Genetic Analysis of protease from *Pseudomonas aeruginosa* Isolated from Different Human Infections

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التحليل الوراثي لبروتييز بكتريا *Pseudomonas aeruginosa* المعزولة من أخماج مختلفة

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SUMMARY

Thirty-two isolates of *Pseudomonas aeruginosa* were isolated from different human infections including wounds, burns, ear and urinary tract infections. The isolates were identified according to morphological, cultural and biochemical characteristics. Some of virulence factors were studied such as antibiotic susceptibility, pyocyanin pigment and extracellular protease production. The results showed that all the Pseudomonal isolates were resistant to ampicillin, amoxicillin, carbencillin, chloramphenicol, cefotaxime**,** erythromycin, lincomycin, penicillin, tetracycline and trimethoprime, whereas they indicated

variable resistance to amikacin**,** ciprofloxacin, gentamicin, neomycin, rifampicin and streptomycin. All isolates produced pyocyanin pigment on King's medium while only sixteen isolates produced extracellular protease on skimmed milk agar.

Four Pseudomonal isolates were selected to study plasmid profile on agarose gel electrophoresis, two of them, *P. aeruginosa* P٢٢ and *P. aeruginosa* P٢٤, had the ability to produce protease and the other two were non-proteases producers isolates (*P. aeruginosa* p١٢ and *P. aeruginosa* P١٦). The results revealed that protease producing isolates harbor a common single plasmid and small plasmids, whereas the non-protease producing isolates harbor small plasmids only.

Plasmid DNA of *P. aeruginosa* P١٦ and *P. aeruginosa* P٢٢ were transformed into competent cells of plasmid less *E. coli* MM٢٩٤ (Rif^r). The results revealed that the small plasmids of Pseudomonal isolate encoding ampicillin and streptomycin resistances were transferred to *E. coli*. Bacterial conjugation was performed between the donor isolates (*P. aeruginosa* P٩ and *P. aeruginosa* P٢٢) and the plasmidless recipient *E. coli* MM٢٩٤ (Rif^r), results showed that the conjugative-plasmids of Pseudomonal isolates encoding to pyocyanin pigment and resistance to all studied antibiotics except lincomycin.

Also plasmid curing from some Pseudomonal isolates (*P. aeruginosa* P٣, *P. aeruginosa* P١٩ AND *P. aeruginosa* P٢٧) was performed by different agents including ٢٪ SDS, ٧٠٠µg/ml ethidium bromide and by high temperature (٤٦ºC). The results showed that high temperature was more efficient in eliminating all plasmids from bacterial cells. Subsequently all cured cells became sensitive to all antibiotics except lincomycin. Over all results of plasmid transferred by

transformation, conjugation and plasmid curing experiments indicate that lincomycin resistance and extracellular protease production encoded by chromosomal genes.

A histopathological study was carried out to understand the effect of extracellular protease as a virulence factor on burned laboratory animals (Albino's rat). The results showed that the protease producing *P. aeruginosa* had the greatest effect on the rat skin structure and morphology which caused complete sloughing of epidermis, associated with severe congestion and thrombosis of dermal blood vessels, Presence of large quantities of inflammatory exudates in the dermis, and destruction of all of the skin appendages like (hair follicles, sebaceous gland……etc). While the non-protease producing *P. aeruginosa* and the wild type of *E. coli* MM٢٩٤ had no effects observed in infected rat skins. On the other hand, the *P. aeruginosa* protease causes severe lung damages (pneumniaties), whereas no infections demonstrated by wild type *E. coli* MM٢٩٤.

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List of Abbreviations

AIDS…….Acquired Immuno Deficiency Syndrome

BSE……..Burned Skin Extract

IFN……Inactivation of gamma Interferon

Inc……Incompatibility group

Kbp……...Kilo base pairs

LPS……..Lipopolysaccharide

MDR…….Multi Drug Resistance

Mob genes……...Mobilization gene

PA toxin……Pseudomonas A toxin

R-plasmid……Resistance plasmid

RTF……..Resistance Transfer Factor

TNF……..Tumor Necrosis Factor

UTI……Urinary Tract Infection

CONCLUSIONS

١- *Pseudomonas aeruginosa* is an opportunistic pathogen that causes human infections, and the most common cause of the nosocomial infections.

٢- It revealed high resistance to most widely used antibiotics (MDR), and some isolates are resistant to all tested (١٨) antibiotics.

٣- Proteases are one of the most virulence factors secreted by this bacterium, and have the lethal effect in post infection of burned patients.

٤- Most of multiple antibiotic resistance genes of *P. aeruginosa* isolates were successfully transformed to *E. coli* MM٢٩٤.

٥- Conjugation processes were done successfully between *P. aeruginosa* isolates and *E. coli* MM٢٩٤, and these indicate that *P. aeruginosa* isolates contain conjugative plasmid which has the ability to transfer to another bacterial species.

٦- Proteases are encoded by chromosomal DNA, which decrease the risk of dissemination from one strain to another by transmissible plasmids.

٧- Among curing agents used (SDS, EB, and elevate temperature at ٤٦°C), the latter appeared to be the more effective agents in curing the antibiotic resistance genes in *P. aeruginosa* isolates studied.

٨- Histopathological examination study revealed that both cloned *E. coli* P٢٢ isolate and proteases producing *P. aeruginosa* P٢٢ isolate (wild type), were the most effective as virulence factors in colonization and infection of burned rat-models.

٩- Proteases producing *P. aeruginosa* isolate are regarded as the most common cause of acute interstitial pneumoniaties and many other infections of lung rat-models.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen of human, belonging to the bacterial family Pseudomonadaceae (Todar, ٢٠٠٤), that is widespread in the environment, a major cause of community acquired infections (Nester *et al*., ٢٠٠١). They are Gram negative, aerobic, motile and rod-shaped bacteria, measuring about (٠.٥ to ٠.٨ µm by ١.٥ to ٣.٠ µm), and occur as single bacteria, in pairs and occasionally in short chains (Todar, ٢٠٠٤). It grows well at ٣٧ - ٤٢°C and they are oxidase positive; many strains produce two types of soluble pigments, the fluorescent pigment (pyoverdin) and the bluish pigment (pyocyanin). Identification is usually based on colonial morphology, oxidase positivity, and presence of characteristic pigments and growth at ٤٢°C (Jawetz *et al*., ٢٠٠١).

P. aeruginosa is a bacterium responsible for severe nosocomial infections, life-threatening infections in immunocompromised persons, and chronic infection in cystic fibrosis patients (Delden and Iglewski, ١٩٩٨). In hospital, the bacterium is the leading cause of nosocomial lung infections and a common cause of wound infections, especially of thermal burns (Nester *et al*., ٢٠٠١). *P. aeruginosa* is responsible for ١٦٪ of nosocomial pneumonia cases, ١٢٪ of hospital acquired urinary tract infections, ٨٪ of surgical wound infections, and ١٠٪ of blood stream infections (Pollack, ١٩٩٥). *P. aeruginosa* is notorious for its resistance to antibiotics, and is, therefore, particularly dangerous and dreaded pathogens. The bacterium is naturally resistant to many antibiotics due to the permeability barriers afforded by its outer membrane lipopoly saccharide (LPS). Also, its tendency to colonize surfaces in a biofilm form makes the cells impervious to therapeutic concentration antibiotics.

Moreover, *Pseudomonas* maintains antibiotic resistance plasmids, both R-factor and resistance transfer factors (RTF), and it is able to transfer these genes (Todar, ٢٠٠٤). *P. aeruginosa* is the bacterium which resist to most widely used antibiotics; it is not unusual for strain of this organism isolated from infections to be resistant to more than ten or more antibiotics. Most strains of *P. aeruginosa* are multidrug resistant, and contain R-plasmid especially clinical strains with different molecular weight (١.٩١

– ٤٠) Mega Dalton. Moreover, it contains high molecular weight plasmids (٦٠ – ١٠٠) Mega Dalton (Tsakris *et al*., ١٩٩٢).

It is a common phenomenon in most general hospitals that the frequency of occurrence of infection due to *P. aeruginosa* is in increase, but undoubtedly there are some relationships to the widespread use of antibiotic therapy. Even the discovery of new antibiotics may fail to solve the problems of antibiotic; the bacteria illustrate resistance to certain antibiotics after its exposure for a while (Kheder, ٢٠٠٢). The bacterium's virulence depends on a large number of cell-associated and extracellular factors (Delden and Iglewski, ١٩٩٨); certain strains release an extracellular slime that is lethal to mice and protects the pathogen from phagocytosis, and the slime is a virulence factor for *P. aeruginosa* (Ross, ١٩٨٣).

