with these pathogens is variable, leading to numerous therapeutic strategies: primary prophylaxis,

eradication, treatment of cystic fibrosis pulmonary exacerbations, and treatment of methicillin-resistant *S. aureus*. Since it is a frequent ethological agent of human diseases and exhibits resistance to a growing number of therapeutic agents, *S. aureus* is also one of the most intensively studied bacterial species (Plata *et al.* 2009).

2.2.2.1 Characterization

S.aureus is a Gram-positive spherical bacterium approximately 1 μm in diameter. Its cells form grape-like clusters. It is often found as a commensal associated with skin, skin glands, and mucous membranes, particularly in the nose of healthy individuals (Crossley and Archer. 1997). It has been estimated that approx. 20–30% of the general population are *S. aureus* carriers (Heyman. 2004).

On a rich medium, *S. aureus* forms medium size "golden" colonies. On sheep blood agar plates, colonies of *S. aureus* often cause β-hemolysis (Ryan and Ray. 2004) The golden pigmentation of *S. aureus* colonies is caused by the presence of carotenoids and has been reported to be a virulence factor protecting the pathogen against oxidants produced by the immune system (Liu *et al*. 2005). Staphylococci are facultative anaerobic capable of generating energy by aerobic respiration, and by fermentation which yields mainly lactic acid.

Staphylococcus spp. is catalase-positive, a feature differentiating them from *Streptococcus spp*., and they are oxidase-negative and require complex nutrients, e.g., many amino acids and vitamins B, for growth. *S. aureus* is very tolerant of high concentrations of sodium chloride, up to 1.7 molar.(konrald *et al*. 2009) Another feature of the Staphylococcus genus is the cell wall peptidoglycan

structure that contains multiple glycine residues in the cross bridge, which causes susceptibility to lysostaphin (Crossley and Archer. 1997).

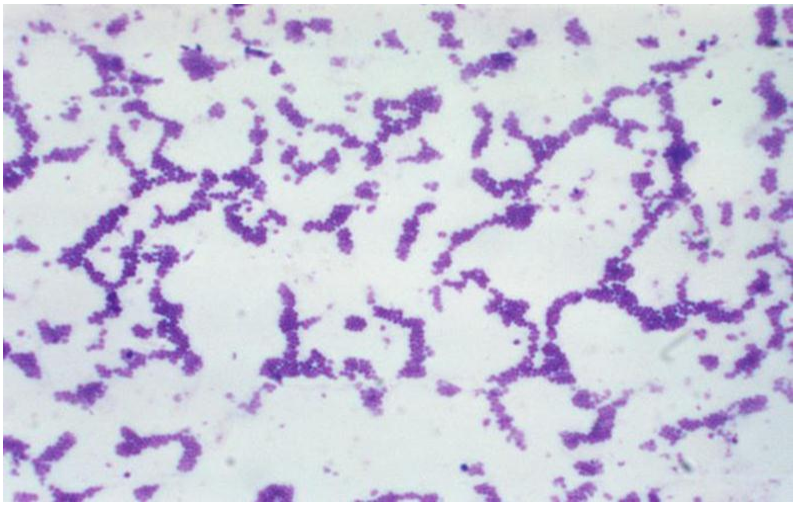


Figure 2.6 *Staphylococcus aureus*

Source:http://vaccinenewsdaily.com/national_institutes_of_health_/318082-nih-studying-newly-emerging-staphylococcus-aureus-strain/

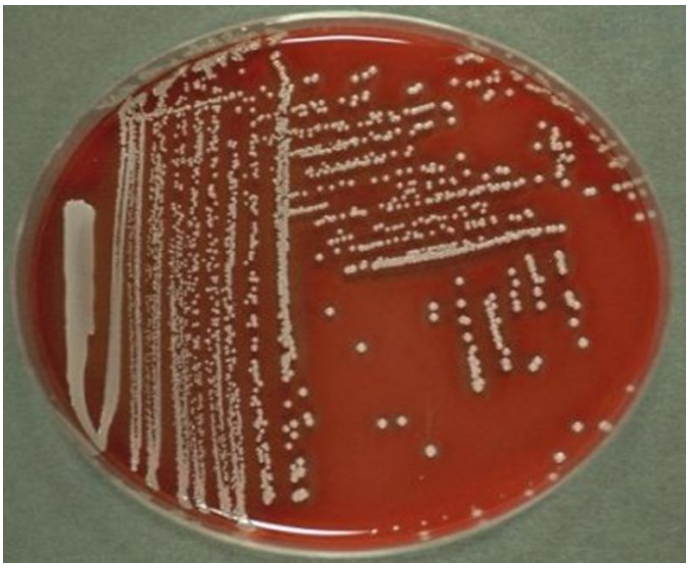


Figure 2.7 *S.aureus* colonies on blood agar

Source:http://pictures.life.ku.dk/atlas/microatlas/veterinary/bacteria/Staphylococcus_aureus/pop1.html

S. aureus produces coagulase which interacts with prothrombin in the blood causing plasma to coagulate by converting fibrinogen into fibrin. (Plata *et al.* 2009).

2.2.2.2 Virulence factors

S. aureus has a variety of virulence factors, which include both structural and secreted products participating in pathogenesis of infection.

2.2.2.2.1 Attachment agents:

S.aureus carries numerous surface proteins named "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) that mediate attachment to host tissues and initiate colonization leading to an infection (Gordon and Lowy. 2008).

Fibronectin binding proteins A and B (FnbpA and FnbpB) participate in attachment of bacterial cells to an extra-cellular matrix component fibronectin, and to plasma clot. Collagen binding protein (CAN), is necessary for adherence of *S. aureus* to collagenous tissues and cartilage and it has been shown that antibodies against CNA block the bacteria attachment to those tissues (Patti *et al.* 1994). Clumping factor A and B (ClfA and ClfB) mediate clumping and adherence of bacterial cells to fibrinogen in the presence of fibronectin. Clumping factors are thought to play a significant role in wound and foreign body infections and it has been shown that ClfA mutant is less virulent than the wild type isogenic strain (Foster and Hook. 1998). Plasma-sensitive surface protein (PlS), once processed by plasmin, participates in binding to both fibrinogen and fibronectin (Hauck and Ohlsen. 2006).

Protein A is a hallmark of *S. aureus* which is encoded by the spa gene and is a cell wall-associated protein that binds to the Fc (Fragment Crystallizable) region of immunoglobin G (IgG). Protein A binds IgG in "wrong orientation" on the surface of *S. aureus* cells which is thought to disrupt opsonization and phagocytosis (Switalski *et al*. 1993). It presents at sites of damage of endothelium, and as a result, it can play a role in adherence and induction of endovascular diseases by *S. aureus* (Hartleib *et al*. 2000). Implanted biomedical device-related *S. aureus* infections depend on the pathogen's ability to attach to the surface of the biomaterial and consequently to form a mucoid biofilm.

Biofilms are complex bacterial populations which are surface-attached and enclosed in a polysaccharide matrix, composed of poly-N-acetylglucosamine (PNAG). PNAG production depends on proteins encoded by the intracellular adhesion (ICA) operon (Fitzpatrick *et al*. 2005). It was reported that 60% of *S. aureus* strains were able to produce biofilm (Arciola *et al*. 2001a, 2001b). There are studies indicate that among clinical isolates of *S. aureus*, only between 45% and 70% (depending on the type of infection) strains were able to form biofilm (Grinholc *et al*. 2007). Those studies also suggested that no correlation exists between biofilm production and the type of staphylococcal infections.

2.2.2.2.2 Exotoxins

S.aureus is capable of secrete toxins that disrupt membranes of host cells. Cytolytic toxins form β-barrel pores in the cytoplasmic membranes and cause leakage of the cell's content and lysis (Foster. 2005). It secrets several cytolytic toxins, among them alpha-hemolysin, beta-hemolysin, gamma-hemolysin, leukocidin, and Panton-Valentine leukocidin (PVL) (Kaneko and Kamio. 2004).

Alpha-hemolysin, encoded by the hla gene, inserts into eukaryotic membranes and oligomerizes into a β-barrel that forms a pore which causes osmotic cytolysis. Alpha hemolysin is particularly cytolytic toward human platelets and monocytes (Menestrina *et al*. 2001).

Pneumonia due to PVL-positive *S. aureus* strains was investigated by Gillet *et al* in 2002, by comparison with PVL-negative *S. aureus* pneumonia. PVL-producing *S. aureus* strains caused hemorrhagic, necrotizing pneumonia that rapidly progressed towards an acute respiratory distress syndrome with hemoptysis and leukopenia, usually in otherwise healthy children and young adults.

In 2002, Dufour *et al*. detected PVL genes in methicillin-resistant *S. aureus* strains isolated from French patients with community-acquired infections. In 2003, Vandenesch *et al*. detected PVL genes in methicillin-resistant *S. aureus* clones from Europe, North America and Oceania. This discovery of PVL genes in highly epidemic clones circulating in the community has further focused the minds of the scientific community on this devastating toxin .

2.2.2.2.3 Superantigen toxin

S. aureus generates a group of powerful immuno- stimulatory proteins implicated in gastroenteritis and toxic shock syndrome. They are resistant to heat denaturation and proteases. These toxins have the ability to cross-link Major histocompatibility complex (MHC) class II molecules located on antigen-presenting cells with T-cell receptors, forming a trimolecular complex. Formation of the complex induces intense T-cell proliferation in an antigen-independent manner resulting in massive cytokine production and release which causes

capillary leak, epithelial damage and hypotension (Baker and Acharya. 2004). The primary function of superantigens is thought to weaken the host's immune system, sufficiently to allow the pathogen to propagate and the disease to progress (Kotzin *et al*. 1993).

2.2.2.2.4 Enzymes

During infection, *S.aureus* produces numerous enzymes, such as proteases, lipases, and elastases, which enable it to invade and destroy host tissues. *S. aureus* is also capable of producing septic shock. It does this by interacting with and activating the host immune system and coagulation pathways. Peptidoglycan, lipoteichoic acid, and α-toxin may all play a role in infection. (Timmerman *et al*. 1993; Heumann *et al*. 1994).

2.2.2.3 Epidemiology of *S.aureus*

The epidemiology of *S. aureus* in CF patients has been investigated in different studies. In order to extend the knowledge of the population of *S.aureus* chronically infecting CF patients, all the isolates should be systematically genotyped with a high degree of discrimination which is difficult using the currently available techniques. The polymorphism of the *Staphylococcus* protein A gene (spa), first used in 1996, by Frenay *et al*, to genotype *S. aureus* and further evaluated by Shopsin *et al*. has proven to be very useful to investigate *S. aureus* genetic diversity.

2.2.2.4 Treatment of *S.aureus*

Some of the first reports of CF lung disease were associated with *S. aureus* infection. Historically, significant improvements in patient longevity have been

associated with the advent of antistaphylococcal therapy (Fitzsimmons. 1993). An early placebo controlled clinical trial that assessed the response to cephalexin as a prophylactic treatment to prevent *S. aureus* in 17 subjects with CF (Loening *et al*. 1979). Several studies have evaluated primary prophylaxis for *S. aureus* using a variety of agents, as reviewed by (Smyth. 2005) Overall, these studies demonstrated a reduction in acquisition of *S. aureus* among children receiving antibiotics. The most serious concern with the chronic use of antistaphylococcal antibiotics is the potential increased risk for infection with *P. aeruginosa*. In 2002, Stutman *et al*. reported increased *P. aeruginosa* acquisition in cystic fibrosis children followed from age 2 to 6 years treated with continuous cephalexin versus placebo. This effect was not observed with flucloxacillin. The UK Cystic Fibrosis Trust Antibiotic Group recommended a 2-week course of antibiotics to eradicate *S. aureus* as an alternative to prophylactic therapy (UK Cystic Fibrosis Trust Antibiotic Report. 2002).

2.2.3 *Acinetobacter baumannii*

A.baumannii is known as multi drug resistance, it can survive under a wide range of environmental conditions and it cause of outbreaks of infections (Fournier *et al.* 2006). This organism is seen less frequently in CF than in immunocompromised patients (Rahal and Urban. 2000).This bacterium is associate with pneumonia, bacteremia, wound infections and urinary tract infections(Maragakis and Perl.2008). Pneumoniacaused by *Acinetobacter* species can present major challenges for physicians, Community-acquired *Acinetobacter* pneumonia can also occur among certain at-risk populations. (Hartzell *et al*. 2007).

2.2.3.1 Characterization

A.baumannii is a Gram-negative, nonmotile, obligate aerobic coccobacilli (Young *et al.* 2007) it belongs to family Moraxellacease and it has been recovered from soil, water, animals, and humans (Fournier *et al.* 2006**).** It is oxidase-negative, catalase-positive and non-fermentative. Colonies are normally smooth, sometimes mucoid Sheep blood agar and MacConkey agar are conventionally used for detection of *A. baumannii* in clinical cultures (Ajao *et al.* 2011).

2.2.3.2 Virulence factors

In the past, *Acinetobacter* was considered to be an organism of low virulence. The occurrence of fulminant community-acquired *Acinetobacter* pneumonia indicates that these organisms may sometimes be of high pathogenicity and cause invasive disease (Peleg *et al.* 2008). It's known as emerging pathogen, attention to it has increased significantly. Most of the studies have been focus on antibiotic resistance mechanisms, but little is known about its virulence factors (Jeremy *et al.* 2012).Attachment and adherence to medical equipment and environmental surfaces appear to be important for *A.baumannii* pathogenesis (Gustavo *et al*. 2011). It is adheres both to biological and abiotic surfaces, on which it is able to form biofilms (Lee *et al*. 2008). Most of studies on *A.baumannii* pathogenesis were focused on the ability of *A.baumannii* to form biofilms on abiotic surfaces (Tomaras *et al*. 2003). This is an important pathogenic feature of many bacteria, facilitating colonization of prosthetic material and contributing to drug resistance and evasion of the host immune system in vivo .Pili and fimbriae are important for initial adhesion, followed by

the production of exopolysaccharide, an important constituent of mature biofilm that suppresses the activity of neutrophil (Nicola *et al*. 2008). *A. baumannii* has a predilection for causing respiratory tract infections, and therefore most published studies on the respiratory infections have focused on *A.baumannii* cytotoxicity of respiratory tract cell lines (Peleg *et al*. 2008).

2.2.3.3 Epidemiology of *A.baumannii*

This pathogen has emerged as a major cause of Health care—Associated Infections (HAI) because of its antimicrobial resistance and its ability to cause large nosocomial outbreaks (Fournier *et al*.2006). A few studies has been investigated the epidemiology of *A.baumannii* isolates colonizing the respiratory tract of hospitalized patients (Al-Dabaibah *et al*. 2012). *A. baumannii* can survive in dry conditions (Hawkey. 1998) and during outbreaks has been recovered from different sites, including bed curtains, furniture and hospital equipment (Van den Broek *et al*. 2006). These observations, and the success that cleaning and disinfecting patients' rooms has had in halting outbreaks, emphasize the role of the hospital environment as a reservoir for *A.baumannii* during outbreaks (Dijkshoorn *et al.* 2007). The emergence of multidrug-resistant (MDR) clinical isolates has been widely reported from hospitals in Europe, North America, Argentina, Brazil, China, Taiwan, Hong Kong, Japan, and Korea (Perez *et al*. 2007) According to survey of University of Maryland Medical Center (UMMC) clinical culture data from 2002 to 2008 suggested that the prevalence of *A. baumannii* did not change substantially in the years before, during or after the study period (Kerri *et al*. 2010).

2.2.3.4 Treatment of *A.baumannii*

A.baumannii is an important and difficult-to-treat pathogen because of its multidrug resistance. Therapy for pneumonia caused by *A.baumannii* should be based on susceptibility results.

Antimicrobial resistance poses great limits for therapeutic options in infected patients, especially if the isolates are resistant to carbapenems, (imipenem, meropenem). Colistin has a good activity against this organism, it has been used because of the increasing incidence of multidrug-resistant *A. baumannii* (Jain and Danziger. 2004).Other therapeutic options has been used include ß-lactamase inhibitors, aminoglycosides, polymixyn and tigecycline (Maragakis and Perl. 2008) Aminoglycosides including Amikacin and tobramycin are the 2 agents that appear to retain activity against many *A.baumannii* isolates (Fishbain and Peleg. 2010).

Figure 2.8. *A.baumannii* on blood agar

Source: http://www.acinetobacter.org/

2.2.4 *Klebsiella pneumoniae*

K. pneumoniae is an important nosocomial pathogen, most frequently causing pneumonia, and urinary tract, wound or blood infections (Brisse *et al*. 2009). It has long been recognized as a possible cause of community-acquired pneumonia (Yu *et al*. 2007). Species of Enterobacteriaceae such as *K.pneumoniae* are not chronic colonizers of the CF patient's airways and appear to play only a minor role in lung infection of these patients (Lipuma. 2010). In 2011, Leao *et al*. have reported the first two CF patients were colonized with KPC-2-producing *K. pneumoniae*. In Epidemic and endemic nosocomial infections caused by *Klebsiella* species, they are leading causes of morbidity and mortality (Cryz *et al.* 1985). Many hospital cases around the world have been linked to *K. pneumoniae*. Therefore, more studies of the strains were important and performed. The whole genome of *K.pneumoniae* have been sequenced, there was evidence showed a significant genomic diversity and sequence acquisition among *K. pneumoniae* pathogenic strains (Wu *et al.* 2009)

2.2.4.1 Characterization

K.pneumoniae is a gram-negative, non-motile, aerobic rod-shaped bacterium, 0.3~1.0μm in diameter, 0.6~6.0μm length long. It is lactose fermenting, Hydogen sulfide production (H_2S) - and indole-negative, has a positive Voges-Proskauer (VP) reaction, it use Citrate (CIT) as a sole carbon source in the media It produces large "sticky" colonies when plated on nutrient media and the suitable growing temperature for it is about 35~45 ºC (Ryan and Ray. 2004).

2.2.4.2 Virulence Factors

K.pneumoniae produces a number of virulence factors that contribute to pathogenesis, including Fimbrial adhesions, O antigens, and capsular antigens (Schabereiter-Gurtner *et al*. 2005)

2.2.4.2.1 Capsule

LPS is a major component of Gram-negative bacterial cell walls and possesses potent immunomodulatory properties (Strieter *et al*. 1990). Since the first identification of *Klebsiella* as a cause of pneumonia by pathologist Karl Friedländer in 1882, Capsular Polysaccharide (CPS) has been established as the species' most distinguishing characteristic and most studied virulence factor (Eisenstadt and Crane, 1994). LPS which contains the O antigen, and CPS, which contains the K antigen (Podschun and Ullmann 1998). Lost or decreased expression of either the O or K antigen renders *K. pneumoniae* less mucoid and less virulent (Yeh *et al*. 2010) The importance of capsule as a virulence factor for *K. pneumoniae* has also been examined using *in vivo* models of colonization and pathogenesis *(*Matthew *et al.* 2005). The capsule is generally considered to be an important virulence factor in *K. pneumoniae*. Its provide protection against desiccation and attack from phages (Schabereiter-Gurtner *et al*. 2005)

2.2.4.2.2 Pili

Pili is protein structures that recognize a wide range of molecular motifs and provide targeting of the bacteria to specific tissue surfaces in the host (Klemm and Schembri. 2000) Most clinical *K. pneumoniae* isolates are able to produce the two Fimbrial adhesions, type 1 fimbriae and type3 fimbriae. Type 1 fimbriae

mediate adhesion to mannose-containing structures on host cells and extracellular matrix and are present in many species of *Enterobacteraceae* (Struve *et al*. 2008). Type 3 fimbriae mediate adhesion to several cell types in vitro (Tarkkanen *et al*. 1990).

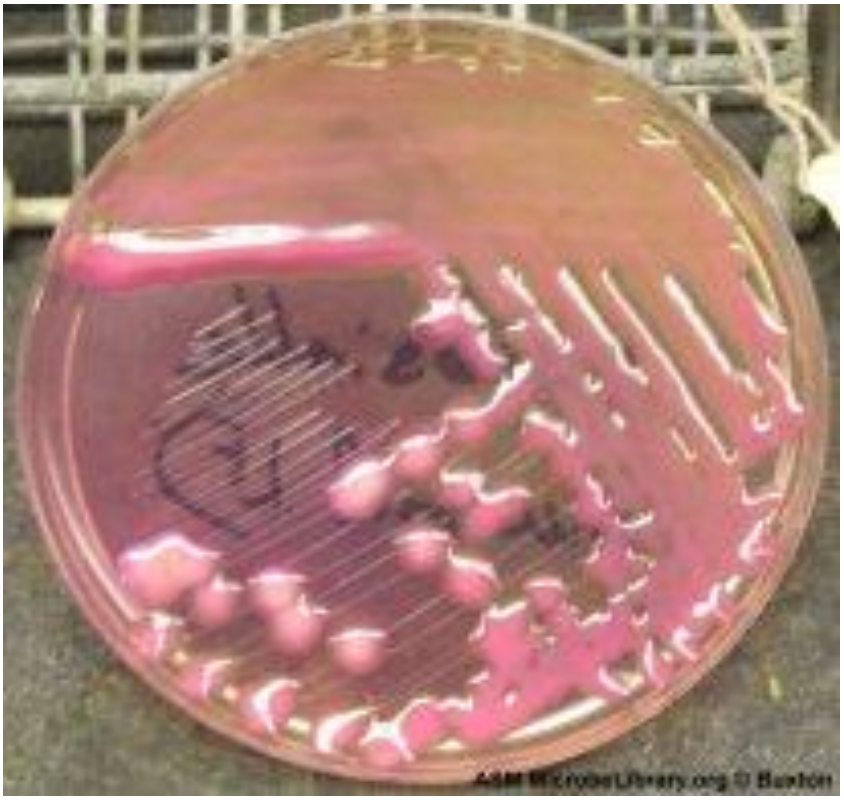


Figure 2.9 Colonies of *Klebsiella pneumoniae* on MacConkey agar.

Source: http://microbitos.wordpress.com/2010/06/14/morfologia-colonial-bacteriana/.

2.2.4.3 Epidemiology of *K. pneumoniae*

K.pneumoniae is present as saprophyte in the nasopharynx (Podschun and Ullmann, 1998). In different countries, *K. pneumoniae* has been ranked among the top ten organisms causing blood stream infection, pneumonia and other invasive infections hospitalized patients (Jones. 2010). The analysis of the phenotypic markers, including biotypes and antimicrobial susceptibility patterns have been used for study the epidemiology of *K.pneumoniae* (Loureiro *et al*. 2001). The availability of sensitive and discriminative tests that permit differentiation between individual and epidemic strains of *K.pneumoniae* may help in the epidemiological studies on the spread of this organism (Ben-Hamouda *et al.* 2003).

2.2.4.4 Treatment of *K. pneumoniae*

K.pneumoniae can be treated by aminoglycosides and cephalosporins; the choice is depend on the patient's health condition, medical history and severity of the disease. Some of *K.pneumoniae* strains are resistant to quinolones and aminoglycosides, which is leaving the therapeutic options limited to tigecycline or Colistin (Nordmann *et al.* 2009). However, Yigit *et al*. (2001) reported that there are *K. pneumoniae* strains that resistant to carbapenems antibiotics. Infections caused by carbapenems resistant strains have few treatment options (Livermore *et al*. 2011) and are associated with mortality rates upwards of 50% (Ben-David *et al.* 2012).

2.3 Antibiotic Resistant in CF Associated Bacteria

Bacterial strains in CF patients are resistant to many antimicrobial agents used in the hospital. These bacteria have the distinctive capacity via multiple mechanisms to become resistant to virtually all the antibiotics available commercially (Falagas and Kasiakou. 2005; Wang *et al*. 2006). World Health Organization also warned the community that multidrug resistant bacteria are emerging worldwide which is a big challenge to healthcare (Sikarwar and Batra , 2011).

General resistance is due to a combination of factors:

*It is intrinsically resistant to antimicrobial agents due to low permeability of its cell wall

*It has the genetic capacity to express a wide repertoire of resistance mechanisms

*It can become resistant through mutation in chromosomal genes which regulate resistance genes.

*It can acquire additional resistance genes from other organisms via plasmids and bacteriophages.

There are three basic mechanisms by which organisms resist the action of antimicrobial agents: restricted uptake and efflux; drug inactivation and changes in targets. (lambert. 2002). Efflux pumps are common components of multidrug-resistant *P.aeruginosa* isolates, and prevent accumulation of antibacterial drugs within the bacterium, extruding the drugs from the cell before they have the opportunity to achieve an adequate concentration at the site of action. The efflux pumps often work together with the limited permeability of the *P. aeruginosa*

outer membrane to produce resistance to β-lactams, fluoroquinolones ,tetracycline, chloramphenicol, macrolides, and aminoglycosides(Schweizer. 2003; Poole and Srikumar. 2001).

The changes in targets mechanisms result from mutational changes in target enzymes which result in maintenance of their vital role in cell metabolism but resistance to the action of selective inhibition by antibiotics (lambert. 2002).

The inactivation of antibiotic mechanism is occurring in Aminoglycoside-resistance in *Pseudomonas spp*. It happens by the genetic expression of enzymes responsible for the modification of the aminoglycosides. There are three specific classes of aminoglycoside-modifying enzymes (AMEs), that have been identified, the N'acetyltransferases, O-phosphotransferases and O-adenyltrnsferases (Poole. 2005). The genes encoding these enzymes have also been identified fused in a "gene-cassette" type structures called integrons, typically fused with β-lactamase genes thus conferring a multiple resistance on the organism (Laurent. 2001). These novel gene-cassettes are thought to migrate between strains carried on small circular plasmids that incorporate into the cell's genome. As a result, strain diversity and the evolution of new Pseudomonas species equipped with varying degrees of antibiotic resistance exist, emerging as serious infections in nosocomial environments.

By 1942, the first penicillin resistant strains of *S.aureus* had been isolated in hospitals (Deurenberg *et al*. 2007) .Within 2 decades, ~80% of both hospital- and community-acquired *S. aureus* isolates were penicillin resistant (Appelbaum. 2006)

The Staphylococcal resistance to penicillin is mediated by penicillinase (a form of β-lactamase) production: an enzyme that cleaves the β-lactam ring of the penicillin molecule, rendering the antibiotic ineffective. Penicillinase-resistant β-lactam antibiotics, such as methicillin, nafcillin, oxacillin and flucloxacillin, are able to resist degradation by staphylococcal penicillinase .The introduction of methicillin in 1961 was rapidly followed by reports of methicillin resistance in *S. aureus*. MRSA strains are found worldwide, and most are multidrug resistant (Appelbaum. 2006). Study of early isolates of MRSA showed that a key genetic component responsible for resistance, mecA, is not native to the *S. aureus* genome. The staphylococcal chromosome cassette mec (SCCmec) has been characterized as a novel, mobile resistance element that differs from both transposons and bacteriophages (Berger and Rohrer. 2002). MRSA typically spreads through clones; however, it is known that the mec gene has been transmitted between *S. aureus* strains and, possibly, between other staphylococcal species (Berger and Rohrer.2002).Antimicrobial resistance among *Acinetobacter* species has increased substantially in the past decade (Lockhart *et al*. 2007). The capacity of *Acinetobacter* species for extensive antimicrobial resistance may be due in part to the organism's relatively impermeable outer membrane and its environmental exposure to a large reservoir of resistance genes (Bonomo and Szabo. 2006). Definitions of multidrug-resistant *Acinetobacter* species vary, referring to a wide array of genotypes and phenotypes, two of the most common definitions of multidrug resistance are carbapenems resistance or resistance to ⩾3 classes of antimicrobials (Falagas *et al.* 2006). Some strains are susceptible only to polymyxins—peptide antibiotics

that are not routinely used because of earlier reports about toxicities (Urban *et al.* 2001).

As we mentioned above the bacteria can acquire additional resistance genes from other organisms via plasmids and bacteriophages. The relationship between certain plasmids and resistance to some antibiotics had been reported previously (Shahcheraghi *et al.* 2003). Approximately 20% of *Klebsiella pneumoniae* in USA are not susceptible to third-generation cephalosporins. Such resistance in *K pneumoniae* to third-generation cephalosporins is typically caused by the acquisition of plasmids containing genes that encode for extended-spectrum β-lactamases (ESBLs), and these plasmids often carry other resistance genes as well(Paterson. 2006). Plasmid-mediated β-lactamase may contribute to resistance in only *P.* aeruginosa strains isolated from 1 of 17 CF patients with chronic lung infections. However, Incompatibility Group P plasmids (In complete plasmids) can transfer chromosomal DNA, which then becomes integrated into the chromosome (Palleroni *et al*. 1994). In addition, free bacteriophages have been demonstrated in sputum from patients with CF (Ojeniyi *et al*. 1991) The increased level of antibiotic resistance and the emergence of such strains highlight the plasticity of *S. aureus* genomes and the remarkable speed of bacterial evolution, especially by horizontal gene transfer including bacteriophages, SCCmec, plasmids and transposons, to allow the bacteria to very rapidly adapt to a specific niche (Rolain *et al*. 2009).

2.4. Phenotype Methods for identification bacteria

Pathogen identification is crucial to confirm bacterial infections and to guide antimicrobial therapy. Clinical laboratories develop reliable methods for bacterial

identification. Identification to the species level typically requires numerous consecutive steps based on defined phenotypic assays. Biochemical tests including oxidase, catalase, coagulase and API 20 are conventional methods that routinely used in Clinical laboratories for identification .The definitive results require 24 to 48 h after isolation, using these methods (Cherkaoui *et al*. 2010).

2.4.1 Isolation & cultivation

Cultivating bacteria is an essential technique in diagnostic microbiology. The culture- based methods have many benefits include the ability to quantify the viable bacteria in a sample, differentiating between other micro-organisms by using their colonial morphology and obtaining a pure sample for further testing, (Minion. 2010), such as biochemical, immunological and molecular tests. Diagnosis of lower respiratory infection in CF patients is commonly by culturing sputum specimens, the infants and some of young CF patients cannot expectorate sputum. Throat swabs, nasopharyngeal suction, oropharyngeal suction and bronchoalveolar lavage specimens have been used for microbiological assessment in these young patients. (Taylor *et al*. 2006) the oropharyngeal suction may help in diagnosis of acute pulmonary infection (Avital *et al*. 1995), on the other hand, Armstrong *et al,* demonstrated that oropharyngeal cultures have only a poor positive predictive value. Kabra *et al*. 2004, showed that throat swabs cultures can be used reliably for identification of airway pathogens. They achieved this result by obtained the throat swabs for culture after physiotherapy. Induced sputum by Hypertonic saline inhalation has been also used for airway infection and inflammation in CF (Claudia *et al*. 2003).

2.4.1.1 Sputum sample

Sputum culture has been used by the respiratory physician to provide insight into the bacteria present in many airway diseases such as pneumonia, CF and Chronic Obstructive Pulmonary Disease (COPD) (Wilson. 2002).

Once a sputum sample obtain, it should be process as soon as possible (Mandell *et al*. 2007). The appearance of the specimen, viscosity, turbidity and color should be reported and described. Gram stain is the first important prosses in sputum examination, dependent on the stain result, specimen will accepted. For evaluation of sputum Gram stain quality many guidelines have been proposed, that could be by using different combinations and cutoffs of squamous epithelial cells (SECs) and/or polymorphonuclear leukocytes (PMNs) per low power field (LPF, 10X objective) (Bartlett and Mundy. 1995). In 2008, Miyashita, *et al* have considered the good quality of sputum sample, if they had numerous polymorphonuclear cells (>25 in a ×100 microscopic field) and few squamous epithelial cells (<10 in a ×100 microscopic field). In study conducted in 2010, the use of a wash technique and quantitative culture of sputum has been shown to reduce the number of contaminants by 100 to 1000 fold and has enhanced the value of sputum samples (Ziyade and Yagci. 2010).

In 2005, Murayama *et al*. have used washing technique by washing the specimen twice, and they have recommended this technique to clarify bacterial pathogens in lower respiratory tract infections among children.

Quantitative culture improves diagnostic accuracy and culture results from expectorated sputum (Philomina. 2009). Liquefaction and homogenization have been used as a quantitative method to ensure, accurate representative; sputum cultures (Margaret. 1980). Dithiothreitol (DTT) was used by Hammerschlag *et al*.

1980, as mucolytic agent to homogenize the CF sputum. In study conducted by Pye *et al*. in 1995, (DTT) was compared to homogenisation with saline or glass beads for recovery of bacteria from sputum samples, The quantity of pathogen recovered was significantly higher from samples homogenised using DTT compared to either saline or glass beads. A poor correlation was observed between the predominant organism identified after qualitative culture of non homogenised sputum and the numbers of bacteria obtained using homogenisation with DTT and quantification (Wong *et al*. 1984). However, according to the report of the UK Cystic Fibrosis Trust Microbiology Laboratory Standards Working Group (September 2010), There is insufficient evidence to properly assess the value of homogenization when processing sputum samples from CF patients.

2.4.2 Antibiotic Susceptibility tests

2.4.2.1 Automated Susceptibility Testing

Over the past 20 years several automated systems have been developed for the identification (ID) and antimicrobial susceptibility testing (AST) of the organisms. The BD Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD) is a instrument for the rapid ID and AST of clinically significant organisms. The ID portion of the panel utilizes conventional, chromogenic and fluorogenic biochemical tests. Phoenix AST is broth-based microdilution utilizing a redox indicator to detect organism growth. Panels are incubated and read by the instrument at 20 minute intervals. The Phoenix also has an expert system for AST interpretation. The Phoenix offers potential advantages compared to other systems including no reagent additions and no need to perform

off line tests (Mccarter *et al*. 2005). The MicroScan is another automated system and it offers a choice of an overnight panel or the more rapid identification-susceptibility panel that uses fluorescent technology. Depending on the laboratory's preference, either a combination identification-susceptibility panel or separate identification and susceptibility panels may be used. The WalkAway system consists of an incubator-reading unit that can read either as a conventional panel or a fluorescent rapid-read panel. Once the panels are placed into the incubator-reader unit, (Chapin and Musgnug 2003) the remainder of the process is fully automated. A bar code reader identifies each panel, incubates it for the appropriate amount of time, and moves the panels to the reading position. Organism identification can be determined within an average of 2.5 hours by rapid methods but may take 6-18 hours with use of conventional testing methodologies. Final results for susceptibilities take an average of 20 hours, with a range of 16.8-27.8 hours depending on type of organism (Sellenriek *et al*. 2005; Chapin and Musgnug 2003). The Phoenix system compared favorably with the MicroScan WalkAway system and is an acceptable alternative for the ID and susceptibility testing of commonly encountered species of Enterobacteriaceae and non-glucose-fermenting gram-negative bacilli (Snyder *et al.* 2008).

2.4.2.2 Disk diffusion method

The principle behind the disk diffusion method is that antibiotic molecules diffuse out from a disk into the agar, creating a dynamically changing gradient of antibiotic concentrations while the organism being tested starts to divide and growth progresses toward the critical mass (Bauer *et al*. 1966). The zone edge is where the concentration of antibiotic begins to inhibit the organism reaching an overwhelming cell mass. At this point, the density of cells is high enough to

absorb antibiotic in the immediate vicinity, thus maintaining concentrations at sub inhibitory levels, and enabling the test organism to grow. For most rapidly growing aerobic and facultative anaerobic bacteria, the critical time it takes for the organism to absorb the antibiotic varies from 3-6 hours, but interpretation by the microbiologist generally occurs between 18 and 24 hours (Kuper *et al*. 2009).

2.5 Genotype Methods for identification bacteria

Molecular biology has the potential to revolutionize the way in which diagnostic tests are delivered in order to optimize care of the infected patients, whether they appear in hospital or in the community (Millar *et al.* 2007), the Genetic techniques have been used in clinical microbiology for detection and identification of microorganisms (Clarridge. 2004). For obtaining an accurate and rapid characterization of bacterial isolates, several molecuolar typing methods have been used, such as Restriction Fragment Length Polymorphism (RFLP), Multiple-Locus Variable-Number Tandem-Repeat (MLVA), Pulsed-Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), Amplified Fragment Length Polymorphism (AFLP) and Random amplification of polymorphic DNA (RAPD PCR) (Yıldırım *et al*. 2011).

2.5.1 DNA isolation from Bacteria

Genomic DNA/RNA isolation is the first and the most important requirement in carrying out molecular biology techniques such as PCR, restriction enzyme analysis, Southern hybridization, genomic DNA library construction (Kalia *et al*.1999) mutation detection or linkage analysis (Aldous *et al*. 2005) as well as DNA microarray gene expression profiling (Omidi *et al.* 2005) Furthermore, bacterial fingerprinting methods such as RAPD, RFLP and

Repetitive Extragenic Palindromic (REP) are the most dominant ways for the evaluation of genetic variations (Yang *et al*. 2009). All these techniques require a reasonable amount of DNA with good enough quality, fidelity and concentration (Li *et al*. 2007)

Various methods have been described for the rapid isolation of chromosomal DNA from prokaryotic cells (Yacoobr and Zealey. 1988).Bacterial DNA can be extracted from the body fluids of patients with suspected bacterial disease and then be amplified by PCR and sequenced to enable detection and bacterial identification (Clarke. 2002). An efficient method of DNA extraction that produces pure, high-quality DNA is crucial to the success of PCR and sequencing reactions and the subsequent treatment of disease (Mcorist *et al.* 2002). The manual methods of DNA extraction are simple and reliable and are suitable for extraction of low numbers of sample. (Smit *et al.* 2000).

In 1989, Pitcher and Saunders, have been used a simple method of extracting DNA using guanidium thiocyanate, a strong protein denaturant, which has been used to lyse a variety of both prokaryotic and eukaryotic cells for nucleic acid extraction, particularly when high nuclease activity was a problem. As the demand for molecular tests increases, new automated methods of DNA extraction have been developed to handle larger numbers of clinical samples (Harris *et al*. 2002). There are a number of DNA extraction kits available, based on a 96-well plate format, which allows integration into the workstation of a robotic liquid handler. This allows a much higher throughput of samples, is less work intensive, and produces PCR-ready bacterial DNA (Harris *et al*. 2002). DNA extraction methods based on the absorption of DNA to metallic beads are also available and, again, can also provide high-throughput DNA extraction (Smit *et al*. 2000).