On the other hand, the study of nosocomial bacteria like *P. aeruginosa* at the molecular genetics level is important, since this bacterium has the ability to transfer its genetic materials especially antibiotic resistant genes to another bacteria through conjugation and also transformation processes which become a great problem in chemotherapy. Controlling of antibiotic resistance gene at the molecular levels such as by using curing agents with appropriate concentration may limit or release the resistance of pathogenic bacteria (Hardy, ١٩٨٦). Most strains of *P. aeruginosa* produce two exotoxins, exotoxin A and exo-enzyme S, and a variety of cytotoxic substances including phospholipases, pyocyanin, rhamnolipids and proteases; an alginate-like exopolysaccharide is responsible for the mucoid phenotype. The importance of these putative virulence factors depends upon the site and nature of infection. Proteases play a key role in corneal ulceration, and are important in burn infection; and associated with chronic pulmonary colonization (Greenwood *et al*., ١٩٩٤; Todar, ٢٠٠٤).

Rao *et al*. (١٩٩٨) documented that the virulence of several bacteria is related to the secretion of several extracellular proteases. Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins. Proteases are degredative enzymes which catalyze the total hydrolysis of proteins. Also, Passodor and Iglewski (١٩٩٥) described that proteases are assumed to play a major role during acute *P. aeruginosa* infection. *P. aeruginosa* produces several proteases including Las B, Las A elastase, and alkaline protease. The role of alkaline proteases in tissue invasion and systemic infections is

unclear; however, its role in corneal infections may be substantial (Howe and Iglewski, ١٩٨٤).

The objectives of the present study are as follows:

١. Isolation and identification of *P. aeruginosa* from different hospitalized patients (ear infections, urinary tract infections, wound and burn infections).
٢. Screening for protease production among each isolate.
٣. Studying the antibiotic susceptibility among each isolate.
٤. Studying the plasmid profiles of some *P. aeruginosa* isolates.
٥. Determination of protease genes loci by transformation, conjugation and curing experiments.
٦. Histopathological study of protease action as a virulence factor in infected animal lab. (Albino's Rat) by the wild strains and *E.coli* MM٢٩٤.

LITERATURE REVIEW

٢-١ *Pseudomonas aeruginosa*.

The genus *Pseudomonas* comprises more than ٢٠٠ species, mostly saprophytes found widely in soil, water and moist environment. *Pseudomonas* is a gram negative , straight or curved but not helical, aerobic, single cell , non-spore forming , motile by polar(monotrichous) flagella, forming pili (Atlas, ١٩٩٥). It belongs to bacterial family

Pseudomonadaceae; the family includes *Xanthomonas*, Z*oogloea* and *Gluconobacter* (Holt *et al*., ١٩٩٤). *Xanthomonas*, together with *Pseudomonas*, comprises the informal group of bacterial known as *Pseudomonas* (Todar, ٢٠٠٤).

Pseudomonas occurs widely in soil, water, plants, and animals. *P. aeruginosa* is frequently present in small numbers in the normal intestinal flora and on the skin of humans and is the major pathogen of the group. Other Pseudomonas species infrequently cause disease. The classification of the Pseudomonas is based on rRNA / DNA homology and common culture characteristics (Jawetz *et al*., ٢٠٠١).

A few species are pathogenic for plant, insects, and animals. *P.aeruginosa* is the species most commonly associated with human disease but *P. mallei* and *P*. *pseudomallei* are also important pathogens in some parts of the world. Several other species of pseudomonas and a number of other glucose non-fermenters are occasionally isolated from human clinical specimens as opportunistic pathogens.

They obtain their energy by aerobic respiration and in some cases by anaerobic respiration (nitrate respiration, denitrification), but never by fermentation. The pseudomonadaceae are chemo-organotrophs, though some are facultative chemoliothotrophs. The metabolic and physiological properties of *Pseudomonas* are characterized by the wide spectrum of substrate these organisms can use; they can even utilize a large number of heterocyclic and aromatic compounds that are not attacked by other bacteria (Schlegel, ١٩٩٣).

Pseudomonas species oxidize sugars in completely and excrete sugar acids (gluconate, ٢-oxogluconate), because of their simple requirements, pseudomonads are ubiquitous. When media containing mineral salts and organic acids or sugars are exposed to air, pseudomonads are usually the first colonizers (Schlegel, ١٩٩٣).

Pseudomonas aeruginosa is an opportunistic pathogen that is widespread in the environment. This means that it exploits some break in the host defenses to initiate an infection (Nester *et al*., ٢٠٠١; Todar, ٢٠٠٤), a major cause of nosocomial infections and an occasional cause of community-acquired infections (Nester *et al*., ٢٠٠١).

It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections, and a variety of systemic infections (Jawetz *et al* ., ٢٠٠١; Todar, ٢٠٠٤). It is also common in patients receiving treatment of severe burns, or other traumatic skin damage (Madigan *et al*., ٢٠٠٠), particularly in patients with sever burns and in cancer and AIDS patients who are

immunosuppressed (Todar, ٢٠٠٤). In addition, this bacteria is found in otitis externa, and infection of the eye, and occurs most commonly after injury or surgical procedures (Mims *et al*., ١٩٩٣; Jawetz *et al*., ٢٠٠١).

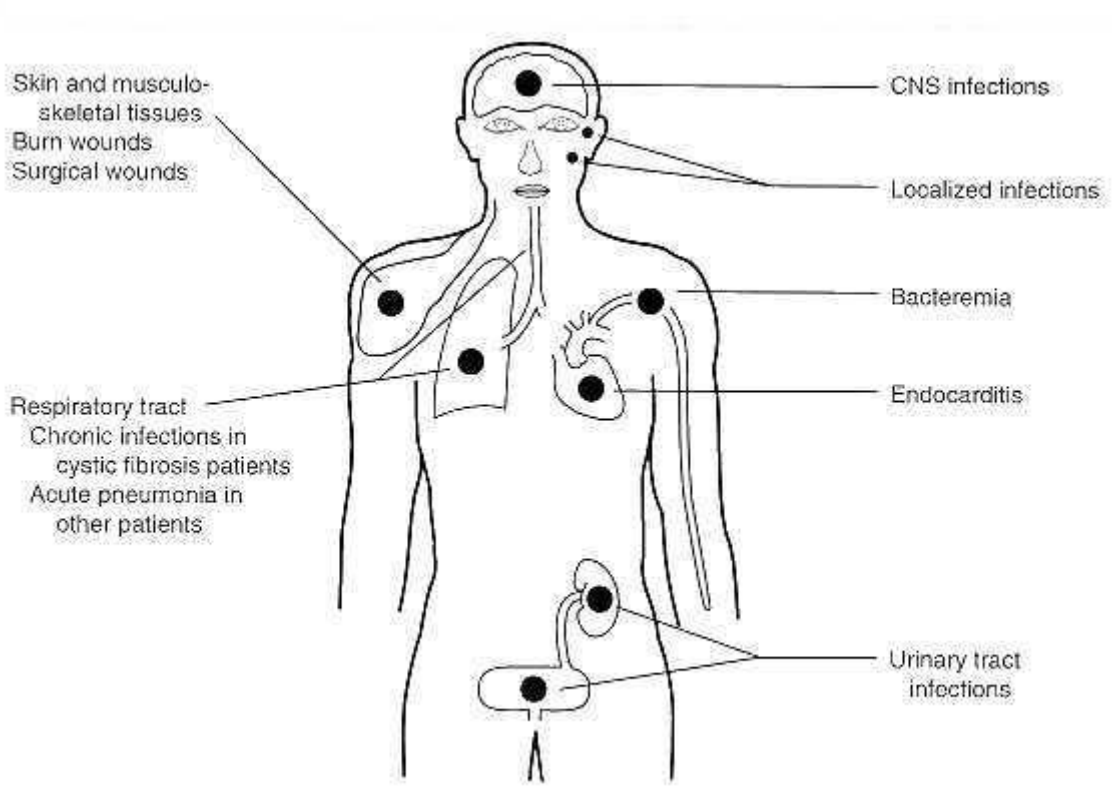


Figure (٢-١): Diverse sites of infection by *Pseudomonas aeruginosa*. This opportunistic pathogen may infect virtually any tissue. Infection is facilitated by the presence of underlying disease (e.g., cancer, cystic fibrosis) or by a breakdown in nonspecific host defenses (as in burns). By (Iglewski, ١٩٩٨).

P. aeruginosa is a gram negative bacilli, oxidase positive, obligate aerobic that growth readily on many types of culture media which sometimes produce sweet grape like odor, except in media with nitrate (Shanson, ١٩٨٩; Greenwood *et al*., ١٩٩٢; Jawetz, ٢٠٠١). Its optimum temperature for growth is ٣٧°C degree, and it is able to grow at temperature as high as ٤٢°C (Holt *et al*., ١٩٩٤; Atlas, ١٩٩٥; Todar, ٢٠٠٤). Almost all strains are motile by means of a single polar flagellum, and can live in sessile biofilm form as a free – swimming cell; pili (fimbriae) extend from the cell surface and promote attachment to host epithelial cells during colonization of mucosal surface (Jawetz *et al*.,٢٠٠١; and Todar, ٢٠٠٤).

P. aeruginosa differs from members of the enterobacteriaceae by deriving energy from carbohydrates by an oxidative rather than a fermentative metabolism (Greenwood *et al*., ١٩٩٢). *P. aeruginosa* isolates may produce three colony types: the most common colonial form is relatively large, low-convex with an irregular surface, an edge that is translucent and

an oblong shape with the long axis parallel to the line of inoculum (Greenwood *et al*., ١٩٩٢). Natural isolates from soil or water typically produce a small, rough colony. Clinical samples, in general, yield one or another of two smooth colony types. One type has a fried-egg appearance which is large, smooth, with flat edges and an elevated appearance. Another type, frequently obtained from respiratory and urinary tract secretions, has a mucoid appearance, which is attributed to the production of alginate slime. The smooth and mucoid colonies are presumed to play a role in colonization and virulence (Todar, ٢٠٠٤). *P. aeruginosa* strains produce two types of soluble pigments, the fluorescent pigment (pyoverdin) and the blue pigment pyocyanin (blue in neutral or alkaline media, red in acid media) , the latter is produced abundantly in media of low-iron content and functions in iron metabolism in the bacterium (Jawetz *et al*., ٢٠٠١; Todar, ٢٠٠٤), and strain producing either pigments are extremely rare , some strains produce the dark red pigment (pyorubin) or the black pigment (pyomelanin) , pyocyanin (from "pyocyaneus") refers to "blue pus" which is characteristics of supportive infections caused by *P. aeruginosa* (Holt *et al*., ١٩٩٤; Atlas, ١٩٩٥; and Todar, ٢٠٠٤).

It is interesting to speculate about why *P. aeruginosa* rarely infects a healthy person, whereas literally millions of individuals have died from diphtheria. The answer is not known, but the low invasive ability of *P. aeruginosa* may result from differences in cell specificity between the Pseudomonas A toxin (PA toxin) and diphtheria toxin.

Most strains of *P. aeruginosa* from clinical infections produce two exotoxins, exotoxin A and exo-enzyme S, and a variety of cytotoxic substances (extracellular enzymes) including elastases, proteases, two hemolysins; a heat-labile phospholipase C and a heat stable glycolipid, also pyocyanin and rhamnolipids; an alginate – like exopolysaccharide is responsible for the mucoid phenotype (Jawetz *et al*.,٢٠٠١). The importance of these putative virulence factors depends upon the site and nature of infection: proteases play a key role in corneal ulceration; exotoxin and proteases are important in burn infection; and phospholipases, proteases and alginate are associated with chronic pulmonary colonization (Greenwood *et al*., ١٩٩٤).

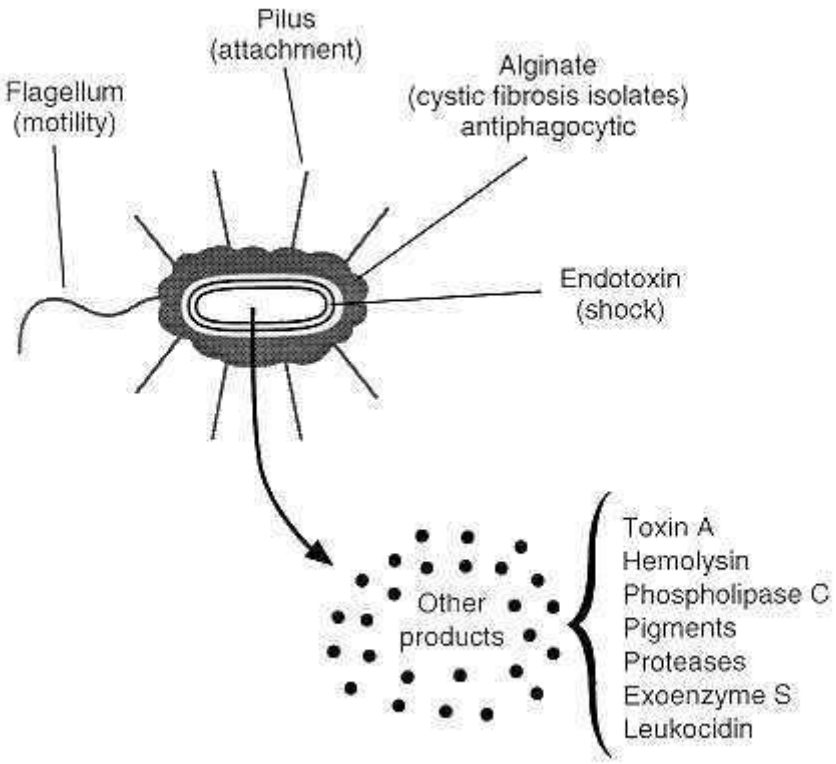


Figure (٢-٢): Virulence factors of *P. aeruginosa*. *P. aeruginosa* has both cell-associated (flagellum, pilus, nonpilus adhesins, alginate/ biofilm, lipopolysaccharide [LPS]) and extracellular virulence factors (proteases, hemolysins, exotoxin A, exoenzyme S, pyocyanin). By Van Delden & Iglewski (١٩٩٨).

The primary reasons for the predominance in nosocomial infections are their wide occurrence, ability to survive outside the human body for long periods, and resistance to antibiotics (Ross, ١٩٨٣).

Special precautions to avoid these infections include the prophylactic administration of an antibiotic, the cleaning of the intestinal tract by enema before the operation, and the use of appropriate aseptic technique during and after surgery. However, the highest incidence of nosocomial infections occurs in association with the urinary tract infections (Ross, ١٩٨٣). An alternative to controlling *P. aeruginosa* burn infections utilizes immunization with vaccines made from endotoxic lipopolysaccharides (Ross, ١٩٨٣; and Rosenthal and Tan, ٢٠٠٢).

٢- ٢ Proteases

Proteases are a large group of enzymes, ubiquitous in nature and found in a wide variety of microorganisms. They are molecules of relatively small size and are compact, spherical structures that catalyze the peptide bond cleavage in proteins (Polgar, ١٩٩٠). These enzymes are important in a number of diverse and crucial biological processes; for example, they are involved in the regulation of metabolism and gene expression, enzyme modification, pathogenicity, and the hydrolysis of large proteins to smaller molecules for transport and metabolism (Rao *et al*., ١٩٩٨).

Proteases are difficult to characterize because of their diversity of action and structure. Originally proteases were classified based on molecular size, charge or substrate specificity. However, with the advent of molecular biology, proteases are now grouped into families based on the following: chemical nature of the catalytic or active sites, mechanism(s) of action, and the evolutionary relationship of their three-dimensional structure (Rao *et al*., ١٩٩٨).

Proteases are broadly divided into either exopeptidases or endopeptidases depending on their site of action. If the enzyme cleaves the peptide bond proximal to the amino or carboxy terminus of the substrate, they are classified as exopeptidases. If the enzyme cleaves peptide bonds distant from the termini of a substrate, they are classified as endopeptidases. Based on the functional group present at the active site and their catalytic mechanism, proteases are then categorized into four groups; serine proteases, aspartic proteases, cysteine /thiol proteases, and metalloproteases. Four classes of endoproteases have been identified in living organisms and three of the four classes of endoproteases have been isolated and purified in bacteria; serine, cysteine, and metalloproteases (Liao *&* McCallus, ١٩٩٨).

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins. Proteases are degradative enzymes which catalyze the total hydrolysis of proteins.

Advances in analytical techniques have demonstrated that proteases conduct highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across the membranes. The current estimated value of the worldwide sales of industrial enzymes is $١ billion of the industrial enzymes, ٧٥٪ are hydrolytic (Godfrey *et al*., ١٩٩٦). Proteases represent one of the three largest groups of

industrial enzymes and account for about ٦٠٪ of the total worldwide sale of enzymes. Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as hemostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. Their involvement in the life cycle of disease- causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS (Rao *et al*., ١٩٩٨). The ability of *Pseudomonas aeruginosa* (as bacterial proteases) to invade tissues depends upon production of extracellular enzymes and toxins that break down physical barriers and damage host cells, as well as resistance to phagocytosis and the host immune defenses (Todar, ٢٠٠٤).

Two extracellular proteases have been associated with virulence that exerts their activity at the invasive stage: elastase and alkaline protease. Elastase has several activities that relate to virulence. The enzyme cleaves collagen, IgG, IgA, and complement. It also lyses fibronectin to expose receptors for bacterial attachment on the mucosa of the lung. Elastase disrupts the respiratory epithelium and interferes with ciliary function. Alkaline protease interferes with fibrin formation and will lyse fibrin. Together, elastase and alkaline protease destroy the ground substance of the cornea and other supporting structures composed of fibrin and elastin. Elastase and alkaline protease together are also reported to cause the inactivation of gamma Interferon (IFN) and Tumor Necrosis Factor (TNF) (Todar, ٢٠٠٤).

٢- ٣ Genetic content of *P. aeruginosa*.

The genetic content means chromosomal DNA and plasmid DNA; Plasmids are small; extrachromosomal circular molecules of DNA that can exist independently of host chromosomes and are present in the most genera of bacteria (they are also present in some yeasts and other fungi).