In cystic fibrosis patients DNA isolation from sputum samples is experimentally simple and is not negatively affected by bacterial viability, competition for nutrients or the presence of growth inhibitory compounds. Various strategies have been described for assessing the presence of mixed bacterial populations of unknown species in different ecological niches (Van Belkum *et al.* 2000).Because acidic polymerases in sputum are known to inhibit DNA polymerases, successful sputum preparation must separate the nucleic acids from the inhibitors. Phenol-chloroform extraction of DNA followed by ethanol precipitation has been used with sputum specimens (Cousins *et al*. 1992). It has been optimized a method to provide an effective extraction of genomic DNA from different types of bacteria with different cell wall structure (i.e. gram negative and gram positive), by lysing the bacterial cells in single step, which is then followed by straight forward isolation of DNA with chloroform–isoamylalcohol and ethanol for precipitation and purification of the genomic DNA (Atashpaza *et al*. 2010).

2.5.2 Polymerase Chain Reaction (PCR)

PCR has been used for the direct detection of many types of infectious agents in clinical samples (Clarke *et al*. 2003) this procedure is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand, so it requires template DNA , two oligonucleotides primers and a heat stable DNA polymerase. This method developed by Kary Mullis in the 1980s (Bartlett and Stirling, 2003). Several conserved genes including rRNA genes (Mccabe *et al*. 1995) heat shock protein gene (Clarke *et al.* 2003) and mcc gene have been employed for the detection and identification of bacteria. The gene target that is most commonly used for bacterial identification

is 16S rRNA (or 16S rDNA), an ~1500 base pair gene that codes for a portion of the 30S ribosome (Reller and Weinstein, 2007) and it is found in all bacteria. PCR with specific 16s rDNA based-oligonucleotide primers have been developed as powerful methods for detecting target bacteria in complex ecosystems (Matsuki *et al*. 2002). In study by Spikler *et al*. in 2004, an accurate PCR assay have been designed based on 16s rRNA sequences data to differentiate *P.aeruginosa* from other *Pseudomonas* species that may also be recovered from CF sputum cultures. In addition to 16s rDNA gene, mec gene, the structure determinists encoding PBP2, has been used as target gene for identification MRSA, this region of gene is highly conserved in methicillin resistance species. (Vannufffel. 1998) Comparing to bacteriophage and capsular typing, PCR is more powerful and reliable molecuolar method for MRSA identification. (Jaffe *et al*. 2000).

2.5.3 Random Amplification of Polymorphic DNA (RAPD- PCR)

It is a random amplification of unknown genomic regions using arbitrary primers. Unlike traditional PCR analysis, RAPD-PCR does not require prior knowledge of the target DNA (Wenjun *et al.* 2009), therefore, with this method a DNA fingerprint may define individual in a very fast and reliable way (Onasanya *et al.* 2003)This technique has been used for characterization *P.aeruginosa* isolates from CF patients (Ortiz-Herrera *et al*. 2004). In study conducted in 1995, the RAPD method was shown to have the ability to distinguish different isolation of *S.aureus* (Van Belkum *et al*. 1995). This technique has also shown to be able to use as a mean to determine the clonal relatedness of the *K.pneumoniae* isolates by their chromosomal polymorphism (Haryani *et al*. 2007). However, there have been reports of inter-laboratory variation and as a result this method is only

particularly useful when comparing results from the same laboratory (Minion. 2010).

2.5.4 Multiple-locus variable number tandem repeat analysis (MLVA)

Variable number tandem repeats (VNTR) are 'head-to-tail)'tandemly) repeated DNA sequences that vary in copy number and are dispersed widely in human and bacterial genomes(Vergnaud and Denoeud. 2000). In this method, the variability in the number of short tandem repeat sequences is utilized to create DNA profiles for epidemiological studies (Schouls *et al*. 2006). It has the advantages of typing methods based on PCR (low cost, short time, and easy to perform) that are independent of equipment and yield unambiguous typing data (Heck. 2009). In addition, its highly reproducible and easily portable among laboratories and it represents a very promising tool for the molecular surveillance of *P. aeruginosa* (Onteniente *et al.* 2003). For *S.aureus* typing a study by Pourcel, *et al* have shown that the discriminatory power of MLVA is higher than those of both MLST and *spa* typing, they reached to this results by improving a MLVA scheme for genotyping *S. aureus* and compared its performance to those of MLST and *spa* typing in a survey of 309 strains (Pourcel *et al*. 2009).

2.5.5 Amplified fragment length polymorphism (AFLP)

AFLP was first described by Vos and Zabeau, 1993, it uses restriction enzymes to digest genomic DNA with two restriction enzymes (usually *MseI* and *EcoRI*) (Wenjun *et al.* 2009) followed by ligation of adaptors to the sticky ends of the restriction fragments. Alternatives to *Mse I* include *Taq I* (which can produce better quality results (Papa *et al*. 2005) and *Tru I* (Bensch and kesson, 2005). *Pst I*, is alternative to *EcoR I* and it might be appropriate for differential gene

expression and some mapping applications (Cervera. 2002). The digestion by restriction enzymes are followed by selective PCR amplification and electrophoresis of a subset of the fragments resulting in a unique, reproducible fingerprint for each individual (Mueller and Wolfenbarger. 1999).This method has the ability to differentiating between closely related strains of the same bacterial species, research has suggested that AFLP analysis may offer the ability to specifically identify different bacterial species (Tarkkanen *et al*. 2009).

Genotyping using AFLP analysis was applied successfully to *P.aeruginosa* epidemiological investigations.(Kenchappa, *et al*. 2005). AFLP has also applied for Acinetobcter spp characterization (Doughari *et al*.2011), *K.pneumoniae* (Van der Zee *et al.* 2003) furthermore, the clonal relationships between *S.aureus* isolates has been examined by AFLP technique (Boerema *et al.* 2006). Different advantages have been observed in the AFLPs such as the small amount of DNA needed, the ability to work without sequence information and high reproducibility (Travis *et al.* 1996) , however the huge quantities of information generated by AFLPs may need automated analysis and therefore computer technology , this feature may limit the use of this method.

2.5.6 Restriction enzymes (REs)

It's an enzyme that cuts double-stranded DNA at specific recognition nucleotide sequences known as restriction sites (Roberts and Murray. 1976). Based on the composition and enzyme cofactor requirements of the REs, the nature of their target sequence, and the position of their DNA cleavage site relative to the target sequence, the REs are categorized into three general groups (Types I, II, III).

All types of enzymes recognize specific short DNA sequences and carry out the endonucleolytic cleavage of DNA to give specific double-stranded fragments with terminal 5'-phosphates (Rao and Sistla. 2004).

• Type I enzymes cleave at sites remote from recognition site; require both ATP and S-adenosyl-L-methionine to function; multifunctional protein with both restriction and methylase activities, These enzymes cut at a site that differs, and is a random distance (at least 1000 bp) away, from their recognition site.

• Type II enzymes cleave within or at short specific distances from recognition site; most require magnesium; single function (restriction) enzymes independent of methylase.

• Type III enzymes cleave at sites a short distance from recognition site; require ATP, They cut DNA about 20-30 base pairs after the recognition site.

The restriction enzyme, *CfoI*, has been used to differentiate between the species associated with CF and COPD respiratory infections (*P. aeruginosa*, *S. aureus, B. cepacia, H. influenzae, S. maltophilia, S. pneumoniae*) (Rogers *et al*. 2003) *CfoI* and *AluI* in silico PCR product digestions showed different base lengths for the *Pseudomonas spp* (Spasenovskia *et al*. 2009), whereas *SmaI* was used to confirm the persistence of MSSA isolates in the sputum of cystic fibrosis patients (Branger *et al.* 1996). In addition, digestion of genomic DNA by the restriction endonuclease *SpeI* and subsequent separation of the restriction fragments by Pulsed-Field Gel Electrophoresis (PFGE) has been found to be a useful method for the typing of *P.aeruginosa* isolates from CF patients (Jung *et al*. 2002). In different studies bacterial strains were purified and digested by different restriction endonucleases such as *EcoRl , EcoRV, Hind III, Spe 1 and*

Bam H1, Which it was a valuable tool to evaluate the degree of genetic relatedness between lineages (Al-Naggar *et al*. 2000) .The REs *Alu*I , *Cfo*I , *Hae*III , *Hin*fI, *Mbo*I , *Msp*I, *Nci*I , *Rsa*I , or *Taq*I , have been used in study by Vaneechoutte *et al*, 1995 to differentiate between *Acinetobacter* species .

2.5.7 Restriction Fragment Length Polymorphism (RFLP)

It is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites (Hiraishi *et al.* 2000). In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis (Hiraishi *et al.* 2000) The resulting output consists of a microbial profile where each detected length is that of specific fragments from the digested PCR product. Each length represents one or more bacteria that have the same restriction fragment length. RFLP profiles can be used for community differentiation, identification of specific organisms in populations and comparison of the relative phylotype richness and community structure (Dunbar *et al.* 2001). This method has been successful in the differentiation of bacterial communities present in many environments, including marine samples, soil samples and sputum samples from CF patients (Mills *et al*. 2003). In 2011, Awad *et al*. have used PCR-RFLP for amplified16S rRNA fragment to conduct genetic fingerprinting and obtain specific molecular markers for chlorpyrifos-degrading bacterial strains.

In 2003, Rogers *et al*, analyzed RFLP amplicons of CF patient sputa and bronchoscopy samples, The analysis indicated the presence of *P. aeruginosa, B.*

cenocepacia, S. aureus, and *H. influenzae* in the CF samples. The PCR-RFLP procedure seems to be a reliable tool for discriminating strains of *B. cepacia* in sputum of CF patients (Pelt *et al*.1999).

Rogers *et al*. 2006 have compared bacterial communities in sputum and mouthwash samples from patients with CF using RFLP and have demonstrated that sputum from the lungs of these patients is not contaminated by bacteria present in the oral cavity and they have also provided evidence that the CF lung can be colonized by certain oral bacterial species. In 2003, Clarke *et al.* have developed novel PCR assay for the detection of *Pseudomonas spp*. from patients with CF by employing conserved primer regions of the groE heat-shock protein domain gene. They found that RFLP analysis of the amplicons of all *P.aeruginosa* isolates demonstrated a single RFLP type that consisted of three bands at approximately 80, 190 and 250 base pair (bp); direct sequencing of the amplicons demonstrated the presence of a single sequence type, indicating the highly conserved nature of this region. Selective amplification of Pseudomonas 16S rDNA by PCR followed by (RFLP) analysis or denaturing gradient gel electrophoresis has been used to detect and differentiate Pseudomonas species from clinical and environmental samples (Duineveld *et al*. 2001). However, PCR-RFLP is a simple, rapid, and nonradioactive approach to detect DNA polymorphism. It has been used frequently for typing a variety of bacteria (Al *et al*. 2005).

2.5.8 Plasmid Profile

Plasmid profile is a method of determining a number and size of plasmids in bacterial isolates (Selimovic *et al*. 2008) Plasmids are extra-chromosomal

molecules of DNA capable of independently replication. Such molecules have been identified in several bacterial genera. Plasmids range in size from less than one Mega Dalton (MDa) to several hundred MDa. One MDa of double-stranded DNA is equivalent to 1500 bp. (Threlfall and Woodford. 1995). In 1976, Meyers *et al*, demonstrated that plasmid DNA could be size fractionated by mobility within an electric field, through an agarose gel; it was an easy and inexpensive technique to perform. Plasmid analysis was applied in two ways: first, by characterizing the number and size(s) of plasmids in the test strains' genome then later, by restriction enzyme generated RFLP profiles.

Two types of plasmids have have medical important:

- Virulence plasmid: Carry determinants of bacterial virulence, e.g. hemolysin genes.
- Resistance plasmids: Carry genetic information bearing on resistance to anti-infective agents (Scrofani *et al*. 1999).

Plasmid profiling has been used as an epidemiological tool during the past years (Poh *et al*. 1988). Plasmids may contain resistance genes for single or multiple antimicrobial agents and these genes may transferred from one bacteria to another (Maithem *et al*. 2012)

This method is simple and requiring a minimum of equipment. In, 2010, Sadeghifard *et al.* have demonstrated that plasmid profiles were significantly associated with antibiotic resistance profiles and the typeability of plasmids can be used reliably to predict antibiotic resistance on molecular bases. *S.aureus* has resistance to different classes of antibiotics (Pantosti *et al.* 2007). The spread of

resistance to antimicrobial agents in *S. aureus* is almost due to plasmids (Mccarthy and Lindsay. 2012) whereas, Nikbin *et al* .2007 have postulated that most of the resistance genes in *P. aeruginosa* are mostly chromosomal. The relationship between certain plasmids and resistance to some antibiotics had been reported previously (Shahcheraghi *et al*. 2003). *A.baumannii* has been known as a multi-drug resistance, plasmid profile has proved to be useful for the study of outbreaks of *Acinetobacter* infections (Bassetti *et al*. 2008).

Chapter Three

Materials and Methods

3.1 Materials

3.1.1 Collection of patients' samples:

The (CF) patients' samples were collected from king Faisal Specialist Hospital & Research Center (Pediatric Clinic) and the (non CF) Patients' samples were collected from King Faisal Specialist Hospital research Center and King Abdul Aziz Hospital & Oncology Center from October 2011 until October 2012.

3.1.2 Culturing of Bacterial samples:

All samples were cultured on the following Media

1- Blood sheep agar

2- MacConkey ager

3- Chocolate agar

All types of media above were obtained from Laboratory of king Abdul Aziz Hospital

3.1.3 Bacterial identification

3.1.3.1 Gram staining

The Gram staining reaction is used to help identify pathogens in specimens and cultures by their Gram reaction and morphology, the following reagents and equipmentes were used in this reaction:

Crystal violet stain, Iodin, Decolonizer (Ethanol) ,Safranin ,Water ,Light microscope , oil, Slides and loops.

3.1.3.2 Biochemical reactions

- **(Catalase test)** 3% Hydrogen peroxide.
- **(Oxidase test)** Oxidase test reagents (powder), item no. R6630, 50 mg/tube.
- API-20E test kit
- **(Coagulase test)** Rabbit plasma.

3.1.4 Antibiotic susceptibility tests

The disk diffusion method was used as susceptibility pattern test. The Antibiotics were used are Amikacin 30 Mg, Ceftazidime 30 Mg, Aztreonam 30 Mg, Piperacillin 100 Mg, Imipenem 10 Mg, Ciprofloxacin 5 Mg, Ampicillin10 Mg, Ceftriaxone 30 Mg, Piperacillin + Tazobactam 30Mg , Cefebmie 30 Mg, Augmentin 30 Mg, Gentamicin30 Mg, Cefoxitin 10Mg, Cephalothin 20 Mg, Cotrimoxazole 30 Mg, Cefotaxime 30 Mg, Colistin 10 Mg, Meropenem 30 Mg, Vancomycin 10 Mg , Teicoplanin 10 Mg, Erythromycin 30 Mg, Clindamycin 10 Mg, Oxacillin 10 Mg , Penicillin 30 Mg.

- Muller –Hinton agar (Merck, Darmstadt, Germany) was used as a test medium for disk diffusion method.

MicroScan® Automated Microbiology System, type TN dried panel, Pos ID 2 (Siemens, Tarrytown, NY) was used for confirmation detection of the isolates and susceptibility patterns for it.

3.1.5 Storage of isolates

Luria-Bertani (LB) Broth**,** Glycerol**.**

3.1.6 DNA Extraction

QIAamp® DNA Mini Kit (50), Cat. No. 4593, Lot No.69483 (QIAGEN, USA).

CTAB buffer, proteinase K, Isopropanol, lysozyme, SD, RNase, Chloroform : Iso Amyl Alcohol (24:1) , 1x TE solution.

Ethanol 99.7-100% V/V was purchased from Sigma Aldrich.

IEC Micromax ventilated microcentrifuge, (DJB Labcare, UK) , Vortex-Genie ® Mixer, (NY, USA) , Ependorf tubes 2ml, Water bath.

3.1.7 PCR

Quick-Load® Iag 2X Master Mix 500 reactions 50 µl vol (NEB, USA)

PCR tubes 0,2 ml, US-PAT. NO. 5,863,791, Lot. A145174Q (Eppendorf AG, Germany).

- Four different species specific primer sets were used for amplifying 16s rDNA genes :

Bacterial Species	Primer Sequence	References
P.aeruginosa	F: 5' GACGGGTGAGTAATGCCTA3'. R: 5' CACTGGTGTTCCTTCCTATA 3'.	Spilker *et al*. 2004
S.aureus	F: 5' TAGATGGATCCGCGC 3'. R: 5' CTTAATGATGGCAACTAAGC 3'.	Giannatale *et al*. 2011
A.baumannii	F: 5' TGGCTCAGATTGAACGCTGGCGGC 3'. R: 5' TACCTTGTTACGACTTCACCCCA 3'.	Vaneechoutte and Dijkshoorn.1995
K.pneumoniae	F: 5' GTAATGTCTGGGAAACTGCC 3'. R: 5'CCACCTTCCTCCAGTTTATC 3'	Arenas *et al*. 2009

Labnet MultiGene™ Gradient PCR Thermal Cycler, 96 well block (128 × 0.2)

3.1.8 Gel electrophoresis

10X TBE Electrophoresis buffer, Lot No. 00080787 (Fermentas, EU).

Agarose, Cat. No. 7581, Lot. No.113773, (USB Ultrapure Corporation Cleveland).

Ethedium bromide, Lot.No.111 K8930 (Sigma).

GelPiolt® 100 bp ladder plus ladder 100 lanes, Cat. No. 239045, Lot No. 133215959 (QIAGEN GmbH, Hilden).

Gel electrophoresis apparatus

Gel Documentation system (Gel Doc-It® 310 Motorized Lens) W\LM-26 Transilluminator.

3.1.9 Restriction Enzymes

Four different restriction enzymes were used:

BamHI , 1000 Unit, Cat# R0136S (NEB, USA).

ApaI , 5000 Unit, Cat# R0114S (NEB, USA).

TaqI, 4000 Unit, Cat #R0149S (NEB, USA).

MspI, 5000 Unit, Cat#R01065 (NEB, USA).

3.1.10 Plasmid isolation

GeneJET Plasmid Miniprep Kit (# K0482, Thermo Scientific, USA).

3.2 Methods

3.2.1 Collection of patients' samples

In the present study, a total of 20 sputum samples from CF patients were collected from king Faisal Specialist Hospital & Research Center (Pediatric Clinic) and a total of 20 sputum samples from Non-CF patients were collected from king Faisal Specialist Hospital & Research Center (Pediatric Clinic) and King Abdul Aziz Hospital & Oncology Center . CF Samples were collected over a period of 1 year starting from October 2011 until October 2012.

3.2.2 Samples Processing

Expectorated sputum samples were collected in a sterile specimen container and transported and processed immediately in the hospital bacteriology

laboratory. The technique of sample selection for Gram stain as well as cultures consisted of teasing out the purulent portion of the sputum, a section of which was then spread thinly over a glass slide. All Gram stains were first screened under a microscope at low power to assess the appropriateness of the sample. Sputum samples were considered of good quality if they had numerous polymorphonuclear cells (>25 in a ×100 microscopic field) and few squamous epithelial cells (<10 in a ×100 microscopic field). Otherwise, the sputum sample was considered to be contaminated with saliva and rejected. Good quality samples were then screened for a predominant bacterial morphological type in an oil immersion field. The presence of a predominant morphotype was considered when Gram stain showed bacteria only or mainly corresponded to the gram morphotype revealed by standard microbiological criteria.

3.2.3 Culturing of patients' samples

Samples were cultured on blood sheep agar, MacConkey agar and chocolate agar by streaking on these media with sterilize loop and then incubated at 37 degree for 24 hours.

3.2.4 Identification of isolates

All samples were identified by the "routine conventional" methods which used in the microbiology laboratory.

3.2.4.1 Gram staining

After preparation and fixation of bacterial film, the slide was flooded with crystal violet and left for 30 seconds, then washed with distilled water. After that, the film was covered with Gram's iodine solution and left for 30 seconds. The

Gram's iodine was washed off and ethanol alcohol (95%) was allowed to flow down at the surface of the slide. This process continues until alcohol become colorless. The slide was washed with a gentle stream of water and stained with safranin for a minute. Finally, it was washed gently for a few seconds and the slide was dried in air and examined with oil immersion lens using few drops of ceder oil. Gram-positive bacteria appeared violet colored while Gram negative appeared red.

3.2.4.2 Biochemical reactions

According to the morphology, Gram stain reaction and odor, the samples were tested by two biochemical tests:

3.2.4.2.1 Oxidase test:

This test identifies organisms that produce the enzyme cytochrome oxidase such as pseudomonas.

1-A small amount of organism was obtained from the culture dish with swab.

2- One drop of oxidase reagent was placed onto the sample on the swab.

3- Positive reactions turn the color to purple directly or within 10 to 30seconds (Cheesbrough 2000).

3.2.4.2.2 Catalase test:

This test is used for differentiate staphylococcus from streptococci.

1- A small amount of growth from culture was placed on clean microscope slide.

2- A few drops of H_2O_2 were added onto the smear then mixed by toothpick.

3- Positive result is the rapid evolution of O_2 as evidenced by bubbling, (positive catalase – staphylococcus, negative catalase – streptococcus) (Cheesbrough 2000).

3.2.4.2.3 Coagulase test

This test is used to identify *S. aureus* which produces the enzyme coagulase.

1- A drop of distilled water was added on a slide to make a thick suspension of test organism

2- A loopful (not more) of plasma was added to the suspension and mixed gently then looked for clumping of the organism within 10 seconds (Cheesbrough. 2000).

3.2.4.2.4 Analytical Profile Index (API 20E)

The API-20E test is for the identification of enteric bacteria. This test was used in this study for identification *K.pneumoniae*.

The API 20E system consists of a plastic strip of 20 individual, miniaturized tests tubes (cupules) each containing a different reagent used to determine the metabolic capabilities, and, ultimately, the genus and species of enteric bacteria in the family Enterobacteraceae.

Each cupule was inoculated with a saline suspension of a pure bacterial culture, rehydrating the dried reagent in each tube. Some of the tubes to be completely filled (tests CIT, VP and gelatin (GEL), whereas (tests Arginine Di Hydolase (ADH), Lysine DeCarboxylase (LDC), Ornithinine DeCarboxylase (ODC), H^2S, UREase (URE) were topped off with mineral oil for anaerobic reactions. The

strip was then incubated in a small, plastic humidity chamber for 18-24 hours at 37°C. After incubation, each tube was assessed for a specific color change indicating the presence of a metabolic reaction that sheds light on the microbes identity.

Interpretation of the 20 reactions, in addition to the oxidase reaction (which is done separately), was converted to a seven-digit code, then looked up in the manual that has the names of bacterial species associated with each seven-digit string of numbers (Janin. 1974).

3.2.4.3 Susceptibility patterns testing

3.2.4.3.1 MIC determination for the isolates

MicroScan® Automated Microbiology System, type TN dried panel, Pos ID 2 (Siemens, Tarrytown, NY) was used for detecting the Minimal Inhibitory Concentration (MIC) (Appindex 1). The interpretation standards for MICs of Clinical and Laboratory Standards Institute (CLSI) were used to determine antibiotics Susceptibilities. The antimicrobials tested for gram negative bacteria were Cefepime, Ceftazidime, Cefotaxime, Gentamicin, Imipenem, Meropenem, Amikacin, and Piperacillin\Tazobactam.

The antimicrobials tested for gram positive bacteria were Teicoplanin, Clindamycin, Gentamicin, Augmentin, Oxacillin, Fox , Penicillin.

3.2.4.3.2 Disk diffusion method

Disk testing was performed as recommended by CLSI (Clinical and Laboratory Standards Institute) The antibiotics disks used for gram negative bacteria were Amikacin 30 Mg, Ceftazidime 30 Mg, Aztreonam 30 Mg,

Piperacillin 100 Mg, Imipenem 10 Mg, Ciprofloxacin 5 Mg,Ampicillin10 Mg, Augmentin 30 Mg, Gentamicin 100 Mg, Cefoxitin 10 Mg, Cephalothin 30 Mg, Cotrimoxazole 25 Mg, Colistin 10 Mg, Meropenem 10 Mg (MAST Diagnostics, Merseyside, UK). The test medium was Muller-Hinton agar (Merck, Darmstadt, Germany). For each isolate, one disk of each antibiotic placed on a suitable distance on the surface of Mueller-Hinton agar plates inoculated with a bacterial suspension equivalent to 0.5 McFarland Standard then they was incubated for 24 h at 37°C.

3.2.5 Storage of Isolates

All isolates were kept in LB broth plus 20% glycerol and stored at -20°C (Luckett *et al*. 2011).

3.2.6 DNA extraction

The Qiamp DNA Mini Kit was used to purify bacterial DNA from Gram negative bacteria. The protocol followed the manufacturer's instructions in the kit. DNA extractions from gram positive bacteria were used by CTAB method (Ibrahim .2011) (Appendix 2)

3.2.7 PCR

PCR is the technique used to amplify specific segment of DNA. This technique is sensitive to contamination, so all tips and tubes were autoclaved, the micropipettes and bench top were cleaned with 70% ethanol.

The PCR reaction for all bacterial species was performed in a final volume 50µl containing 2µl genomic DNA for each sample, 0.5µl from forward primer, 0.5 reverse primer, 25µl master mix and 22µl ddH_2O.

3.2.7.1 16s rDNA gene PCR amplification for *P.aeruginosa*

In order to amplify 612 bp fragment of 16 s rDNA target gene the following primer were used:

- PA-F: 5' CGGCCCAGACTCCTACGGG 3'.
- PA-R: 5' TTACCGCGGCTGCTGGCAC 3'.

Amplification of the target gene was carried out under following condition:

After an initial denaturation for 2 min at 95°C, 25 cycles were completed,

each consisting of 20 s at 94°C, 20 s at 58°C and 40 s at 72°C. A final extension of 1 min at 72°C was applied. Finally reaction was allowed to cool at 4°C (Spilker *et al*. 2004).

3.2.7.2 16s rDNA gene PCR amplification for *S.aureus*

In order to amplify 916 bp fragment of 16 s rDNA target gene the following primer were used:

- S.A-F: 5' TAGATGGATCCGCGC 3'.
- S.A-R: 5' CTTAATGATGGCAACTAAGC 3'.

Amplification of the target gene was carried out under following condition:

After an initial denaturation for 2 min at 95°C, 25 cycles were completed,

each consisting of 20 s at 94°C, 20 s at 58°C and 40 s at 72°C. A final extension of 1 min at 72°C was applied. Finally reaction was allowed to cool at 4°C (Giannatale *et al*. 2011).

3.2.7.3 16s rDNA gene PCR amplification for *K.pneumoniae*

In order to amplify 1069 bp fragment of 16 s rDNA target gene the following primer were used:

- K.P-F: 5' GTAATGTCTGGGAAACTGCC 3'.
- K.P-R: 5'CCACCTTCCTCCAGTTTATC 3'

Amplification of the target gene was carried out under following condition:

After an initial denaturation for 1 min at 94°C, 40 cycles were completed,

each consisting of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C. A final extension of 3 min at 72°C was applied. Finally reaction was allowed to cool at 4°C (Arenas *et al*. 2009).

3.2.7.4 16s rDNA gene PCR amplification for *A.baumannii*

In order to amplify 1500 bp fragment of 16 s rDNA target gene the following primer were used:

- ACI-F: 5' TGGCTCAGATTGAACGCTGGCGGC 3'.
- ACI-R: 5' TACCTTGTTACGACTTCACCCCA 3'.

Amplification of the target gene was carried out under following condition:

After an initial denaturation for 5 min at 95°C, 35 cycles were completed,

each consisting of 45 s at 95°C, 45 s at 48°C and 1 min at 72°C. A final extension of 7 min at 72°C was applied. Finally reaction was allowed to cool at 4°C (Vaneechoutte and Dijkshoorn.1995).

3.2.8 Gel electrophoresis:

Gel electrophoresis was used to check PCR products after amplification, in RFLP for genotyping after sample digestion by restriction enzymes and to check the present or absent of the plasmid in the strains.

After PCR amplification, the PCR products were analyzed by 2% agarose gel electrophoresis in a TBE buffer stained with ethidium bromide at120 v for 1 hour. Amplified products were visualized using a Gel Documentation System by comparison with a molecular size marker (GelPilot 100 bp plus ladder 100 lanes).

3.2.9 RFLP-PCR for *P.aeruginosa* isolates

The amplified 612 bp PCR product was digested by *BamHI* enzyme. The enzymatic digestion was preform in a final volume of 20 µl, using the following components: 5µl of the PCR product, 2µl of the enzyme buffer, 1µl of the *BamHI*, .5µl of the bovine serum albumin (BSA) and 11.5 µl of the ddH_2O. The reaction was incubated at 37°C for 4 hours then the enzyme inactivated by incubated at 80 °C for 20 min. The digestion products were than analyzed by 2% agarose gel electrophoresis stained with ethidium bromide at 120 v for 1 hour (Burns *et al*. 2001).

3.2.10 RFLP-PCR for *S.aureus* isolates

The amplified 900 bp PCR product was digested by *ApaI* enzyme. The enzymatic digestion was preform in a final volume of 20 µl, using the following components: 5µl of the PCR product, 2µl of the enzyme buffer, 1µl of the *ApaI*, .5µl of BSA and 11.5 µl of the ddH_2O. The reaction was incubated at 37°C for 2 hours then the enzyme inactivated by incubated at 80°C for 20 min. The digestion

products were than analyzed by 2% agarose gel electrophoresis stained with ethidium bromide at 120 v for 1 hour. (Carles-Nurit *et al*. 1992)

3.2.11 RFLP-PCR for *A.baumannii* isolates

The amplified 1500 bp PCR product was digested by *MspI* enzyme. The enzymatic digestion was preform in a final volume of 20 µl, using the following components: 5µl of the PCR product, 2µl of the enzyme buffer, 1µl of the *MspI*, and 12µl of the ddH_2O. The reaction was incubated at 37°C for 1 hour. The digestion products were than analyzed by 2% agarose gel electrophoresis stained with ethidium bromide at 120 v for 1 hour. (Vaneechoutte and Dijkshoorn. 1995)

3.2.12 RFLP-PCR for *K.pneumoniae* isolates

The amplified 1069 bp PCR product was digested by *TaqI* enzyme. The enzymatic digestion was preform in a final volume of 20 µl, using the following components: 5µl of the PCR product, 2µl of the enzyme buffer, 1µl of the *TaqI*, .5µl of BSA and 11.5 µl of the ddH_2O. The reaction was incubated at 65°C for 1 hour then the enzyme inactivated by incubated at 80°C for 20 min. The digestion products were than analyzed by 2% agarose gel electrophoresis stained with ethidium bromide at 120 v for 1 hour. (Arenas *et al*. 2009)

3.2.13 Plasmid patterns of isolates

GeneJET Plasmid Miniprep Kit (# K0482, Thermo Scientific, USA) was used to purify plasmid DNA. The protocol followed the manufacturer's instructions in the kit. After purification the products were analyzed by 2.5% agarose gel electrophoresis stained with ethidium bromide at 50 v for 2 hour .

Chapter Four

Results

Molecular biology techniques for correct detection and identification of bacteria is now widely used in clinical microbiology and also developed for identification of isolates obtained from CF patients. Hence, correct identification of bacteria help in the selection of appropriate treatment for CF airway infections.

4.1 Identification of the isolated bacteria:

4.1.1 Purification

Purification of bacteria isolated from CF and non CF patients was carried out using streaking method where a total of 49 pure isolates (25 isolates from CF and 24 isolates from non CF patients) were obtained.

4.1.2 Classical identification

Identification of the pure isolates from patients was carried out using cell and colony morphology, Gram staining and biochemical profiling. (Table4.1 & 4.2). Results indicated that the most common isolated strain in CF patients was *P.aeruginosa* (56%) followed by *S.aureus* and *A.baumannii* (16%) and *K.pneumoniae* (12%). Results also indicated that the most common isolated strain in non CF patients was *P.aeruginosa* and *A.baumannii* (37.5%) followed by *S.aureus* (16.64%) and *K.pneumoniae* (8.32%)

Table 4.1 Bacterial Species in Cystic Fibrosis Patients

NO.	G	Specimen	Bacterial species
P1	F	Sputum	*P.aeruginosa*
P2	F	Sputum	*S.aureus*
P3	F	Sputum	*P.aeruginosa*
P3*	F	Sputum	*S.aureus*
P4	F	Sputum	*P.aeruginosa*
P5	F	Sputum	*P.aeruginosa*
P5*	F	Sputum	*K.pneumoniae*
P6	M	Sputum	*P.aeruginosa*
P7	M	Sputum	*P.aeruginosa*
P7*	M	Sputum	*A.baumannii*
P8	M	Sputum	*P.aeruginosa*
P9	M	Sputum	*P.aeruginosa*
P10	M	sputum	*S.aureus*
P11	M	Sputum	*P.aeruginosa*
P11*	M	Sputum	*K.pneumoniae*
P12	M	Sputum	*A.baumannii*
P12*	M	Sputum	*P.aeruginosa*
P13	M	Sputum	*S.aureus*
P14	M	Sputum	*P.aeruginosa*
P15	M	Sputum	*A.baumannii*
P16	M	Sputum	*P.aeruginosa*
P17	M	Sputum	*P.aeruginosa*
P18	M	Sputum	*P.aeruginosa*
P19	M	Sputum	*K.pneumoniae*
P20	M	Sputum	*A.baumannii*

P= Patient G= Gender M=Male F=Female * Patient has 2 isolates.

P.aeruginosa=Pseudomonas aeruginosa S.aureus=Staph aureus K.pneumoniae=Klebsiella pneumoniae A.baumannii = Acinetobacter baumannii

Table 4.2 Bacterial Species in Non-Cystic Fibrosis Patients

NO.	G	Specimen	Bacterial species
P1	F	Sputum	*P.aeruginosa*
P2	F	Sputum	*S.aureus*
P3	F	Sputum	*P.aeruginosa*
P4	F	Sputum	*P.aeruginosa*
P5	F	Sputum	*P.aeruginosa*
P6	M	Sputum	*P.aeruginosa*
P7	M	Sputum	*P.aeruginosa*
P7*	M	Sputum	*A.baumannii*
P8	M	Sputum	*P.aeruginosa*
P8*	M	Sputum	*A.baumannii*
P9	F	Sputum	*P.aeruginosa*
P10	M	Sputum	*S.aureus*
P10*	M	Sputum	*A.baumannii*
P11	F	Sputum	*P.aeruginosa*
P11*	F	Sputum	*K.pneumoniae*
P12	F	Sputum	*A.baumannii*
P13	M	Sputum	*S.aureus*
P14	M	Sputum	*S.aureus*
P15	M	Sputum	*A.baumannii*
P16	F	Sputum	*A.baumannii*
P17	M	Sputum	*A.baumannii*
P18	M	Sputum	*A.baumannii*
P19	M	Sputum	*K.pneumoniae*
P20	M	Sputum	*A.baumannii*

P= Patient G= Gender M=Male F=Female * Patient has 2 isolates. *P.aeruginosa=Pseudomonas aeruginosa S.aureus=Staph aureus K.pneumoniae=Klebsiella pneumoniae A.baumannii = Acinetobacter baumannii*

4.2 Antibiotic Resistance Patterns

4.2.1 Disk diffusion method:

The antibiotic resistance patterns among CF and non CF strains were done using the Antibiotic-disk assay with different types of antibiotics to determine the antibiotic resistance patterns of used strains. The used antibiotic disks used in this study were: Ampicillin (AP), Augmentin (AUG), Gentamicin (GM), Cefoxitin (FOX) Cephalothin (KF), Cotrimoxazol (TS), Amikacin (AK), Ceftazidime (CAZ) Aztreonam (ATM), Piperacillin (PRL), Imipenem (IMI) Ciprofloxacin (CIP), Ceftriaxone (CRO) Piperacillin+Tazobactam (TZP) Cefotaxime (CTX) Cefebime (FEB), Meropenem (MEM) Colistin (CS). Tables (4-3, 4-4, 4-5, and 4-6) and Figs. (4, 4-1, 4-2, 4-3, 4-4, 4.5, 4-6) for Gram negative *Pseudomonas aeruginosa*, *Acinetobacter baumannii, Klebsiella pneumonia* and Table (4.6) and Figs (4-7, 4-8) for Gram positive *S.aureus* strain which indicated the Resistance sensitive and intermediate resistances of all used samples.

Table 4-3, 4-4 and 4-5 indicated the number of Gram negative CF *Pseudomonas aeruginosa*, *Acinetobacter baumannii, Klebsiella pneumonia* samples in their ability to develop antibiotic resistance. Some samples of *Pseudomonas aeruginosa* could strong resist to some antibiotics used it resist to KF, FOX, AP, AUG, TS, AK followed by other isolated samples other CF samples could resist the chosen antibiotics with various number of samples Table (4-3) and Figs (4-A, 4-1 and 4-2).

In case of *Acinetobacter baumannii* samples it were resist to all used antibiotic except one sample are sensitive to IMI as indicated in Table (4-4) and Figs (4-B, 4-3,4-4).

The antibiotic resistance patterns of *Klebsiella pneumonia* also indicated most of samples are resist most of used antibiotics. Samples also are intermediate as indicated in

Table (4-5) and figs. (4-C, 4-5,4-6). *S.aureus* Gram positive samples resist most of used antibiotics except FOX, PG, TE and VA; some samples were sensitive to most used antibiotics as indicated in Table (4-6) and Figs. (4-D, 4-7, 4-8).

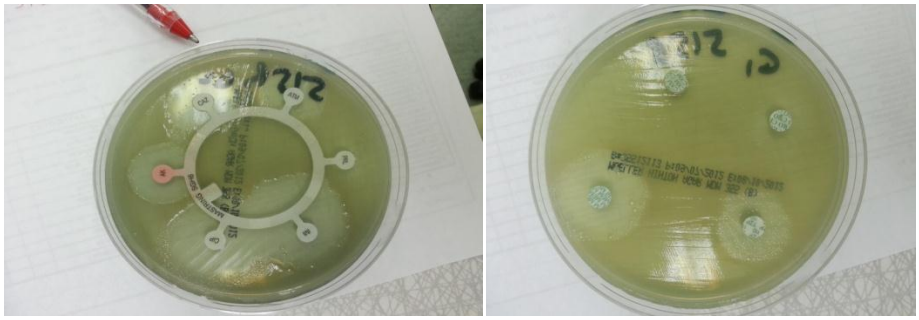
Results also indicated that the non CF of all strains are sensitive than CF samples for most of used antibiotics comparing with CF samples. The previous results are probably indicated the differences between the CF and Non CF samples in their Antibiotic resistance pattern among them may due to the Frequent and inappropriate use of antibiotics.