Plasmids must have the ability to replicate independently; they can replicate autonomously in the cell, and must have at least one origin of replication (Ori site), where replication begins (Jawetz *et al*., ٢٠٠١). Their genetic information is not essential to the host,

and the bacteria that lack them usually function normally. Plasmids play a significant role in bacterial adaptation and evolution. They serve as important tools in studies of molecular biology. They vary in size from a few thousand to hundreds of thousands of base pairs. The numbers of copies also vary among plasmids, the bacterial cell can harbor more than one type (Hardy, ١٩٨٦). Thus, a cell can contains, for example, two different types of plasmid, with hundreds of copies of one plasmid type, and only one copy of the other. Incompatible plasmids cannot co-exist together, after few generations of bacterial growth, one or other is lost; plasmids are classified into incompatibility groups (Inc, group) on this bases (Hardy, ١٩٨٦).

Plasmids may be classified in terms of their mode of existence and spread. An episome is a plasmid that can exist either with or without being integrated into the host's chromosome. Some plasmids, conjugative plasmids, have genes for pili and can transfer copies of themselves to other bacteria during conjugation. Because of pathogenic nature of *P. aeruginosa* and, its resistance to most antibiotics and the success of this species as a common hospitalized pathogen organisms, therefore many studies have been conducted to study these organisms, including their plasmids, which are responsible for inactivation of antibiotics (Grinsted *et al*., ١٩٧٤; O'hara and Kono, ١٩٧٥;; Bissonnette and Roy, ١٩٩٢; and Smalla *et al*., ٢٠٠٠), and they extracted many R-plasmids that confer resistance to a number of antibiotics such as penicillin, rifampicine, carbencillin, neomycin, kanamycin, tetracycline, chloramphenicol, sulfonamide and streptomycin. It was found that most of these plasmids are transferable to either another species or other genus with different molecular weight. Datta *et al*., (١٩٧١) divided R-factor into two classes fertility inhibition (fi^-) and (fi^+). The fi^+ factor inhibit F-mediated conjugation, fi^-ones do not. The fi^+group of R-factor are related to F in that they determine the synthesis of sex pili similar to those of F. Among fi^- R-factor, one group has been distinguished which produces sex pili similar to those of the transmissible colicine factor I (I pili).

Holloway, (١٩٦٩) reported that an R-factor which is derived from strains of *P. aeruginosa* was highly resistant to carbencillin and appeared similar to R-factor, RP٤, which came from *P. aeruginosa* strain S٨, and this R-factor is fi^- which gives no evidence of production of (I pili). Grinsted *et al*. (١٩٧٤) found that PR١ which is a group of genes specifying resistance to carbencillin, neomycin, kanamycin, and tetracycline originated in *P. aeruginosa* is freely transmissible strains of *P. aeruginosa*, *E. coli* and *Proteus mirabilis*. Olsen and Shipley (١٩٧٣) isolated R١٨٢٢ from specifying multiple drug resistance, and were transferred to a variety of species of bacteria including Enterobacteriaceae.

Another type of plasmids found in *P. aeruginosa* is called sex plasmid and has the ability to transfer through the conjugation process (Chandler and Krishnapillai, ١٩٧٧; Bradley, ١٩٨٣; Smalla *et al*., ٢٠٠٠). The third type of plasmid available in *P. aeruginosa* is mercury resistance plasmid that confers resistance to mercury and is often mediated by sex-plasmid, or R-plasmid (Marques *et al*., ١٩٧٩), or mercury and organomercury (Clark *et al*., ١٩٧٧) and mercurial and silver compounds. In addition, there are Tol-plasmids about ٢٧٠ kb which enable the cells to grow in toluene, xylene as a sole carbon source, or for degradation of organic compounds, and carry genes for resistance to oil materials (Pattnakik *&* Subramyam, ١٩٩٥). Chakrabarty, (١٩٧٦) reported that the size of p١, p٢ and RP١ plasmid in *P. aeruginosa* are ٦٥٦.٢ bp,١٦٤٠٦.٢ bp and ٢٤٩٣٧.٥ bp respectively, while for p٣٩ is ١٦٤٠٦.٢ bp. Iton *et al*., (١٩٨٤) found that the size of Rk٢ plasmid in *P. aeruginosa* is ٦٠ kb. (Tsakris *et al*., ١٩٩٢) reported that the size of antibiotic resistance plasmid in *P. aeruginosa* ranged between ٣٢٣١.٨ – ١٦١٥٩.٢ bp, and found that the size ranged between ٣٠٧ – ٧٢٨٧.٨ bp.

٢- ٤ Antibiotic resistance of *P. aeruginosa*.

P. aeruginosa is notorious for its resistance to antibiotics and is, therefore, a particularly dangerous and dreaded pathogen; there is much attention paid to the study of antibiotic resistance in *P. aeruginosa*. Saunders and Grinsted (١٩٧٢) found that the R- factor (RP١) which originated in *P. aeruginosa* ١٨٢٢ confers resistance to carbencillin, neomycin, kanamycin, tetracycline, rifampicin, streptomycin and nalidixic acid. Marques *et al*. (١٩٧٩) found that, out of ٧١ strains isolated from soil and water, ٧٪ were resistant to gentamicin, and all strains studied were resistant to ampicillin, tetracycline, chloramphenicol, nalidixic acid and streptomycin. Additionally, they found that the strains isolated from soil were more resistant to antibiotics than those isolated from surface water.

In another study, Jibran, (١٩٨٦) studied on *Pseudomonas* species which were isolated from wounds and found that among ٨٣ isolates, ٧١٪ were sensitive to gentamicin, but all isolates were resist to ampicillin, cephalooridine and tetracycline, and very high degree of resistance to chloramphenicol, streptomycin and kanamycin. Moreover, Zhu (١٩٩٢) in China, found that the strains isolated were resistant to those antibiotics used for long time therapy, and some results were obtained with patients of chronic disease in Netherlands hospitals (Mouton *et al*., ١٩٩٣). *P. aeruginosa* are resistant against more antibiotics used in Tunisian hospitals, and also in Malaysian hospitals (Pallilo and Salleh, ١٩٩٢).

Tassios *et al*. (١٩٩٨) found that ١٠٪ out of ٨٨ isolates of *P. aeruginosa* from ١١ Greek hospitals were resisting to all antibiotics and ٩١٪ were multidrug resistant. Al-Najjar and Al-

Hadithi (١٩٧٩) found that ٢٠٪ of *P. aeruginosa* isolated from various clinical materials in Basra hospital were resistant to gentamicin; however they showed high level of resistance to ampicillin, chloramphenicol, and cephalothin. ١٣.٣٪ of the isolates were found to be resistant to all tested drugs including streptomycin, kanamycin, and nitrofuration. Bouza *et al*. (١٩٩٩) found that out of ١٠١٤ isolates from ١٣٦ hospitals in Spain that ٢٣٪ were resistant to ciprofloxacin, ٣١٪ to gentamicin the most active against antibiotics was amikacin, similar results obtained in France and Italian (Bonfiglio *et al*., ١٩٩٨).

Abd Al-Amir, (١٩٩٩) showed that out of ٩٠ *Pseudomonas* species resistant to heavy metals isolated from sewage water, hospital and soil in Baghdad, percent of resistance were ٧٨, ٧٣.٣ and ٧٣.٣ for nitrofuration, ampicillin and streptomycin respectively, while for gentamicin was ٥٠٪. Since, *P. aeruginosa* is a natural inhabitant in the soil, living in association with the Bacillus, Actinomycetes and molds; it has developed resistance to a variety of naturally occurring antibiotics, due to the permeability barrier afforded by its outer membrane lipopolysaccharide (LPS) (Kono and O'hara, ١٩٧٥ and Godfrey *et al*., ١٩٨٤). Its tendency to colonize surface in a biofilm form, makes the cell impervious to therapeutic of antibiotics. Godfrey *et al*. (١٩٨٤) reported that the outer membrane of *P. aeruginosa* has long been considered as a barrier against βeta-lactam antibiotics.

Pseudomonas maintains antibiotic resistance plasmid (R-factor). These plasmids are transmissible to sensitive bacteria and make them acquire resistance to antibiotics, and have the ability to genetic recombination through conjugation, transformation, and transduction (Kono and O'hara, ١٩٧٥; and Foster, ١٩٨٣). Moreover, there are other genes belonging to antibiotics resistance located on chromosome (Kono and O'hara, ١٩٧٥; and Williams *et al*., ١٩٨٠). Multidrug active efflux systems have recently been recognized in a number of bacteria as efficient mechanisms of resistance in *P. aeruginosa*, by which antibiotics are expelled from the cells by membrane transporter proteins, the so-called drug-efflux pumps (Hamzephour *et al*., ١٩٩٥; Wadman *et al*., ١٩٩٩; and Lomovskaya *et al*., ٢٠٠١). A number of bacteria which posses active efflux pumps enabling them to avoid the deleterious effects of noxious agents, and active efflux proteins have been shown to contribute significantly to multidrug resistance in *P. aeruginosa* (Nikaido, ١٩٩٤).