Table 4.3 Antibiotic Sensitivity in *P.aeruginosa*

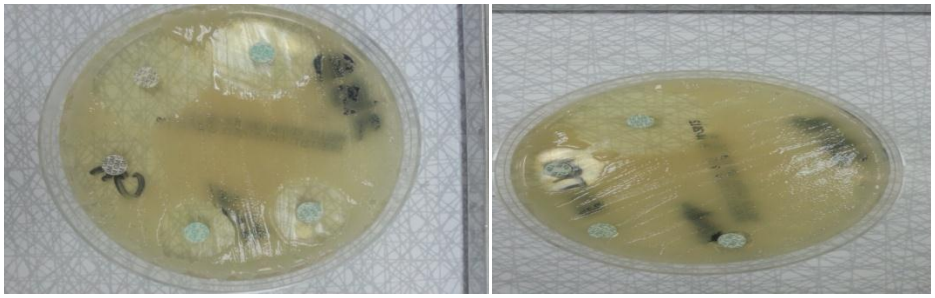
Antibiotics	No.CF Isolates			No. Non CF Isolates		
	R	S	I	R	S	I
AP	13	1	0	9	0	0
AUG	13	1	0	9	0	0
GM	4	8	2	4	3	2
FOX	14	0	0	8	1	0
KF	14	0	0	9	0	0
TS	10	4	0	8	1	0
AK	10	2	2	2	5	2
CAZ	2	11	1	5	3	1
ATM	2	10	2	6	1	2
PRL	7	7	0	7	2	0
IMI	2	12	0	2	6	0
CIP	4	10	0	4	5	0
CRO	8	4	2	8	1	0
TZP	2	9	3	7	2	0
CTX	5	7	2	7	1	1
FEB	3	10	1	7	1	1
MEM	1	12	1	3	6	0

AP: Ampicillin AUG: Augmentin GM: Gentamicin FOX: Cefoxitin KF: Cephalothin TS: Cotrimoxazol AK: Amikacin CAZ: Ceftazidime ATM: Aztreonam . PRL: Piperacillin. IMI: Imipenem. CIP: Ciprofloxacin. CRO: Ceftriaxone. TZP: Piperacillin+Tazobactam. CTX: Cefotaxime. FEB: Cefebime. MEM: Meropenem.

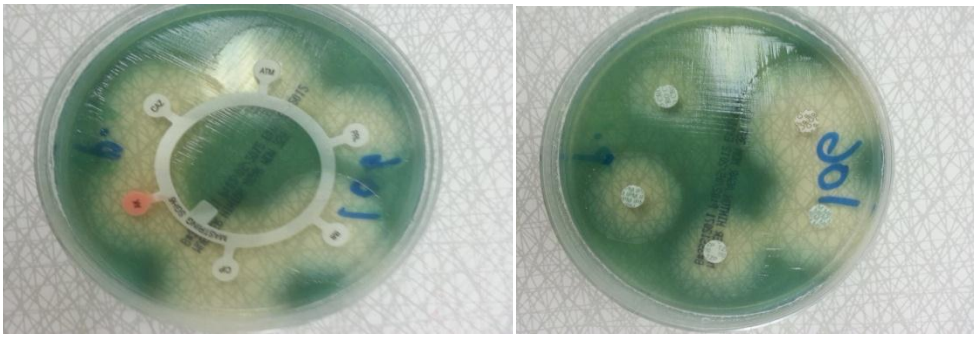
S= Sensitive I= Intermediate R= Resistance



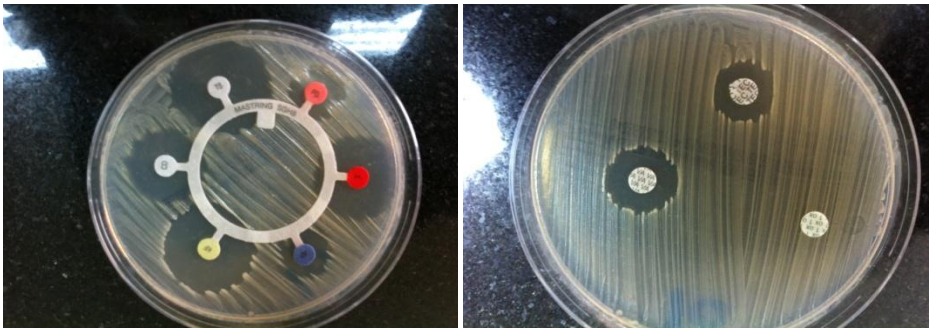
A



B

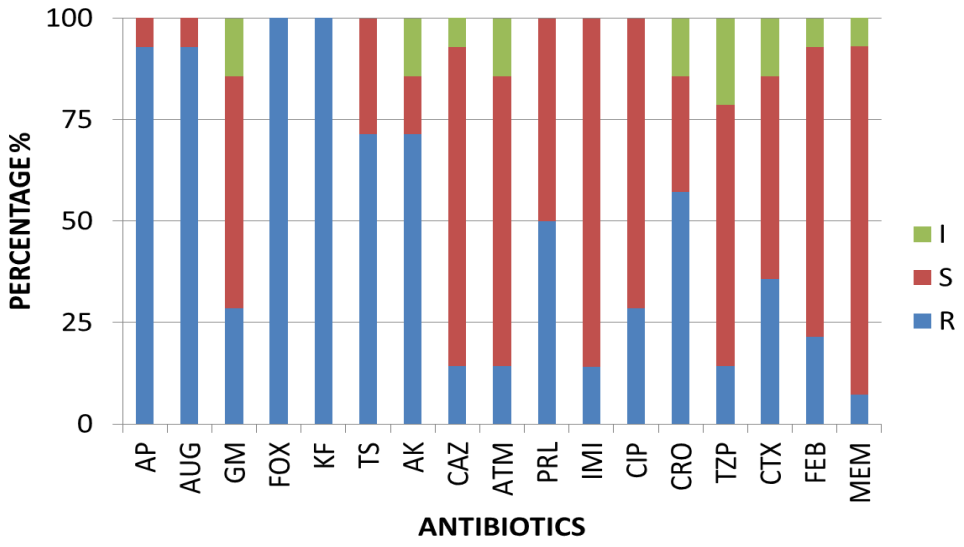


C



D

Figure: (4): Disk Diffusion Assay test



S= Sensitive I= Intermediate R= Resistance

Figure 4.1 Antibiotic susceptibility patterns of *P.aeruginosa* isolates in CF patients

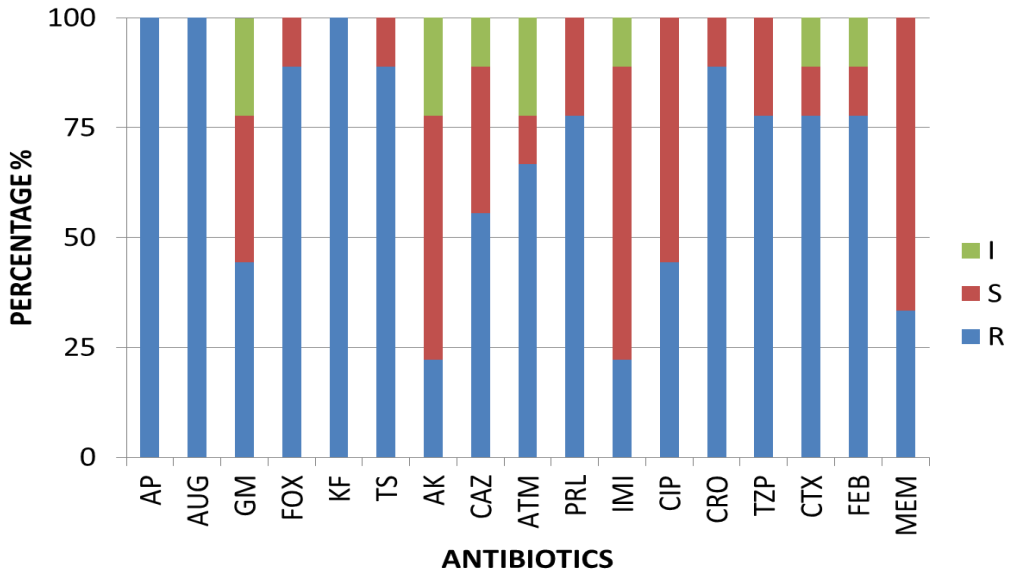


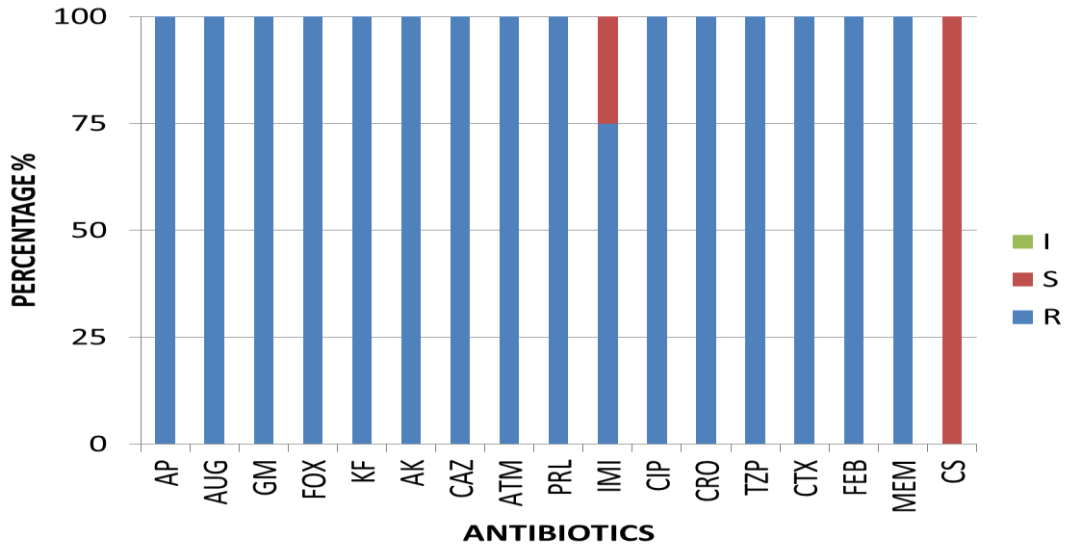
Figure 4.2 Antibiotic susceptibility patterns of *P.aeruginosa* isolates in non-CF patients.

Table 4.4 Antibiotic Sensitivity in *A.baumannii*

Antibiotics	No. CF Isolates			No. Non CF Isolates		
	R	S	I	R	S	I
AP	4	0	0	8	1	0
AUG	4	0	0	8	1	0
GM	4	0	0	8	1	0
FOX	4	0	0	8	1	0
KF	4	0	0	8	1	0
TS	4	0	0	8	1	0
AK	4	0	0	8	1	0
CAZ	4	0	0	8	1	0
ATM	4	0	0	8	1	0
PRL	4	0	0	8	1	0
IMI	3	1	0	8	1	0
CIP	4	0	0	9	0	0
CRO	4	0	0	8	1	0
TZP	4	0	0	8	1	0
CTX	4	0	0	8	0	1
FEB	4	0	0	8	1	0
MEM	4	0	0	8	1	0
CS	0	4	0	0	9	0

AP: Ampicillin AUG: Augmentin GM: Gentamicin FOX: Cefoxitin KF: Cephalothin TS: Cotrimoxazol AK: Amikacin CAZ: Ceftazidime ATM: Aztreonam . PRL: Piperacillin. IMI: Imipenem. CIP: Ciprofloxacin. CRO: Ceftriaxone. TZP: Piperacillin+Tazobactam. CTX: Cefotaxime. FEB: Cefebime. MEM: Meropenem. CS:Colistin.

S= Sensitive I= Intermediate R= Resistance



S= Sensitive I= Intermediate R= Resistance

Figure 4.3 Antibiotic susceptibility patterns of *A.baumannii* isolates in CF patients

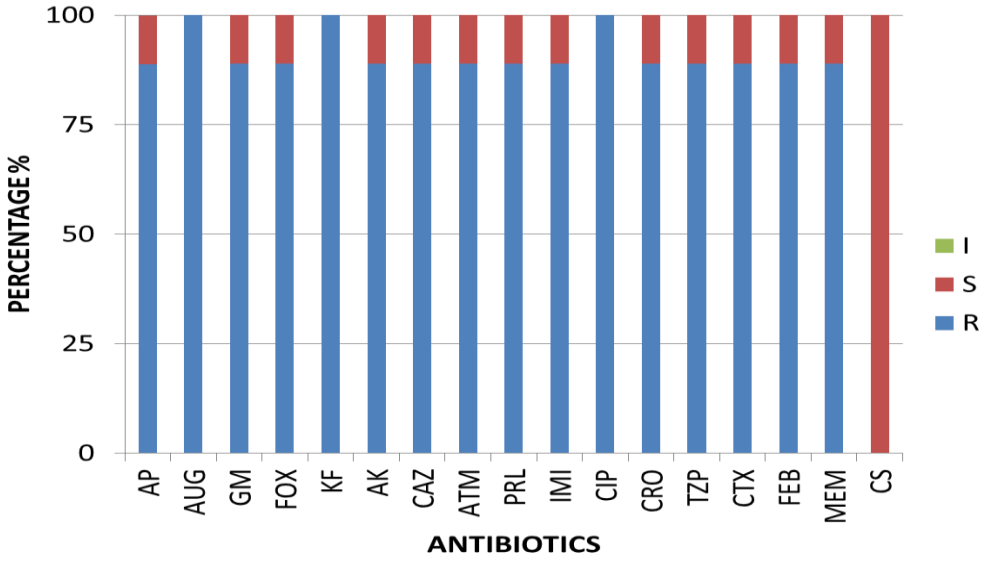


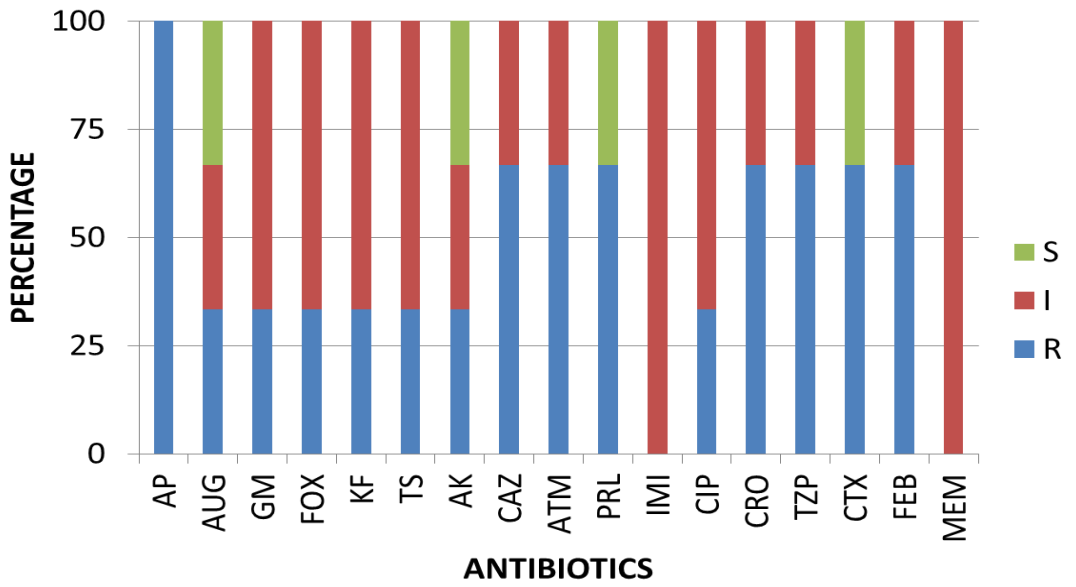
Figure 4.4 Antibiotic susceptibility patterns of *A.baumannii* isolates in non-CF patients.

Table 4.5 Antibiotic Sensitivity in *K.pneumoniae*

Antibiotics	No. CF Isolates			No. Non CF Isolates		
	R	S	I	R	S	I
AP	3	0	0	2	0	0
AUG	1	1	1	1	1	0
GM	1	2	0	0	2	0
FOX	1	2	0	0	2	0
KF	1	2	0	0	2	0
TS	1	2	0	0	2	0
AK	1	1	1	0	2	0
CAZ	2	1	0	1	1	0
ATM	2	1	0	0	2	0
PRL	2	0	1	1	1	0
IMI	0	3	0	0	2	0
CIP	1	2	0	0	2	0
CRO	2	1	0	0	2	0
TZP	2	1	0	1	1	0
CTX	2	0	1	0	1	1
FEB	2	1	0	0	2	0
MEM	0	3	0	0	2	0

AP: Ampicillin AUG: Augmentin GM: Gentamicin FOX: Cefoxitin KF: Cephalothin TS: Cotrimoxazol AK: Amikacin CAZ: Ceftazidime ATM: Aztreonam . PRL: Piperacillin. IMI: Imipenem. CIP: Ciprofloxacin. CRO: Ceftriaxone. TZP: Piperacillin+Tazobactam. CTX: Cefotaxime. FEB: Cefebime. MEM: Meropenem**.**

S= Sensitive I= Intermediate R= Resistance



S= Sensitive I= Intermediate R= Resistance

Figure 4.5 .Antibiotic susceptibility patterns of *K.pneumoniae* isolates in CF patients

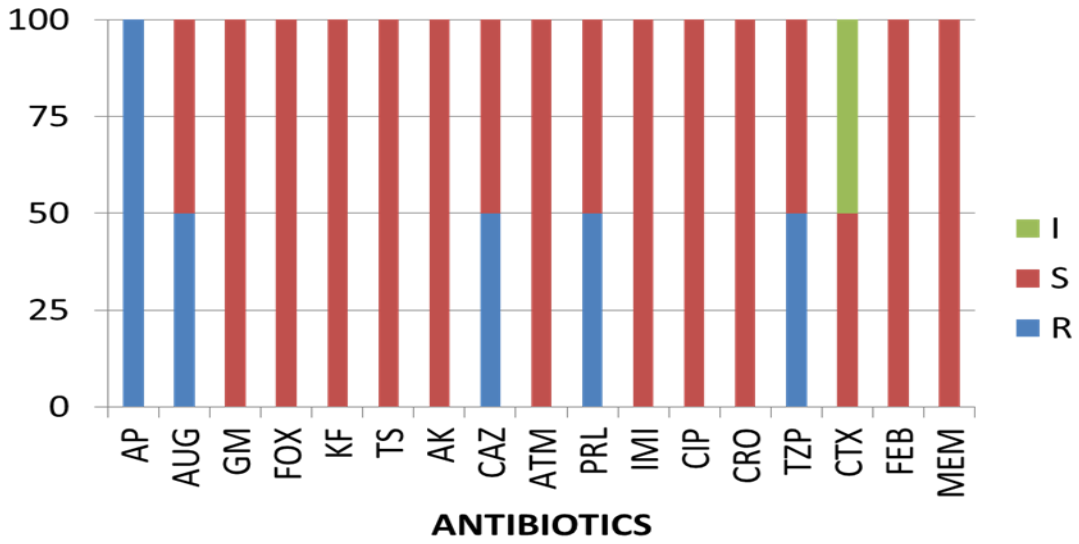
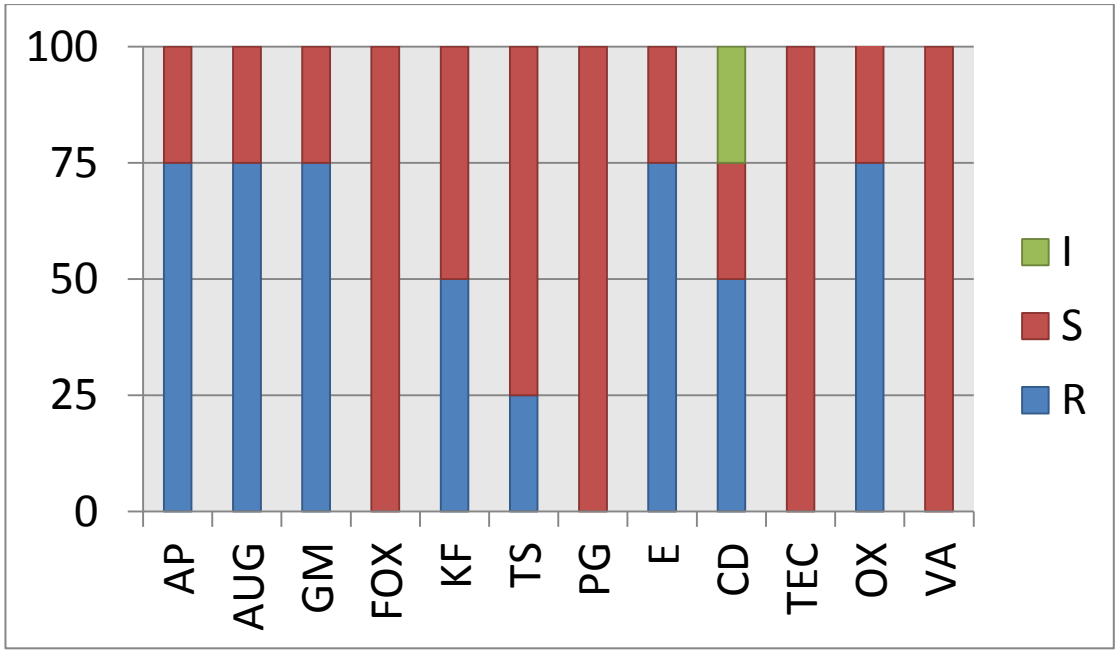


Figure 4.6 Antibiotic susceptibility patterns of *K.pneumoniae* isolates in non CF patients .