Early *P. aeruginosa* of over expressing an outer membrane protein of ٥٠ kDa (OprK), the OprK phenotype was associated with enhanced resistance to quinolones, tetracycline, chloramphenicol, and streptomycin. The gene coding for OprK was recognized as being part of an operon composed of three genes encoding respectively. OprK is homologous to outer membrane export proteins; MexB a ١٨٠-kDa cytoplasmic membrane protein acting as active efflux pump; and MexA a ٤٠ kDa periplasmic component though to link OprK and MexB

(Pool *et al*., ١٩٩٣). Recently another protein efflux system was described in *P. aeruginosa* which found in cytoplasmic membrane fraction, associated with resistance to tetracycline, chloramphenicol and to some β-lactams such as carbencillin (Li *et al*., ١٩٩٤).

٢- ٥ Histopathological study of *P. aeruginosa* extracellular proteases infected rat (skins and lungs).

Burned skin seems to be especially susceptible to infection by *P. aeruginosa* Nathan *et al*. (١٩٧٣). The pathogenesis of *P. aeruginosa* infections have been studied in experimentally produced burns in a burned mouse model (Stiertz, ١٩٧٥). From these studies it was demonstrated that the burned skin site allowed for the initial colonization and proliferation of the organism in vivo. Haidaris and Michael (١٩٧٨) who showed that mice immunized with elastase or its toxoid showed increased resistance to experimental *P. aeruginosa* infections, suggest that this protease contributes to the pathogenesis of the organism. Thus, it appears that protease is a virulence-enhancing factor rather than a major virulence factor. The data presented by Pavlovskis and Wretlind (١٩٧٩) indicate that to some extent these effects may be neutralized by antiprotease serum. These observations should provide a broader basis for studies leading to immunoprophylaxis and treatment of pseudomonas infections.

٣-١ Materials and substances:

Table (٣-١): Apparatus and Tools.

Name	Company	Country
Autoclave	Betec	Iran
Autoclave	Webeco-Bad, Schwastan	Germany
Auto vortex MIXER	Stuart scientific Co. Ltd.	England
Balance	Sartorius	Germany
Biofuge	Keudro Heraeus	Germany
Centrifuge	Hettich EBH. ٢٠	Germany
Cold centrifuge	Keudro Heraeus	Germany

Electrophoresis Unit	Akhtarian, mod. SH-٥٠٣	Iran
Electrophoresis constant power supply	CONSORT-E٨٦٣ ٦٠٠ Volt – ٢٥٠ m Ampere	Belgium
Incubator	WTB binder	Germany
Light microscope	Olympus optical Co., LTD	Japan
Micropipette (٢-٢٠ micro liter)	Sigma	Germany
Micropipette (١٠-١٠٠ micro liter)	Costar	Cambridge,U.S.A
Micro syringe	KLOEHN Co., INC WHITTER, CALIF	U.S.A
Microprocessor pH meter	HANNA (pH-٢١١)	Germany
Oven	WTB binder	Germany
Oven	Philip HARRIS	England
Sensitive balance	HANGPING JA ١٠٠٣	Japan
Shaker	Labover	Germany
Shaker incubator	Stuart orbital incubator, S ١٥٠	U.K
Spectrophotometer	BEJING RAYLEIGH ANALYTICAL, MC	China

Name	Company	Country
Ultra violet transilluminator	UVP	Upland, U.S.A
Water bath	TAFESA	W. Germany
Medical Deep Freezer	SANYO	Japan
Millipore Filter (٠.٤٥, ٠.٢٢)	Sartorions Membrane Filter W.	Germany
Digital Camera	SONY	Japan

٣-١-١ Materials

Table (٣-٢): Materials and their manufacture companies

Materials	Company
١- Chemical Material Calcium chloride, sodium chloride, sodium hydroxide, Sodium acetate,	

ammonium chloride, potassium chloride, magnesium sulphate anhydrate, glacial acetic acid, K_2HPO_4, Na_2HPO_4, Tris base, phenol, chloroform, Sodium dodecyl sulphate, Na_2-EDTA, Agarose ,low melting agarose.	BDH
Glycerol, Ether, Aceton, Ethanol, Isoamylalcohol.	Ajax
٢-Sugars and Amino acid D-glucose, Sucrose, lactose, Thiamine, yeast extract.	BDH
٣-Dyes Ethidium bromide, Bromothymol blue, India ink.	BDH
٤-Enzymes *EcoR* I T_4 DNA ligase Proteinase K^+ Lysozyme(powder)	 Fermentas Takara CinnaGen CinnaGen
٥-Other material Loading buffer api E٢٠ system	 Takara BioMerieux SA, Company

٣-١-٢ Plasmids and standard bacterial strains

Table (٣-٣): Standard bacterial strains and plasmid vectors

plasmids and bacterial strains	Genotype	Source
Escherichia coli HB١٠١	*hsd* R^-, *hsd* M^-, *recA*$^-$, *leu*$^-$, *pro*$^-$, *gal*$^-$, Sm^r	Institute of Genetic Engineering & Biotechnology post graduate studies Baghdad University
Escherichia coli MM٢٩٤	*hsd* R^-, *hsd* M^+, *end AI*, *pro*$^-$, *thi*$^-$, Rif^r	Biology Department Sulaimani University
pACYC ١٨٤	*tra*$^-$,Tc^r, Cm^r	CinnaGen – Company

		Tehran
Lambda DNA cut by *Hind* III & *EcoR* I		Fermentas

hsd R $^{-}$: lack of restriction system.

hsd M $^{-}$: lack of modification system.

hsd M $^{+}$: presence of modification system.

rec A $^{-}$: lack of recombination system.

end AI : lack of endo nuclease activity.

gal $^{-}$: have no ability to ferment galactose.

tra $^{-}$: lack of transconjugant genes.

pro $^{-}$: proline, ***lue*** $^{-}$: Lucien, ***thi*** $^{-}$: thymine.

Sm^{r} :streptomycin, **Rif^{r}** :rifampicin, **Tc^{r}** :tetracycline, **Cm^{r}** :chloramphenicol resistances.

٣-١-٣ Buffer and solutions

٣-١-٣-١ Antibiotic solution:

Antibiotic solution was prepared according to (Al-zaaq, ١٩٨٧). The antibiotic solution sterilized by Millipore filter ٠.٢٢, and stored at ٤ °C.

Table (٣-٤): Antibiotic solutions.

Antibiotics	Symbol	Stock solution	Final concentration	Solvent	Company

		mg/ml	µg/ml		
Ampicillin	Ap	١٠	١٠٠	Distill Water	Oxoid/England
Chloramphenicol	Cm	٢.٥	٥٠	Aceton	Oxoid
Kanamycin	Km	١٠	٥٠	D.W	Sigma/Germany
Rifampicin	Rif	٢.٥	١٠٠	Aceton	Sigma
Streptomycin	Sm	١٠	١٠٠	D.W	Oxoid
Tetracycline	Tc	٢	٢٠	Ethanol/Water ٥٠٪	Oxoid
Trimethoprim	Tp	٥	٥٠	Aceton	Sigma

Table (٣-٥): Antibacterial discs and symbolization.

Antibiotics	Symbol	Concentration µg / ml	Company
Amikacin	Ak	٣٠	Oxoid England
Amoxicillin	Ax	٢٥	Oxoid England
Ampicillin	Ap	١٠	Oxoid England
Carbencillin	Car	١٠٠	Oxoid England
Ciprofloxacin	Cip	٥	Oxoid England
Chloramphenicol	Cm	٣٠	Oxoid England
Cefotaxime	Ctx	٣٠	Sigma Germany
Erythromycin	Ery	١٥	Sigma Germany
Gentamycin	Gm	١٠	Oxoid England
Kanamycin	Km	٣٠	Sigma Germany
Lincomycin	Lin	١٠	Oxoid England
Neomycin	N	٣٠	Sigma Germany
Penicillin	Pi	٥	Sigma Germany
Rifampicin	Rif	٣٠	Sigma Germany
Streptomycin	Sm	١٠	Oxoid England
Tetracycline	Tc	٣٠	Oxoid England
Trimethoprim	Tp	٣٠	Sigma Germany

٣-١-٣-٢ Saline EDTA

The solution was prepared by dissolving ٠.١٥ mol of sodium chloride, ٠.١ mol of EDTA in distilled water and the pH was adjusted to ٨ then volume was completed to ١ liter and sterilized by autoclave (Murmur, ١٩٦١).

٣-١-٣-٣ Lysozyme solution

This solution was prepared freshly by dissolving ١٠mg of Lysozyme in ١ml STET buffer (Sambrook *et al*., ١٩٨٩).

٣-١-٣-٤ TE buffer

The buffer was prepared by dissolving ٠.٠١ mol of tris-base and ٠.٠٠١ mol of EDTA in distilled water then pH was adjusted to ٨, the volume completed to ١liter and sterilized by autoclave (Sambrook *et al*., ١٩٨٩).

٣-١-٣-٥ ٢٥٪ SDS solution

The solution was prepared by dissolving ٢٥gm of SDS in ١٠٠ml of saline STE buffer (Pospiech and Neuman, ١٩٩٥).

٣-١-٣-٦ EDTA-Sodium acetate

The solution was prepared by dissolving ٣ mole of sodium acetate, ٠.٠٠١mole of EDTA in distilled water, the pH was adjusted to ٧ and the solution completed to one liter with distilled water and sterilized by autoclave (Sambrook *et al*., ١٩٨٩).

٣-١-٣-٧ Chloroform- isoamyl Alcohol solution

The mixture was prepared in a ratio of ٢٤:١ (chloroform: isoamyl Alcohol) and stored in a dark bottle at ٤°C (Sambrook *et al*., ١٩٨٩).

٣-١-٣-٨ Phenol saturated with Tris buffer

The mixture was prepared by dissolving the phenol in a water bath ٦٨°C, ٠.١٪ of antioxidant ٨-hydroxyquinoline was added to the melting phenol, then an equal volume of buffer solution of ٠.٥ mol tris-Hcl pH ٨ was added, mixed well by mechanical vortex and then the solution was let to settle. The upper aqueous layer was separated from the lower layer which is the phenol. The upper layer was discard by pasture pipette and then added an

equal volume of ٠.١ molar tris-Hcl pH ٨ to the phenol and this process was repeated till the pH of phenol became more than ٧.٨, the phenol mixture was stored under a thin layer of tris solution in a dark bottle at ٤°C (Sambrook et al., ١٩٨٩).

٣-١-٣-٩ Phenol: chloroform: isoamyl alcohol solution

Phenol, chloroform, isoamyl alcohol were mixed together at a ratio of ٢٥:٢٤:١ and stored under a layer of ٠.١molar tris-Hcl solution pH ٨ in a dark bottle at ٤°C (Sambrook *et al*., ١٩٨٩).

٣-١-٣-١٠ TBE buffer

The buffer was prepared by dissolving ٠.٠٨٩ mole of tris-base, ٠.٠٨٩ mole of boric acid and ٠.٠٠٢ mole of Na_2-EDTA in distilled water. The pH was adjusted to ٨ and the volume completed to a liter then sterilized by autoclave (Sambrook *et al*., ١٩٨٩).

٣-١-٣-١١ STET buffer

The buffer was prepared by dissolving ٠.٠٥ M tris-base, ٠.٠٥ M of EDTA and ٠.٠٥ M of NaCl in D.W, and then the pH adjusted to (٨). The volume of the solution was completed to ١ L with the distilled water, and they sterilized by autoclave (Sambrook *et al*., ١٩٨٩).

٣-١-٣-١٢ Ethidium bromide dye

The dye solution was prepared by dissolving ٠.٥ gm of Ethidium bromide in ١٠ ml D.W, and stored in a dark bottle (Sambrook *et al*., ١٩٨٩).

٣-١-٣-١٣ Extracted solutions

Solution I:

It was prepared by mixing ١ ml ٢٠٪ (w/v) glucose, ٠.٥ ml ٠.٢٥M EDTA pH ٨ and ١٧.٦ ml D.W.

Solution II:

It was prepared by mixing ٨.٨ ml H_2O (Water), ٠.٢ ml ١٠M NaOH and ١ ml ١٠٪ SDS.

Solution III (٣M Sodium acetate) pH ٤.٨:

Aliquot of ٢.٤ gm of Sodium acetate.٣$H_٢O$ was dissolved in ٢٥٠ ml of D.W; pH was adjusted to ٤.٨ by glacial acetic acid, then the volume was completed to ٥٠٠ ml (Sambrook *et al*., ١٩٨٩).

٣-١-٣-١٤ Normal saline

It was prepared by dissolving ٠.١٥ moles of sodium chloride and ٠.١ mole of $KH_٢PO_٤$ in distilled water, the pH was adjusted to ٧.٢ by adding sodium hydroxide. Then the volume was completed to a liter, and then sterilized by autoclave.

٣-١-٣-١٥ Oxidase Reagent

One gram of tetramethyl-p-phenylenediamine dihydrochloride was dissolved in ١٠٠ ml of sterilized distilled water and used for the identification of oxidase enzyme in *P. aeruginosa*.

٣-١-٤ Culture media:

٣-١-٤-١ Ready media

Were prepared according to the recommendations of the manufacturing companies and sterilized by auto cleaving.

١. Brain-Heart infusion agar (BHF), (Mast diagnostics company; U.K).
٢. Brain-Heart infusion broth (BHI), (Mast diagnostics company; U.K).
٣. Eosin methylene blue agar (EMB), (Difco U.S.A).
٤. MacConKey agar (Difco-U.S.A).
٥. Muller-Hinton agar (Difco-U.S.A).
٦. Triple sugar iron agar (Mast diagnostics U.K).
٧. Nutrient agar (Mast diagnostics U.K).

٣-١-٤-٢ Cetrimide agar (Hawkey and Lewis, ١٩٨٩)

It was prepared by mixing each of peptone ٢٠ gm, $MgCl_2$ ١.٥ gm, K_2SO_4 ١٠ gm, cetrimide (N-acetyl-N, N, N-trimethyl ammonium bromide) ٠.٣ gm, agar ١٥ gm, and dissolved in ١٠٠٠ml of distilled water.

٣-١-٤-٣ Gelatin medium (**Krieg,** ١٩٨٤).

It was prepared by dissolving ١.٣ gm of nutrient broth and ٤gm of gelatin in ١٠٠ml D.W, mixed very well, then dispensed in test tubes and autoclaved.

٣-١-٤-٤ Mannitol salt agar

It was prepared by mixing mannitol ١٠ gm, nutrient agar ٢٨ gm, NaCl ٧٠ gm, phenol red ٠.٠٢٥ gm and distilled water ١٠٠٠ ml, all are dissolved in water with heating, sterilized by autoclave at ١٢١ °C for ١٥ min., after cooling to (٥٠-٥٥ °C), and the medium was dispensed into sterile petredish.

٣-١-٤-٥ Preservation medium

It was prepared by mixing nutrient broth ١٣ gm, glycerol ٥٠ ml, and distilled water ١٠٠٠ ml, the pH was adjusted to ٧, autoclaved and distributed in ٥ ml amounts in screw cap bottles (Cruickshank *et al*. ١٩٧٥).

٣-١-٤-٦ Skim milk agar

It was prepared by mixing skim milk ١٠ gm and ٩٠ ml of D.W, the pH of skim milk adjusted at ٧, then autoclaved at ١٢١°C for ٥ min. also ١٥ gm of agar mixed with D.W and auto cleaved at ١٢١°C for ١٥ min. the volume completed to ١٠٠٠ ml, then in sterile condition mixed with skim milk.

٣-١-٤-٧ Urease activity test medium

It was prepared by mixing peptone١ gm**,** glucose١ gm**,** NaCl ٥ gm, KH_2PO_4 ٢ gm, phenol red ٠.٠١٢ gm**,** agar ١٢ gm and D.W ١٠٠ ml, all were dissolved in water and autoclaved at ١٢١°C for ١٥ min., after cooling to ٥٠°C, ٥٠ ml of sterile filtered ٤٠٪ urea solution was added aseptically in sterile bottles or screw cap tubes and allowed to set in a sloped position (Chesbrough, ١٩٩١).

٣-١-٤-٨ King No.١ medium

It was prepared by mixing $MgCl_2$ ١.٥ gm**,** K_2HPO_4 ١.٥ gm**,** K_2SO_4 ١.٥ gm, peptone ٢ gm**,** agar ١٠ gm, and all dissolved in ١٠٠٠ ml of D.W, later sterilized by autoclave at ١٢١°C for ١٥ min., and then dispensed in sterile petridishes.

٣-١-٤-٩ Minimum medium M_9

It was prepared by dissolving ٩ gm of Na_2HPO_4, ٣ gm of KH_2PO_4, ٠.٥ gm of sodium chloride, and ١ gm of NH_4Cl in ٩٨٨ ml deionized distilled water and the pH adjusted to ٧.٤ then sterilized by autoclave then ٢ ml of ١ molar $MgSO_4$ and ١٠ ml of ٢٠٪ glucose solution was added if the medium was used as solid medium ٢٪ agar would be added together with each casamino acid final concentration ٥mg/ml, thiamine ٥٠mg/ml (Sambrook *et al*., ١٩٨٩).

٣-١-٤-١٠ SOC liquid medium

It was prepared by dissolving ٢ gm, ٠.٥ gm٠.٠٠٥ gm of trypton, yeast extract, and sodium chloride respectively in ٩٠ ml distilled water and then ١ ml of ٠.٢٥ molar of potassium chloride was added, the pH was adjusted to ٧ and the volume completed to ١٠٠ ml with D.W before using, ٠.٥ ml of ٢ molar magnesium chloride, and ١ ml of ٠.٢٥ molar of glucose sterilized by filtering were added in case of using the medium as solid culture, ٢٪ of agar will be added (Sambrook *et al*., ١٩٨٩).

٣-١-٤-١١ Luria broth (LB)

١٠ gm of trypton, ٥gm yeast extract, and ١٠ gm sodium chloride were dissolved in ٩٥٠ ml distilled water. The pH adjusted to ٧ and the volume was completed to a liter with D.W, then sterilized by autoclave (Sambrook *et al*., ١٩٨٩).