Table 4.6 Antibiotic Sensitivity in *S.aureus*

Antibiotics	No. CF Isolates			No. Non CF Isolates		
	R	S	I	R	S	I
AP	3	1	0	4	0	0
AUG	3	1	0	2	0	2
GM	3	1	0	1	3	0
FOX	0	4	0	0	4	0
KF	2	2	0	1	2	1
TS	1	3	0	0	4	0
PG	0	4	0	2	2	0
E	3	1	0	3	1	0
CD	2	1	1	2	2	0
TEC	0	4	0	0	4	0
OX	1	3	0	0	4	0
VA	0	4	0	0	4	0

AUG:Augmentin AP:Ampicillin GM:Gentamicin FOX:Cefoxitin KF:Cephalothin TS:Cotrimoxazol PG:Penicillin E:Erythromycin CD:Clindamycin OX:Oxacillin TEC:Teicoplanin V:Vancomycin



S= Sensitive I=Intermediate R=Resistance

Figure 4.7 Antibiotic susceptibility patterns of *S.aureus* isolates in CF patients

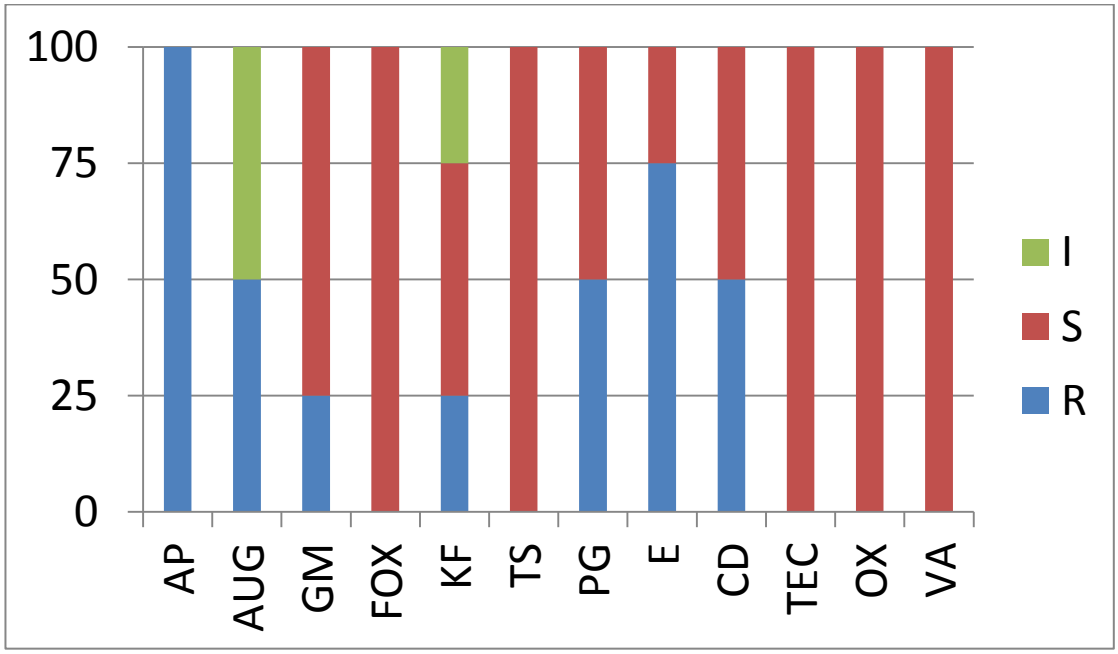


Figure 4.8 Antibiotic susceptibility patterns of *S.aureus* isolates in non CF patients

4.2.2 MICs method

The use of instrumentation for (MIC) can standardize the reading of end points and often produce susceptibility test results in a shorter period than manual readings because sensitive optical detection systems allow detection of subtle changes in bacterial growth**.** The MicroScan WalkAway (Siemens Healthcare Diagnostics) is a large self-contained incubator/reader device that can incubate and analyze 40–96 microdilution trays and it was used in this study. The gram positive isolates were tested by 7 different antibiotics revealed the results indicated in Table (4.7) and figure (4.9) All gram negative isolates were exposed to different antibiotic revealed the results indicated in Table (4.8), (4.9) , (4.10) and Figure (4.10), (4.11), (4.12).

Results indicated that *S.aureus* Gram positive samples resist GM with 87% then CD and 62% but it was sensitive to PG and TEC 100%. *P. aeruginosa* was sensitive to Gm with 100% and also sensitive to IMI and MEM with 95%, but it was resistance to TZP with 52% and CTX with 43%. *A.baumanni*i was the most resistance strain for all used antibiotics with 76% and it was resistant to GM with 100%. *K.pneumoniae* was sensitive to GM, CAZ,IMI and MEM with 100% then AK , FEB and TZP with 80%.

Table 4.7 MICs Susceptibility of *S.aureus* isolates

	TEC	AUG	OX	CD	PG	FOX	GM
S %	100	62.5	100	75	100	100	87.5
R %	0	37.5	0	12.5	0	0	12.5
I %	0	0	0	12.5	0	0	0

TEC:Teicoplanin AUG:Augmentin GM:Gentamicin FOX:Cefoxitin PG:Penicillin CD:Clindamycin OX:Oxacillin

S= Sensitive I=Intermediate R=Resistance

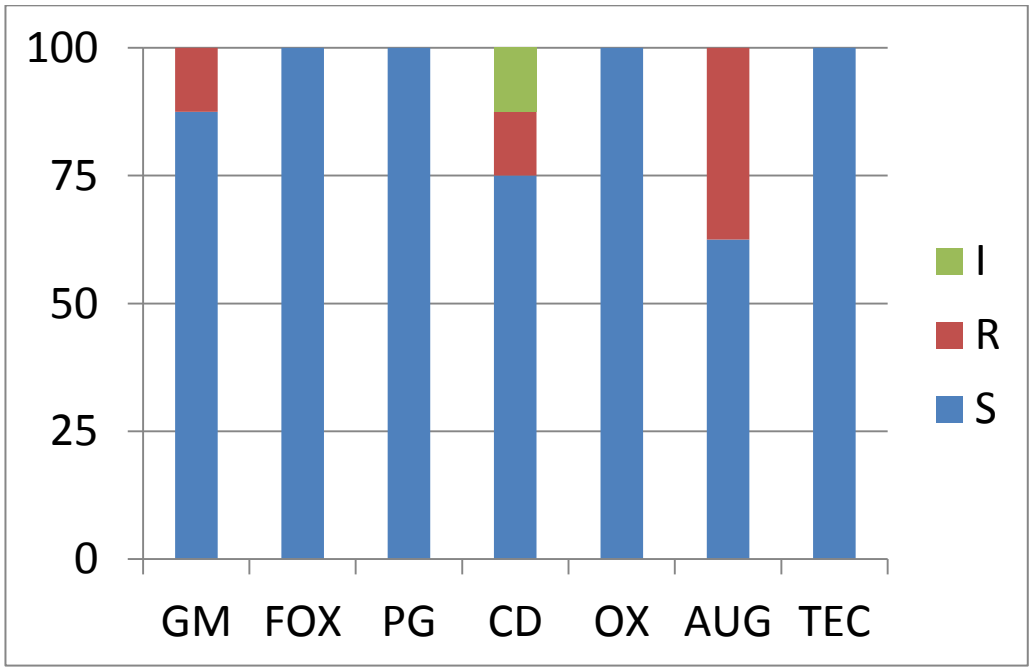


Figure 4.9 MICs Susceptibility patterns of *S.aureus* isolates

Table 4.8 MICs Susceptibility of *P.aeruginosa* isolates

	AK	IMI	MEM	CTX	CAZ	FEB	TZP	GM
S %	43.4	95.6	95.6	34.7	30.38	65.1	30.38	100
R %	39.06	4.34	4.34	43.4	39.06	21.7	52.08	0
I %	17.36	0	0	8.68	0	13.02	17.36	0
IB %	0	0	0	13.02	30.38	0	0	0

GM: Gentamicin AK: Amikacin CAZ: Ceftazidime IMI: Imipenem. TZP: Piperacillin+Tazobactam. CTX: Cefotaxime. FEB:Cefebime. MEM: Meropenem.

S= Sensitive I= Intermediate R= Resistance IB=Inducible Beta-lactamase

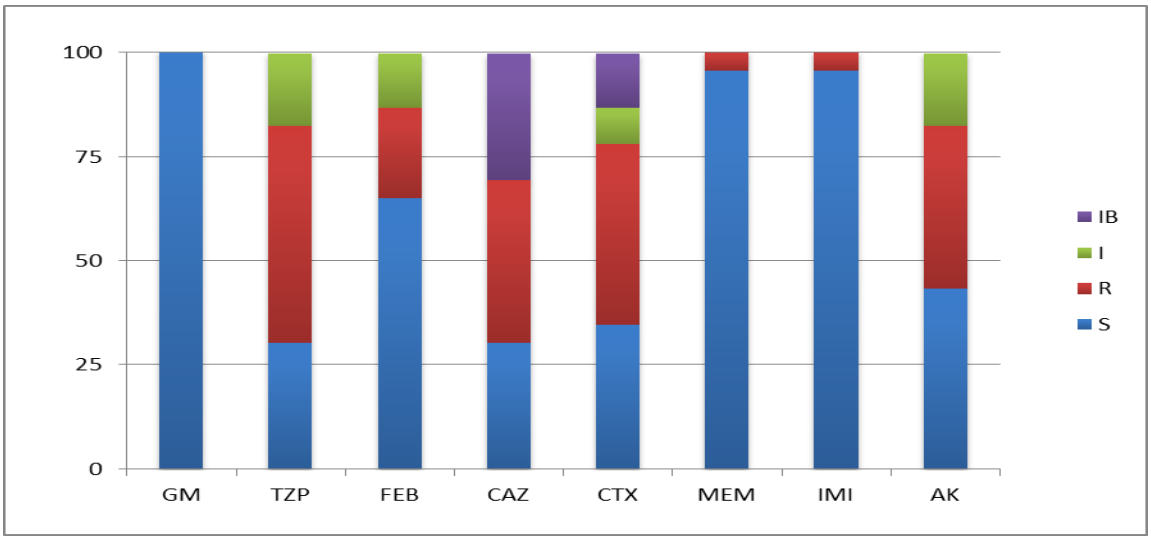


Figure 4.10 MICs Susceptibility patterns of *P.aeruginosa* isolates

Table 4.9 MICs Susceptibility of *A.baumannii* isolates.

	AK	IMI	MEM	CTX	CAZ	FEB	TZP	GM
S %	23.07	23.07	15.4	23.07	23.07	23.07	23.07	0
R %	76.92	76.92	76.92	76.92	76.92	76.92	76.92	100
I %	0	0	7.7	0	0	0	0	0
IB %	0	0	0	0	0	0	0	0

GM: Gentamicin AK: Amikacin CAZ: Ceftazidime IMI: Imipenem. TZP: Piperacillin+Tazobactam. CTX: Cefotaxime. FEB:Cefebime. MEM: Meropenem.

S= Sensitive I= Intermediate R= Resistance IB=Inducible Beta-lactamase

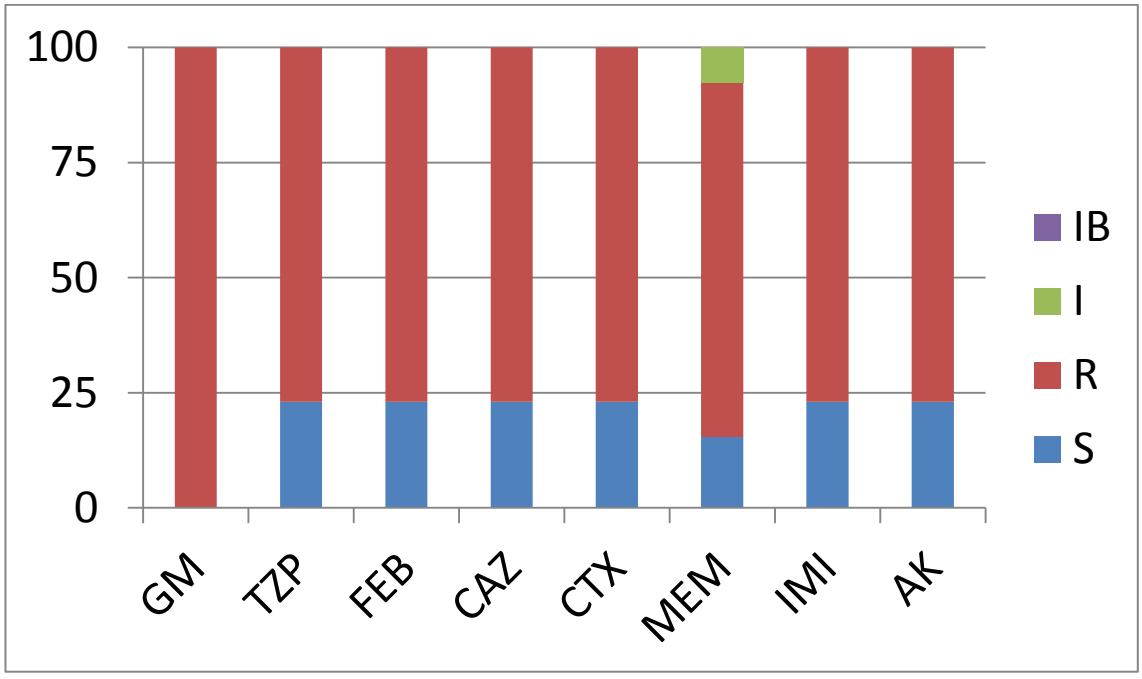


Figure 4.11 MICs Susceptibility patterns of *A.baumannii* isolates.

Table 4.10 MICs Susceptibility of *K.pneumoniae* isolates.

	AK	IMI	MEM	CTX	CAZ	FEB	TZP	GM
S %	80	100	100	60	100	80	80	100
R %	20	0	0	0	0	20	20	0
I %	0	0	0	40	0	0	0	0
IB %	0	0	0	0	0	0	0	0

GM: Gentamicin AK: Amikacin CAZ: Ceftazidime IMI: Imipenem. TZP: Piperacillin+Tazobactam. CTX: Cefotaxime. FEB:Cefebime. MEM: Meropenem.

S= Sensitive I= Intermediate R= Resistance IB=Inducible Beta-lactamase

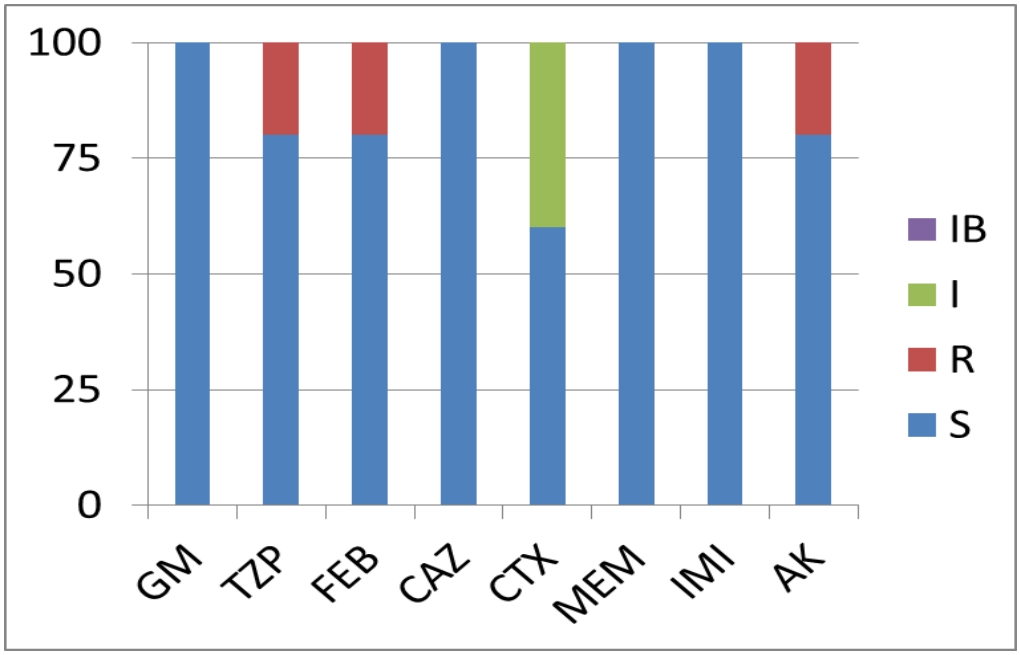


Figure 4.12 MICs Susceptibility patterns of *K.pneumoniae* isolates.

4.3 DNA isolation

Genomic DNA, extracted from pathogenic bacterial strains was used as templates for PCR amplification with two different methods. The results showed that using the Qiamp mini Kit was very efficient method for DNA extraction from Gram negative bacteria and manual CTAB method was very efficient method for DNA extraction from Gram positive bacteria.

4.4 PCR Analysis

Four primer pairs were used in this study. These primers targeted the variable regions in the 16S rRNA gene and every primer pair was specific for each species. PCR assays employing this primer pair produced DNA products of the predicted size (Figure: (4.13), (4.14), (4.15), (4.16) By using these primers targeting 16S rRNA gene. Results showed Amplified fragment 612 bp, 900 bp 1500 bp, 1069 bp for *P.aeruginosa*, *S.aureus*, *A.baumannii* and *K.pneumoniae* respectively.

4.5 RFLP among *P.aeruginosa* isolates

Restriction digestion pattern of 16S rDNA PCR product using *BamHI* enzyme revealed identical digestion pattern among all *P.aeruginosa* isolates two bands, 400 bp and 120 bp as shown in Figure (4.17).

4.6 RFLP among *S. aureus* isolates

Restriction digestion pattern of 16S rDNA PCR product using *ApaI* enzyme revealed identical digestion pattern among all *S.aureus* isolates two bands, 600 bp and 300 bp] as shown in Figure (4.18)

4.7 RFLP among *A.baumannii* isolates

Restriction digestion pattern of 16S rDNA PCR product using *MspI* enzyme revealed different digestion pattern among the strains .One genotype produce three bands with 900 , 500 and 100 bp ,While the other genotypes produce four bands with 700, 400,180 and 120 as shown in Figure (4.19).

4.8 RFLP among *K.pneumoniae* isolates

Restriction digestion pattern of 16S rDNA PCR product using *TaqI* enzyme revealed identical digestion pattern among all *K.pneumoniae* isolates three bands, 600, 240 and 200 bp) as shown in Figure (4.20).

M N 1 2 3 4 5 6 7 8 9 10 11 12 13

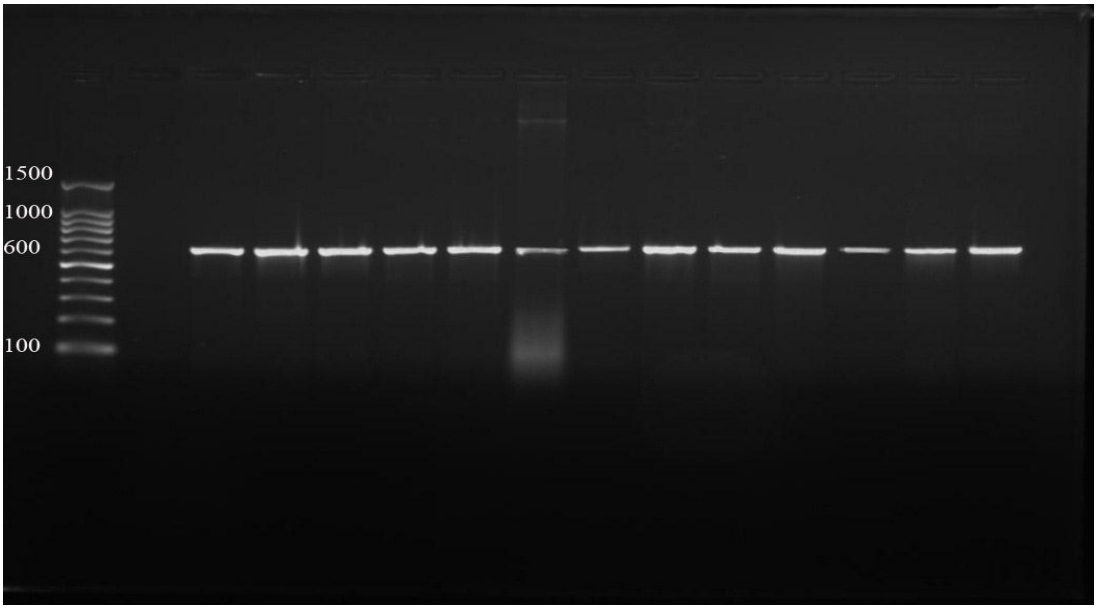


Figure 4.13 PCR products for 16s rDNA gene of *P.aeruginosa* isolates. PCR products including positive and negative control were resolved in 2% agarose gel stained with ethidium bromide showed amplified fragments at 612bp

Lane 1-6: CF isolates

Lane 7-12: Non CF isolates

Lane13: Positive control (*P.aeruginosa* Atcc 27853)

Lane M: DNA molecuolar marker

Lane N: Negative control (*P.fluorescens*)

M 1 2 3 4 5 6 7 8 9 N

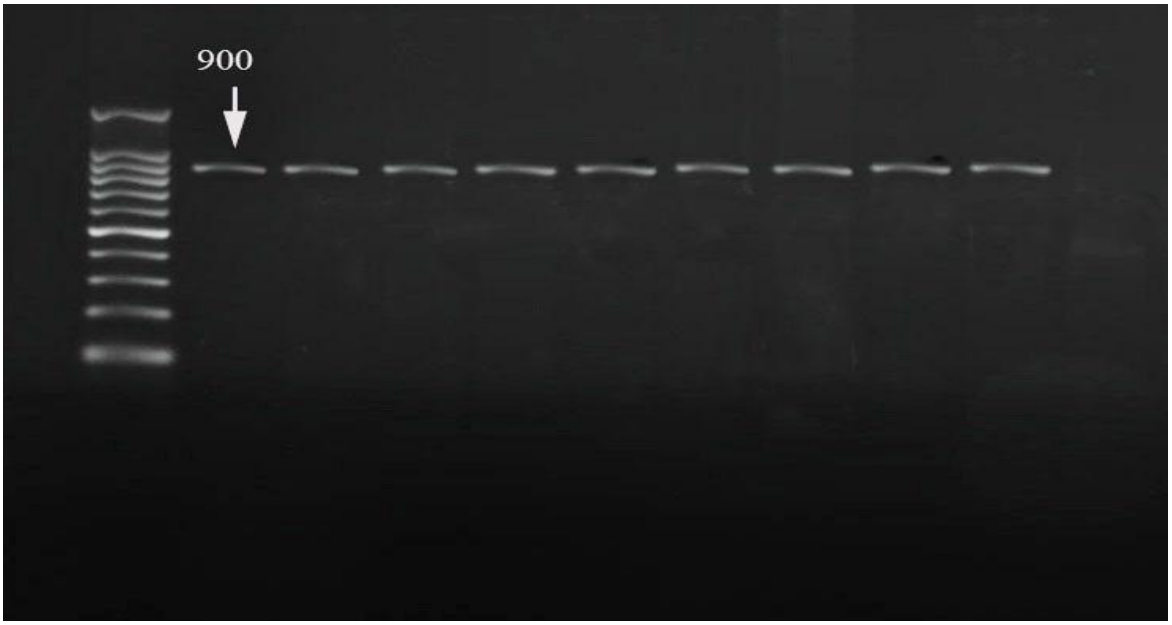


Figure 4.14 PCR products for 16s rDNA gene of *S.aureus* isolates. PCR products including positive and negative control were resolved in 2% agarose gel stained with ethidium bromide showed amplified fragments at 900 bp.

Lane 1-4: CF isolates

Lane 5-8: Non CF isolates

Lane9: Positive control (*S.aureus* Atcc 25923)

Lane M: DNA molecuolar marker

Lane N: Negative control (MRSA)

C 1 2 3 4 5 6 7 8 9 10 11 12 13 M

Figure 4.15 PCR products for 16s rDNA gene of *A.baumannii* isolates. **PCR products including negative control were resolved in 2% agarose gel stained with ethidium bromide showed amplified fragments at 1500 bp.**

Lane 1-4: CF isolates

Lane 5-13: Non CF isolates

Lane M: DNA molecuolar marker

Lane C: Negative control (*A. haemolyticus*)

M 1 2 3 4 5 6 N

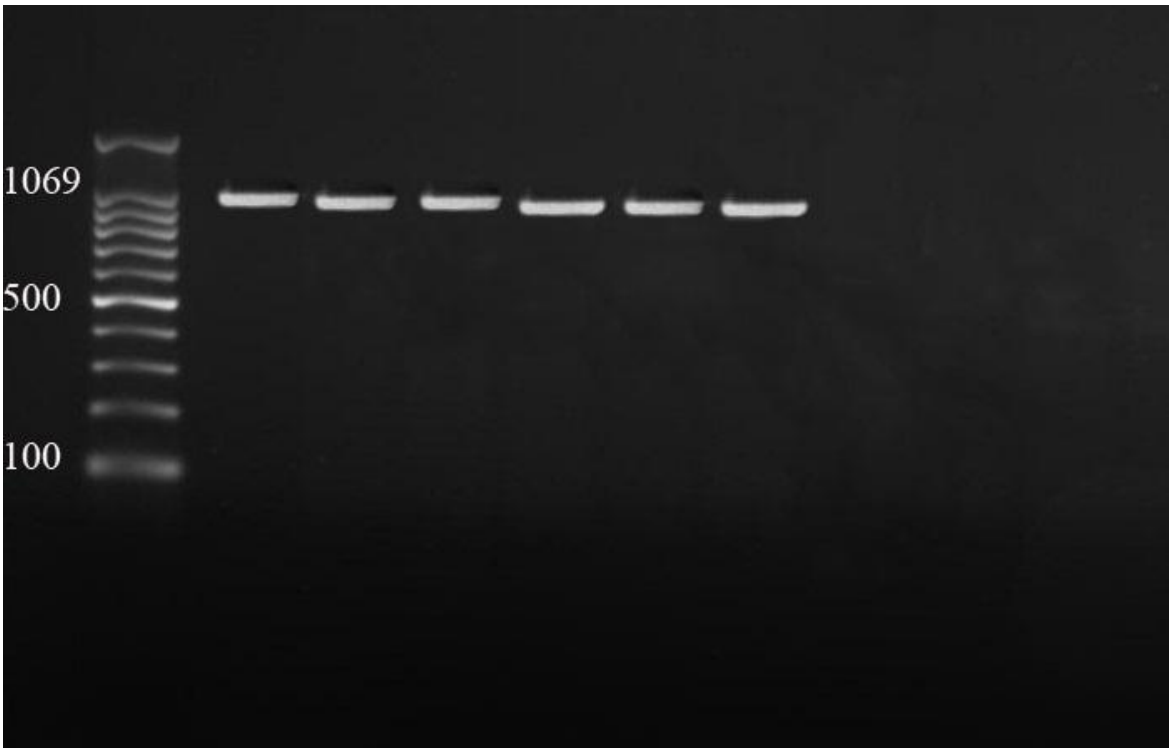


Figure 4.16 PCR products for 16s rDNA gene of *K.pneumoniae* isolates. PCR products including negative control were resolved in 2% agarose gel stained with ethidium bromide showed amplified fragments at 1069 bp.

Lane 1-3: CF isolates

Lane 4-5: Non CF isolates

Lane M: DNA molecuolar marker

Lane N: Negative control (*K.* oxytoca)

Lane C: Positive control (*K.pneumoniae* Atcc 700603)

M 1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 4.17 RFLP for *P.aeruginosa* isolates. After digestion with *BamHI* using 2% agarose gel.

Lane 1-8: CF isolates

Lane 9-13: Non CF isolates

Lane M: DNA molecuolar marker

M 1 2 3 4 5 6 7 8 C M

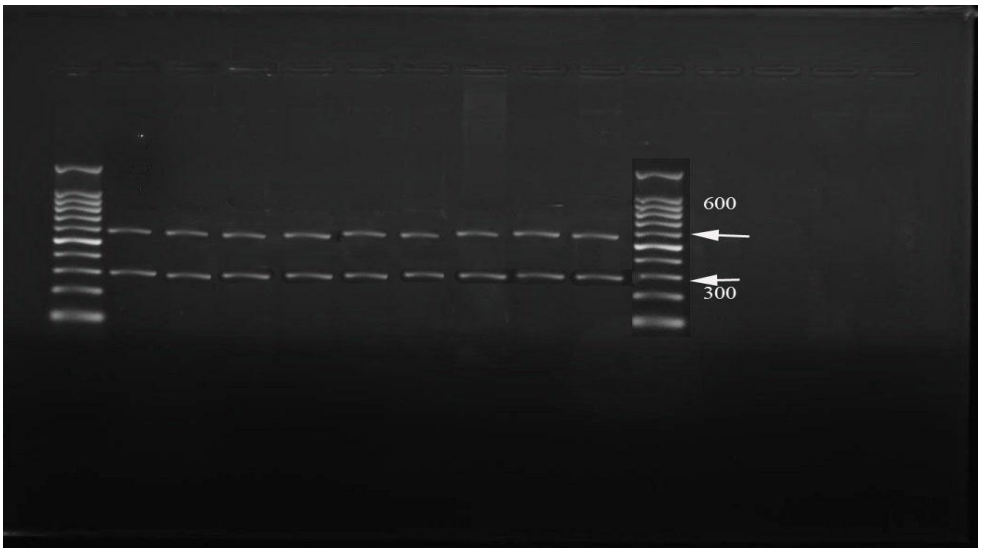


Figure 4.18 RFLP for *S.aureus* isolates .After digestion with *ApaI* digestion using 2% agarose gel.

Lane 1-4: CF isolates

Lane 5-8: Non CF isolates

Lane M: DNA molecuolar marker

Lane C: Positive control *S.aureus* (Atcc 25923)

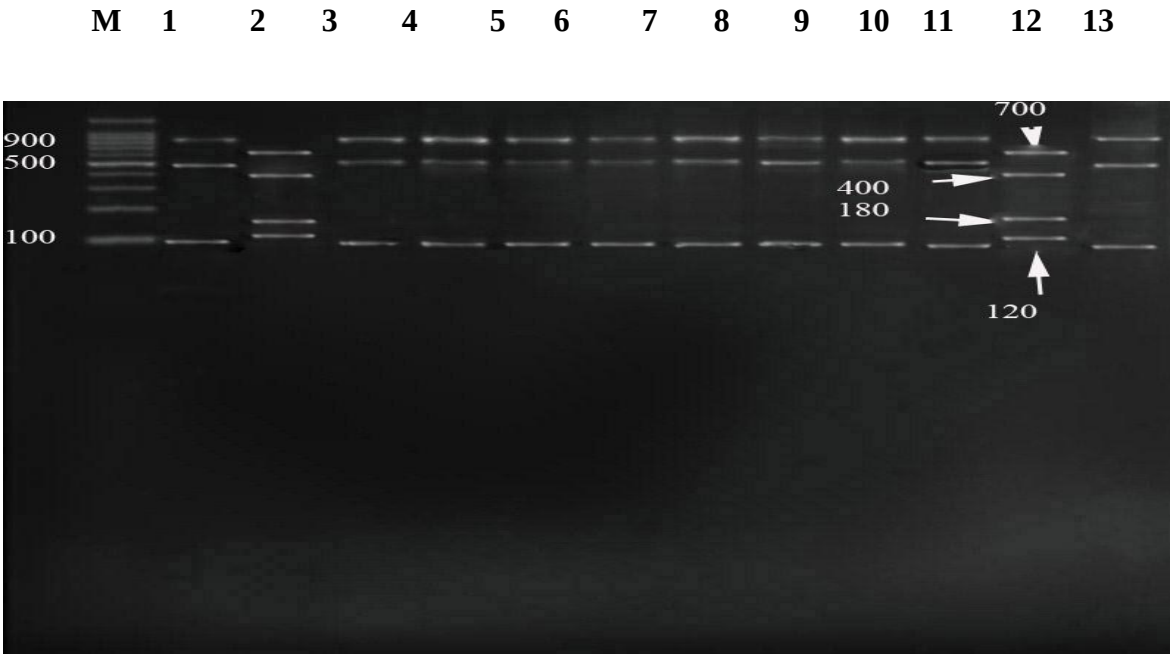


Figure 4.19 RFLP for *A.baumannii* isolates .After digestion with *MspI* digestion using 2% agarose gel.

Lane 1-4: CF isolates

Lane 5-12: Non CF isolates

Lane M: DNA molecuolar marker

M 1 2 3 4 5 C

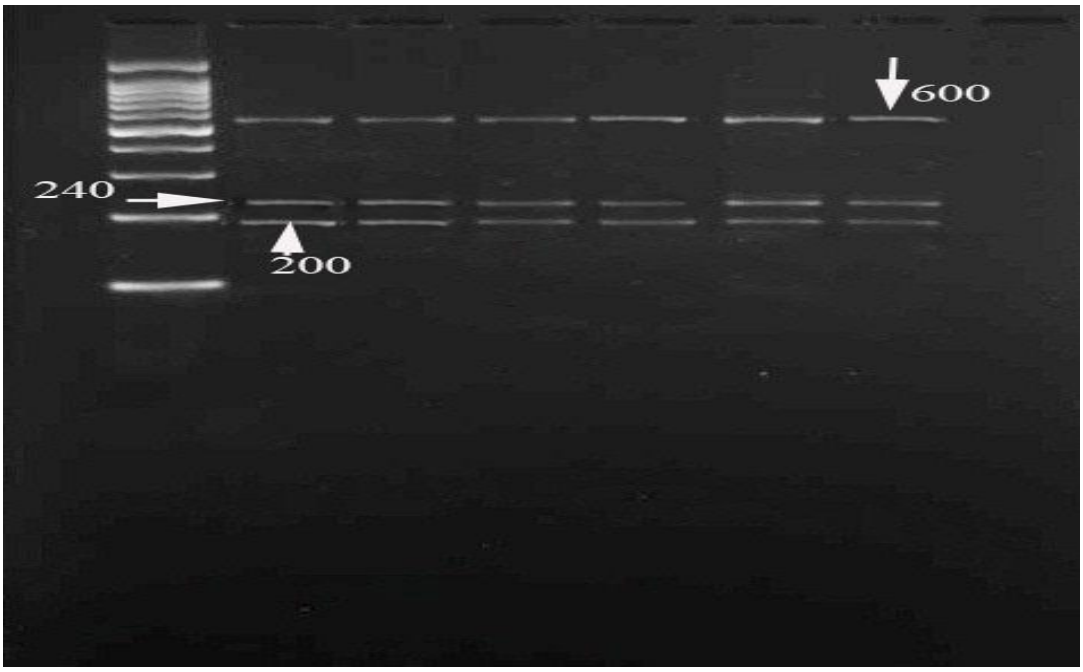


Figure 4.20 RFLP for *K.pneumoniae* isolates. After digestion with *TaqI* using 2% agarose gel.

Lane 1-3: CF isolates

Lane 4-5: Non CF isolates

Lane M: DNA molecuolar marker

Lane C: Positive control *K.pneumoniae* (Atcc 700603)

4.9 Plasmid Patterns in *P.aeruginosa* isolates

Plasmids were found in 4 (28.5 %) CF strains and 5 (55.5%) non CF strains with an average of 2 plasmids per strains; their relative molecular mass ranged from 23,130 bp to 4,361 bp. No plasmids were found in 10 (71.4%) CF strains and 4 (44.4%) non CF strains. Figure (4.23) show the profiles of plasmids isolated from strains of *P.aeruginosa*.