٣-٢ Methods

٣-٢-١ Sterilization

All the media, solutions and glasswares used in this work were autoclaved at ١٢١°C for ١٥ min.

٣-٢-٢ pH of culture media

The pH of all culture media was adjusted to ٧.٢ – ٧.٤ using pH meter.

٣-٢-٣ Sample collection

Clinical samples were collected from Teaching, Emergency and General Hospitals of Sulaimaniya City, Kurdistan Region, IRAQ, since January and February ٢٠٠٥. The swab samples were took from infected sites of patients including (Burns, Ear and Wounds) while urine samples were taken from patients suffering from urinary tract infections (UTI). The patients were carefully educated to collect a proper sample by themselves; sterile dry wide necked leak proof containers were used for urine collection, collected mid-stream urine and were directly transferred to laboratory.

٣-٢-٤ Bacteriological examination

٣-٢-٤-١ Isolation of *Pseudomonas aeruginosa*

The samples collected from different human infections were transferred to the laboratory and activated using brain heart infusion broth. After activation inoculated on the MacConKey agar, a single colonies were selected, for more purification inoculated on the selective medium cetrimide agar, and oxidase test was done, positive isolates, and microscopically Gram negative rod shape, identified provisionally as *Pseudomonas aeruginosa*, subcultured on nutrient agar slants, after incubation at ٣٧ °C for ٢٤ hr., stored at ٤ °C, till other bacteriological tests were done (Chesbrough, ١٩٩١).

٣-٢-٤-٢ Identification of *Pseudomonas aeruginosa*

٣-٢-٤-٢-١ Biochemical tests

٣-٢-٤-٢-١-١ Urease activity (Production)

Urease is an enzyme which hydrolyzes urea [$(NH_2)_2CO$] to carbon dioxide (CO_2) and ammonia (NH_3), medium inoculated by stabbing with a sterile straight inoculation wire and incubated at ٣٧ °C for ٣-٥ days.

٣-٢-٤-٢-١-٢ Triple sugar iron test (TSI-test)

Test tubes containing (TSI) medium were inoculated with test organism by stabbing using a sterile straight wire, and incubated at ٣٧ °C for ٢٤-٤٨ hr.

٣-٢-٤-٢-١-٣ Oxidase test

The overnight colony of the test organism was smeared by a glass spatula or a platinum loop onto filter paper and ٢-٣ drops of oxidase reagent were added. Oxidase positive species gave a violet coloration immediately or within ١٠ seconds (Atlas *et al*., ١٩٩٥).

٣-٢-٤-٢-١-٤ Catalase test

A ٢٤ hr. old colony of the test organism was transferred by a sterile loop onto a clean slide then one or two drops of catalase test reagent were placed on the slide mixed well on the slide. Positive results were indicated by air bubble formation as a result of oxygen production (Atlas *et al*., ١٩٩٥).

٣-٢-٤-٢-١-٥ Motility test

Semi solid motility medium was inoculated by stabbing and incubated at ٣٧ °C, for ٢٤.hr, spreading of bacterial growth through the medium was considered as positive results (Atlas *et al*., ١٩٩٥).

٣-٢-٤-٢-١-٦ Growth at ٤°C and ٤١°C

Tested bacterium was inoculated to nutrient agar plates and incubated at both (٤ °C and ٤١ °C), for ٢٤hr (Atlas *et al*., ١٩٩٥).

٣-٢-٤-٢-١-٧ Pyocyanin production

Tested bacterium was inoculated to King No.١ medium using streaking technique, and incubated at ٣٧ C° for ٢٤ hr. coloring the plates with greenish-blue color as a result of pyocyanin production indicated positive result (King *et al*., ١٩٥٤).

٣-٢-٥ Biochemical Test

The api ٢٠E Micro tube system was used. This system is a standardized, miniaturized version of conventional procedures for the identification of Enterobacteriaceae and other Gram negative bacteria. It is designed for the performance of ٢١ standard biochemical tests for a single colony on plating medium.

٣-٢-٥-١ Performance of the api ٢٠E test

The system was used to identify the bacterial isolates according to the recommendations of the manufacture companies. Analytical profile index (API) test strips, produced by (BioMerieux SA, Lyon, France) can be used to identify members of the Enterobacteriaceae. These consist of a series of miniature capsules on a molded plastic strip, each of which contains a sterile dehydrated medium in powder form (Ball, ١٩٩٧). The results of the API system yield a seven-digit biotype number from which identification can be made (Atlas,

١٩٩٥). Here we used API ٢٠E system for confirmed identification & typing of (٣٢) isolated *P. aeruginosa* which we previously identified by biochemical tests as mentioned before.

Methodologies in accordance with the manufacturer's instructions were prepared by BioMerieux as follows :

٣-٢-٥-٢ Preparation of the strip

An incubation box (tray and lid) was prepared by about ٥ml of distilled water distributed into the honeycombed wells of the tray to create a humid atmosphere.

٣-٢-٥-٣ Preparation of the inoculums

A single well isolated colony was transferred with the aid of a pipette to the sterile test tube containing ٥ml of sterile distilled water, and then carefully emulsified to achieve a homogeneous bacterial suspension.

٣-٢-٥-٤ Inoculation of the strip

- Using sterile pipette, tube and cuple of tests [**CIT**], [**VP**] and **[GEL**] were filled with the bacterial suspension.
- Only the tubes of the other tests were filled with the same bacterial suspension.
- Anaerobiosis was created in the tests **ADH**, **LDC**, **ODC, H_2S**, and **URE** by overlying with mineral oil.
- Incubation box was closed and incubated at ٣٧°C for ٢٤ hours.

٣-٢-٥-٥ Reading of the strip

After incubation time results were recorded as shown in table (٣-٧).

All spontaneous reactions were recorded on the sheet and the tests which require the addition of reagents were revealed as follows :

- TDA test: one drop of **TDA** reagent was added to the **TDA** tube. A dark brown color indicates positive test.
- IND test: one drop of **IND** reagent was added to the **IND** tube. After ٢ minutes a red color indicates positive test.
- VP test: one drop of each **VP**١ and **VP**٢ reagents were added to the **VP** tube. After ١٠ minutes a pink or red color indicates positive test.

٣-٢-٥-٦ Identification

After reading the result identification was done by changing obtained results of the reactions into a numerical profile as follows :

On the result sheet, the tests are separated into ٧ groups of ٣ and a number ١, ٢, and ٤ is indicated for each. By adding the numbers corresponding to positive reactions within each group, a ٧-digit profile numbers were obtained for the ٢٠ tests of the api ٢٠ E strip. The Oxidase reaction constitutes the ٢١st test and has a value of ٤ if it is positive.

Then comparison between obtained numbers with numbers that find in the analytical profile index was used for identification of the tested bacterium.

٣-٢-٦ Maintenance and storage of bacterial culture

The culture was maintained by subculture on nutrient agar slant and incubated for ٢٤ hours at ٣٧°C, then kept in the fridge at ٤°C. Stock cultures were subculture on to fresh slant at monthly intervals through a liquid medium. In order to store isolates for a long time without loosing their genetical characteristics, nutrient agar plates streaked with bacteria, after appearance of growth at ٣٧°C, one ml of nutrient broth was added to the surface of plate and the growth harvested, then transferred to small vials containing sterilized ١ ml ٨٠٪ glycerol, then stored at -٢٠°C (Ausubel *et al*., ١٩٨٧).

٣-٢-٧ Antibiotic susceptibility test

٣-٢-٧-١ Antibiotic susceptibility test by disk-diffusion method

A single colony of *P. aeruginosa* was transferred to a fresh test tube which contained ٥ ml Brain-Heart infusion broth, then incubated for ٢٤ hours at ٣٧°C. After incubation time (١٠٠ µl) of the inoculums was transferred to a Muller-Hinton agar plate, the inoculums were streaked

by a sterile straight wire all over the surface of the medium three times; after ١٠-١٥ min. antibiotic discs were plated on the medium using a sterile forceps then incubated at ٣٧°C for ٢٤ hours. After incubation the inhibition zone diameters were determined as measured in millimeters (mm), comparison was done with standard inhibition zone (Bauer *et al*., ١٩٦٦).

٣-٢-٧-٢ Antibiotic resistance (Susceptibility) test

Muller-Hinton agar was used as growth medium; after sterilization and cooling to ٥٠°C, a final concentration of antibiotic under study was added, the medium with antibiotic was mixed and poured into sterile Petri-dishes in ٢٠ ml quantities. The antibiotic agar plates were inoculated with isolated single colony of *P. aeruginosa* by streaking method, and then incubated at ٣٧°C for ٢٤ hours. Next day antibiotic sensitivity and resistance of isolates to several antibiotics were recorded. (Sambrook *et al*., ١٩٨٩; and Baron and Finegold, ١٩٩٠).