4.10 Plasmid Patterns in *S.aureus* isolates

Plasmids were found in 2 (50 %) CF strains and 2 (50%) non CF strains with an average of 2 plasmids per strains; their relative molecular mass ranged from 20,000 bp to 4300 bp. No plasmids were found in 2 (50 %) CF strains and 2 (50%) non CF strains. Figure (4.21) show the profiles of plasmids isolated from strains of *S.aureus.*

4.11 Plasmid Patterns in *A.baumannii* isolates

Plasmids were found in 3 (75 %) CF strains and 4 (44.4 %) non CF strains with 1 plasmid per strains; their relative molecular mass ranged from 15,130 bp to 4,361 bp. No plasmids were found in 1 (25 %) CF strains and 5 (55.6%) non CF strains. Figure (4.22) show the profiles of plasmids isolated from strains of *A.baumannii.*

4.12 Plasmid Patterns in *K.pneumoniae* isolates

Plasmids were found in 3 (100 %) CF strains and 2 (100%) non CF strains with an average of 2 plasmids per strains; their relative molecular mass ranged from 23,130 bp to 6,557 bp. Figure (4.24) show the profiles of plasmids isolated from strains of *K.pneumoniae*.

-

M 1 2 3 4 5 6 7 8 C

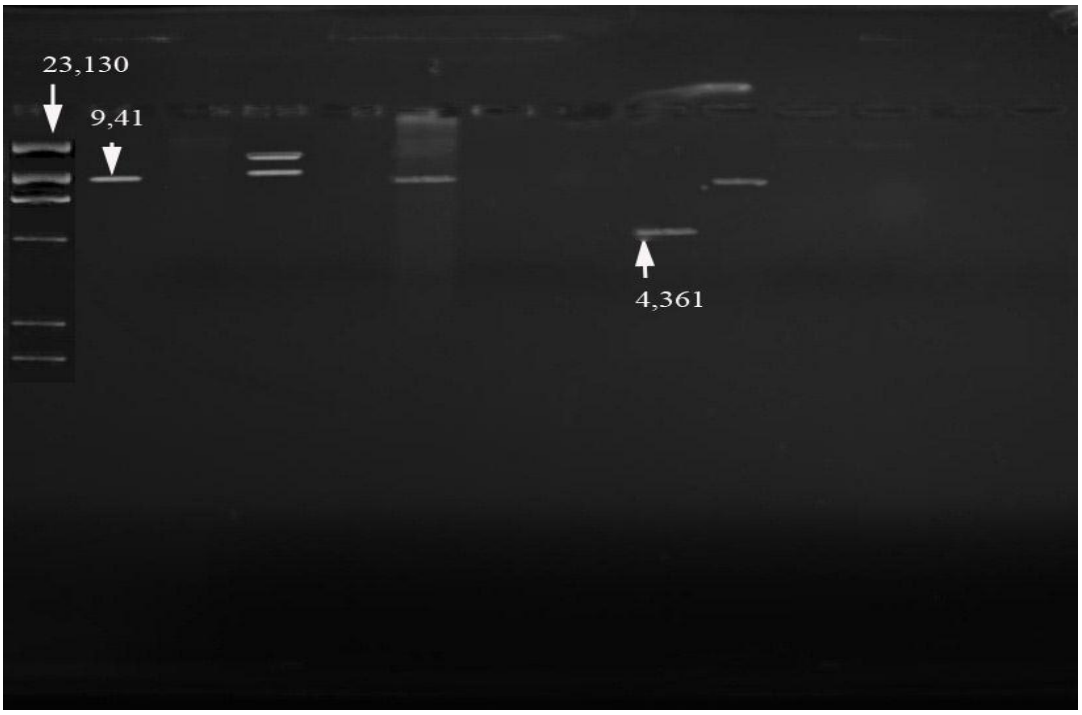


Figure 4.21 Plasmid profile of *S.aureus* isolates

Lane 1-4: CF isolates

Lane 5-8: Non CF isolates

Lane M: Lambda DNA HindIII digest as a marker

Lane C: Positive control *S.aureus* (Atcc 25923)

M 1 2 3 4 5 6 7 8 9 10 11 12 13

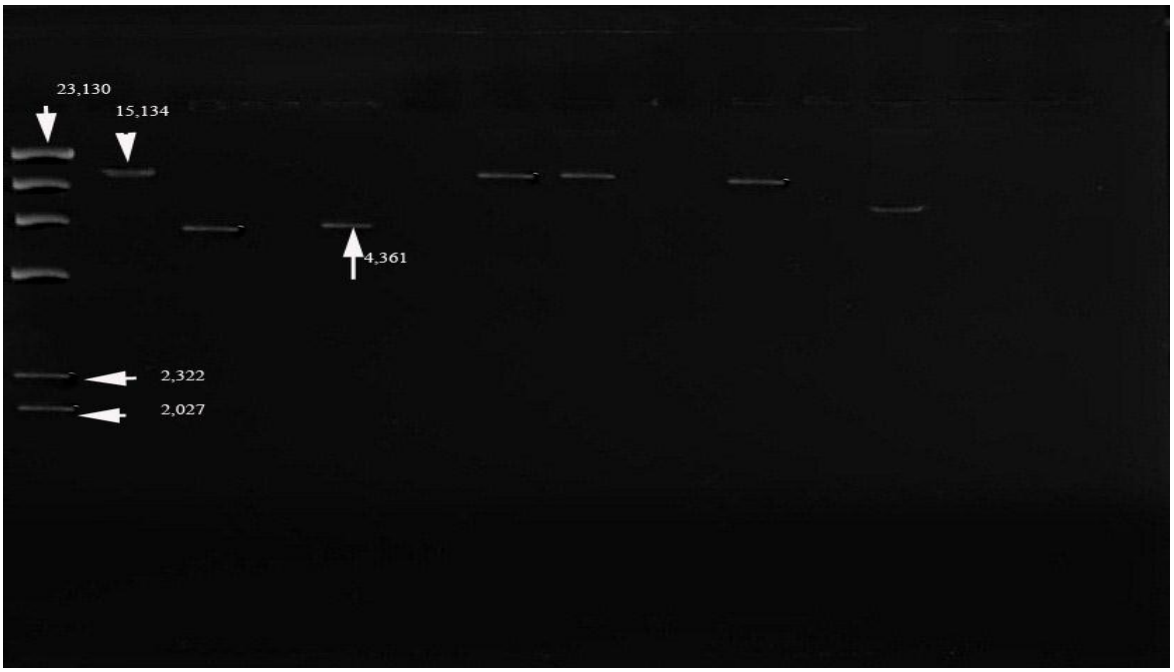


Figure 4.22 Plasmid profile of *A.baumannii* isolates.

Lane 1-4: CF isolates

Lane 5-13: Non CF isolates

Lane M: Lambda DNA HindIII digest as a marker

1 2 3 4 5 6 7 8 9 10 11 12 C M

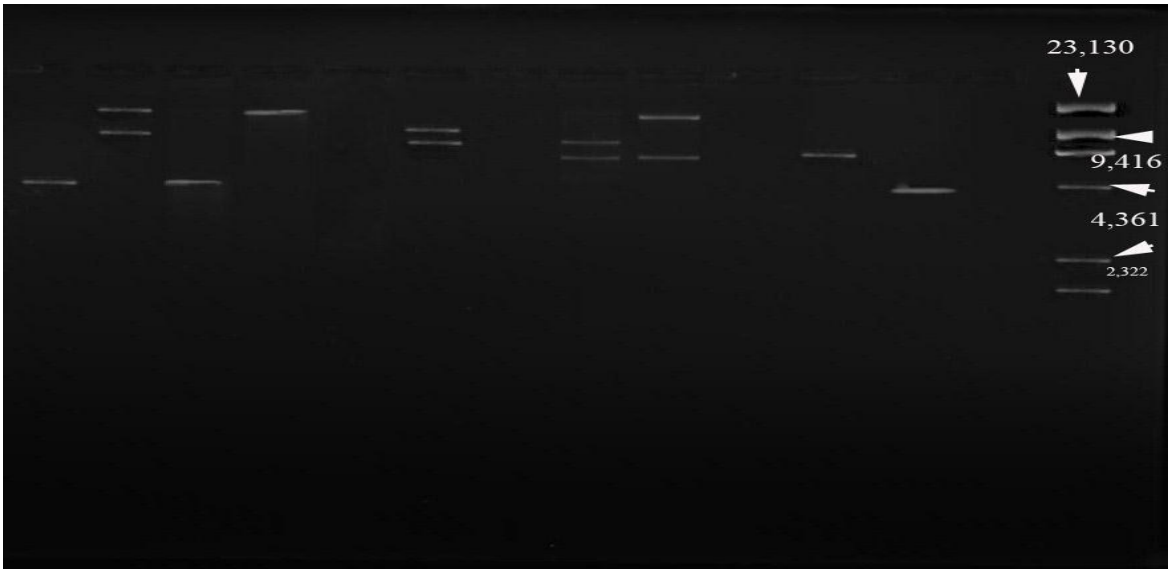


Figure 4.23 Plasmid profile of *P.aeruginosa* isolates.

Lane 1-5: CF isolates

Lane 6-12: Non CF isolates

Lane M: Lambda DNA HindIII digest as a marker

Lane C: Positive control (*P.aeruginosa* Atcc 27853)

M 1 2 3 4 5 6

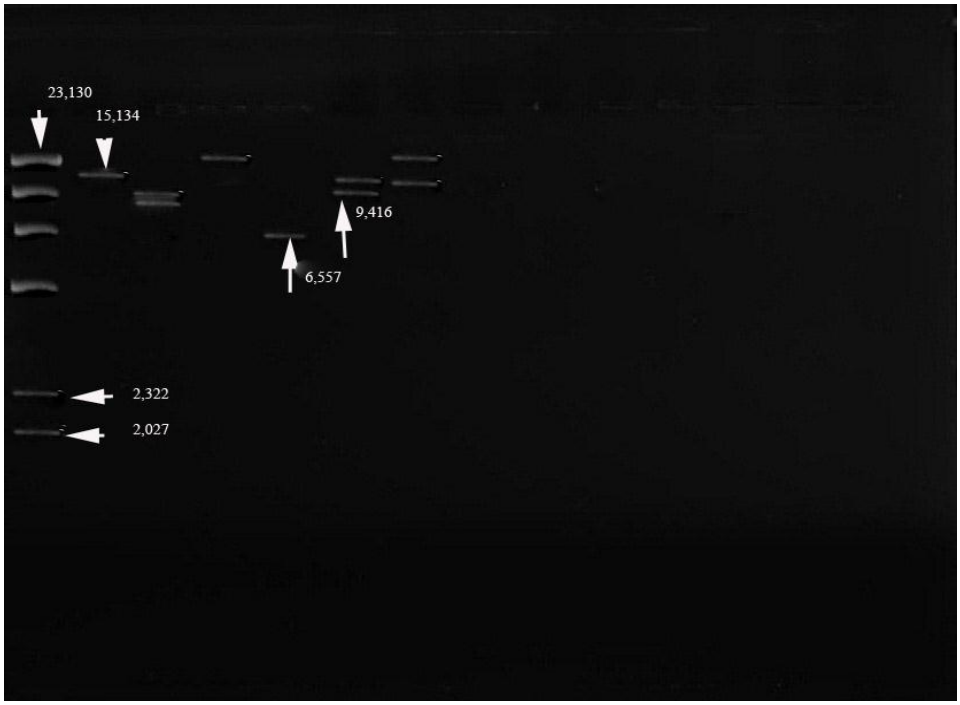


Figure 4.24 Plasmid profile of *K.pneumoniae* isolates.

Lane 1-3: CF isolates

Lane 4-5: Non CF isolates

Lane M: Lambda DNA HindIII digest as a marker

Lane C: Positive control (*K.pneumoniae* Atcc 700603)

Chapter Five

Discussion

CF is the most common lethal inherited disease in white persons (Davis *et al.* 1996). Chronic airway inflammation and infection are the greatest causes of morbidity and mortality in CF patients (Gibson *et al.* 2003) Lungs of CF patients are often colonized or infected in infancy and early childhood with organisms, such as *Staphylococcus aureus* and *Haemophilus influenzae*, that may damage the epithelial surfaces, leading to increased attachment of, and eventual replacement by *P. aeruginosa* (Lyczak *et al.* 2002). Approximately 30,000 Americans have CF, and there are an estimated 1,000 new cases diagnosed each year (Cystic Fibrosis Foundation) and there are more than 5,000 registered CF patients in the UK (McCormick *et al.* 2002). The incidence of CF in Saudi Arabia was reported to be 1 in 4243 children (Nazer *et al.* 1989).The Microbiological analysis of clinical specimens has relied traditionally on cultivation prior to identification but at the same time molecular biological approaches have gained widespread acceptance for the study of bacterial communities in samples (Horner-Devine *et al.* 2004). This study was conducted to investigate the bacterial pathogens colonizing patients with cystic fibrosis in jeddah and explored the possibility of amplification of the 16S rDNA followed by restriction analysis to identify 4 different species assioated with CF infection. The data obtained in this study found that *P.aeruginosa* present in 56% (14 of 25) of CF samples and 37 % (9 of 24) in non CF samples, being the

highest rate of colonization in agreement with Cystic Fibrosis Foundation Report, USA, in 2006 and Cystic Fibrosis Foundation Report, UK, in 2010 . *S.aureus* was isolated from 16% of CF samples and 16.64 % of non CF samples. As reported in previous studies on the prevalence and antimicrobial susceptibility of bacterial isolates from CF patients in USA, Germany, and South America, we found that *S. aureus* and *P.aeruginosa* are the most frequent pathogens in this population (Lambiase *et al*. 2006). In 2010, Paixão *et al*, found that the prevalence of *S. aureus* was highest (70.7%) among children and in older age the prevalence of *S.aureus* isolates declined as the prevalence of *P. aeruginosa* increased. *A.baumannii* is an un usual organism in CF patients (Rahal and Urban, 2000). In this study *A.baumannii* present in 16% of CF samples (4 of 25) and 37.5% in non CF samples (9 of 24).In comparing this result with study in USA was indicated in 2010 , *A.baumannii* was found in 5 patients out of 53 U.S patients (LiPuma. 2010) which is give evidence of the infrequency of this species in these patients . There is paucity of reports regarding colonization of *K. pneumoniae* in CF patients (Leão *et al*, 2011; Bittar *et al*, 2008) .It's present in 12% of CF samples (3 patients) and 8.32 % in non CF samples (2 patients), it was the lowest prevalent microorganism isolated from our patients. In contracts with this result, *K. pneumoniae* was the second most pathogen isolated from CF patients in Brazil (Khanbabaee *et al*. 2012).Antibiotic therapy reduces the morbidity of CF lung disease. Although the treatment of lung infection in CF patients is based on the patient's age, the colonizing organisms, and the severity of the patient's pulmonary exacerbation, choosing antibiotic according to the resistance pattern of the strains is highly recommended (Doering *et al*. 2000). The following antibiotic susceptibility results were observed in our study; for *P.aeruginosa* isolates were recorded a high resistance of Ampicillin 95 % and

Augmentin 95.6 then Ceftriaxone 69.44%, Aztreonam, 60% and Cefotaxime 52%. The *P.aeruginosa* isolates were most susceptible to Meropenem 78%, Imipenem 78% and Ceftazidime 60%. The susceptibility to Meropenem and Imipenem were higher in other study, Meropenem 82% and Imipenem 84%. (Nazik *et al*. 2007) The differences of susceptibility patterns between this and other studies may be related to different in the isolated strain and the use of infection control practices. The *S.aureus* isolates showed 100% sensitive to Vancomycin, Teicoplanin and cefoxitin (a measure of methicillin resistance). The susceptibility test results showed ampicillin and Augmantin to be the least effective agents with 75% bacterial resistance, this have been widely reported for *S. aureus* from various sites of healthy subjects (Onanuga and Onalapo, 2008) and nosocomial infections (Hoerlle and Brandelli. 2009). *A.baumannii* isolates have a propensity to readily develop resistance to second and third generation antibiotics such as cefotaxime, ciprofloxacin, and giving rise to therapeutic problems (Jain and Danziger. 2004) As higher generation antibiotics are being developed to overcome problem of resistance against available antibiotics, *A.baumannii* are developing mechanisms to resist newer antimicrobials(Patwardhan *et al*. 2008) .In this study *A. baumannii* isolates showed resistance to both old and new generation antibiotics.

Database availability of 16s *rDNA* genes for several bacteria species provides fundamental information for comparative studies by using bioinformatics tools, which can predict accurately gene polymorphism among species correlating with phenotypic features. In our study 16s rDNA species specific primers have used for identification all isolates. Selective amplification of *Pseudomonas* 16S rDNA by PCR followed by restriction fragment length polymorphism analysis has been used to detect and differentiate *Pseudomonas* species from clinical and environmental

samples (Porteous *et al.* 2002). Karpati and Jonasson, 1996, used a conserved 16S rDNA primer with a *Pseudomonas* genus-specific primer in a PCR assay to detect *Pseudomonas* DNA in CF sputum. 16S rDNA-based PCR assay provide simple, and reliable identification of tested isolates in this study and differentiate them from other phylogenetically closely related species .16s rDNA PCR assay has 100% sensitivity and specificity for it intended target species. In comparing these results with other studies we found that 16S rDNA gene is highly conserved on *Klebsiella* genus (Arenas *et al*. 2009). In study by Kurupati *et al*, the species specific primers targeting this gene were investigated by testing *K. Pneumoniae* and 65 negative-control organisms and the target sequence was shown to be highly specific for *K. pneumoniae*, as it failed to detect any other bacteria. In similar to our results, *P.aeruginosa* 16S rDNA– based PCR assay show sensitivity and specificity (Spilker *et al*. 2004) *A.baumannii* 16s rDNA gene has also the specificity that give 100% results for identification this species (Vaneechoutte and Dijkshoorn 1995). Studies RFLP shown that it's a powerful taxonomic tool for bacterial identification at the species level and used extensively in the 19's (Cascón *et al*.1997; Mollet *et al*.1997). In our study among the 23 *P.aeruginosa* isolates 1 *BamHI* RFLP pattern was detected, The low variability of *Pseudomonas* 16S RFLP patterns has been reported earlier (Laguerre *et al.* 1994). *S.aureus* isolates also has identical *ApaI* RFLP pattern. On the other hand, variability in RFLP pattern was found amongst *A.baumannii* isolates and this was similar to study by (Hernández *et al*. 2011).

In order to determine whether the observed multi drug resistance pattern in the isolates was plasmid or chromosomal mediated, the isolates were screened for the presence of plasmids. One or more plasmids were isolated from *S.aureus* in this study and this was agree with study by Piccinini and Zecconi in 2001. Plasmid

DNA was detected in only 39 % of *P.aeruginosa* isolates in our study. Therefore it is postulated that most of the resistance genes in *P. aeruginosa* are mostly chromosomal. We can verify this postulate by Plasmid curing. This result is sustaining a result obtained by Nikbin *et al*. 2007, they found the plasmid in 29 % of their *P.aeruginosa* isolates. In *A.baumannii* isolates plasmid was found in 7 isolates and 6 isolates had no plasmids .Since all isolates, including the 6 isolates that supposedly carry no plasmids, presented multi resistance to drugs, some of the resistance markers of the *A.baumannii* possibly are chromosomally located, in agreement with previous studies (Bergogne-Bérézin *et al.*1996).

In Conclusion, Our results show that CF patients in Jeddah had uncommon species in CF patients (*A.baumannii*. *K.pneumoniae*) also the PCR-RFLP technique has High sensitivity for identifying species associated with infection in CF patients. Different numbers and sizes of Plasmids were detected in the isolates and the appearance of the plasmid in the isolates was vary from Species to another.