٣-٢-٨ Total (Chromosome & Plasmid) DNA extraction by salting out method

١- LB agar containing ١٠ mg/ml antibiotic was inoculated by single colony of a bacterium isolates, and then incubated at ٣٧°C for ٢٤ hours. After that the bacterial cells were harvested. The harvested colonies were put in test tube that contains ٥ ml of STE buffer. Centrifugation of test tube was performed at (٣٠٠٠ rpm) for ١٥ min. Supernatant was discarded and pellet resuspended in ٥ ml of STE buffer, and mixed by vortex; these steps were repeated (٢,٣ and ٤) times in order to wash bacterial cells very well. Finally the cells were stored in ٥ ml STE buffer at ٤°C for ١٨hr.

٢- Aliquot of ٦٠٠µl of ٢٥٪ SDS solution was added to the bacterial suspension and incubated in water bath at ٥٥ °C for ٥ min.

٣- Aliquot of ٢ ml of ٥ M NaCl was added to the extract and then the extract was mixed by inversion, and left to cool to ٣٧°C.

٤- Aliquot of ٢ µl of proteinase K (٢٠mg/ml) was added to the extract solution and incubated in water bath at ٣٧°C for ٣٠ min.

٥- Aliquot of ٥ ml of mixture (Phenol: Chloroform: Isoamylalcohol) was added to the extracted solution then mixed gently by inversion at ٢٥°C for ٣٠ min. After that the extract solution and separated by centrifugation at (١٠٠٠٠ rpm) for ١٥ min.

٦- Aqueous phase that contains total DNA was separated from the organic phase by centrifugation at (١٠٠٠٠ rpm) for ٣٠ min. The DNA extract was precipitated by isopropanole (v/v ٠.٦) and mixed by inversion then the DNA was collected by the pasture pipette, washed in ٧٠٪ cold ethanol, then dissolved in (١-٢ ml TE buffer at pH ٨), after that stored at (-٢٠°C). (Pospiech and Neuman, ١٩٩٥).

٣-٢-٩ Extraction of plasmid DNA

One method used for extraction of plasmid DNA as in the following:

٣-٢-٩-١ Plasmid DNA extraction by alkaline lysis

١. A single colony of bacterial isolates was grown in ١٠ ml of LB broth containing ٥٠ µg/ml ampicillin and incubated at ٣٧ C° for ٢٤ hr. with shaking. Bacterial cells were harvested by centrifugation at ١٠٠٠٠ for ١٠ min.
٢. The pellet was resuspended in ١٠ ml of EST/saline buffer.
٣. The suspension was centrifuged at ١٠٠٠٠ rpm for ١٠ min. (the step ٢, ٣ repeated twice)
٤. The pellet was resuspended in ٠.٢ ml of solution I, then transferred to sterile eppendorf tube and left for ٥ minutes at room temperature.
٥. Aliquot of ٠.٤ ml of solution II was added to the mixture and then the eppendorf tube was gently inverted for many times and omitted in ice bath for ١٥ min.
٦. Aliquot of ٠.٣ ml of cold solution III was added to the mixture above and the eppendorf tube was inverted for many times before putting in the ice bath for ٥ min.
٧. The solution was centrifuged at ١٤٠٠٠ rpm for ٥ min.
٨. Aliquot of ٠.٥ ml of the supernatant was transferred to the new sterile eppendorf, then an equal volume of phenol-chloroform-isopropanole solution was added and mixed very well then the solution was objected to centrifugation as in the previous step.
٩. The upper layer was transferred to the new sterile eppendorf, and both the middle and lower layers were omitted. This step was repeated till the protein was excluded from the solution.
١٠. Aliquot of ٣ M of cold sodium acetate in ratio of ٠.١ volumes was added to the supernatant that contained plasmid DNA and mixed very well.
١١. Double volume of cold absolute ethanol was added to the suspension and mixed gently and then omitted at -١٠ C° for two hr.

١٢. The mixture was centrifuged at ١٢٠٠٠ rpm for ١٥ min. then the ethanol was excluded from the solution, and the pellet was washed by ٧٠٪ ethanol and then centrifuged as in the previous step.

١٣. The eppendroff was inverted on sterile filter paper and the pellet dissolved in ٥٠µl of TE buffer and stored at -٢٠ C°, (Kado and Liu, ١٩٨١).

٣-٢-٩-٢ Determination of total DNA concentration by spectrophotometer (Sambrook *et al*., ١٩٨٩).

To determine the concentration of extracted total DNA of *P. aeruginosa* isolates, ٠.١ ml of prepared plasmid DNA was diluted by ١ ml of TE buffer, then optical density at (٢٦٠ nm) was recorded using UV light spectrophotometer (Bejing Rayleigh Analytical, Mc, {China}). TE buffer was used as blank (standard solution), and concentration of plasmid DNA was calculated as follows:

Conc. of DNA µg/ml = optical density at ٢٦٠_{nm} × dilution factor × ٥٠ µg/ml.

٣-٢-١٠ Agarose Gel electrophoresis

Agarose Gel was prepared according to the method of Sambrook *et al*. (١٩٨٩); agarose gel was prepared by using ٠.٧ gm agarose powder in ١٠٠ ml TE buffer ,heated to boiling and cooled to ٤٥°C, then ١٠µl of ethidium bromide was added to the final concentration ٠.٥ mg/ml. The gel was poured on a glass plate (١٠ x ٢٠ cm) and the comb was inserted then the gel was allowed to set, the comb was removed and the gel soaked in a gel tank congaing ٠.٥ X Tris-Borate buffer.١٥ µl of plasmid DNA samples were mixed to ٣µl of loading buffer dye. Finally ١٠µl of this mixture was loaded in to the wells, then the gel tank was covered by lid. Electrophoresis was run at ٦volt/cm. The gels were illuminated with ultraviolet transilluminator, and then photographed by digital camera.

٣-٢-١١ Bacterial Conjugation

The transmissible ability of DNA plasmid of *P. aeruginosa* was tested according to Olsen *et al*. (١٩٩٢) methods as follows :

Day one:

١- A single colony of donor strain was inoculated into a ٥٠ ml nutrient broth containing appropriated antibiotic.

٢- A single colony of *E.coli* MM٢٩٤ Rif^{r} was inoculated into a ٥٠ ml nutrient broth containing ١٠٠ µg/ml of rifampicin.

٣- Both were incubated overnight at ٣٧°C with vigorous shaking or until OD ٥٩٠ ≈ ٠.٥٤.

Day two: Surface mating

١- The (٥×١٠^{٧} of donor cell and ١×١٠^{٨} recipients) were mixed in sterile eppendorf.

٢- The pellet was suspended in ٥٠µl of LB and the cells were transferred to the ٠.٢٢ µm Millipore filter on LB agar plate.

٣- The LB agar plate which contained Millipore filter was incubated at ٣٧°C for ١-٢ hr.

٤- The cells were resuspended by placing filter in a tube containing ٠.٥ ml of ٠.٨٥٪ saline and agitating of the tube was done on a vortex.

٥- Serial dilution was made١/١٠ dilution to ١٠$^{-٨}$ of the mating mixture.

٦- Spreading was done by ٠.١ ml of dilution ١٠$^{-١}$ to ١٠$^{-٦}$ on LB agar containing rifampicin (١٠٠µl/ml) and trimethoprim (٥٠µl/ml).

٧- Spreading of ٠.١ml of original overnight culture of donor and recipient on the same medium was done to determine the frequency of spontaneous mutation to antibiotic resistance.

٨- Spreading of ٠.١ ml of dilution ١٠$^{-٦}$ and ١٠$^{-٨}$ on LB agar containing rifampicin (١٠٠µl/ml) was done to estimate the number of recipient cells.

٩- The plates were incubated at ٣٧°C.

Control:

A- The ٠.١ ml of donor cells was transferred to the rifampicin plate.

B- Transfer ٠.١ ml of recipient cell *E.coli* MM٢٩٤ was transferred to the Plates containing antibiotic of donor strain.

C- The plates were incubated overnight at ٣٧°C.

Day three:

Colonies grown on selective media were counted and calculation was done for the number of bacteria per ml in mating mixture exhibiting the phenotype rifampicin (total recipient) and Rif^r, Tp^r (transconjugant).

Calculate the frequency of Transconjugant cells per donor or per recipient cells=

No. of transconjugant cells / ml

No. of recipient cells / ml

٣- ٢-١٢ Genetic transformation

٣- ٢-١٢-١ Preparation of competent cells

١- A starting culture was prepared by adding freshly isolated single colony to ٥ml of Nutrient broth medium. Grown overnight at ٣٧°C.

٢- Aliquot of ٠.٣ ml of starting culture was inoculated in to ٦٠ml of fresh Nutrient broth medium and the cells were grown at ٣٧°C for ٢ hours.

٣- The culture was chilled on ice for ٥-٧ minutes cell suspension centrifuged at ٦٠٠٠rpm /١٥min in a sterile ١٠٠ml glass centrifuge tube.

٤- Supernatant was discarded, the pellet was resuspended in half of the original culture volume, (٣٠ml) of an ice-cold sterile solution of ٠.١M $CaCl_٢$.

٥- Cell suspension was placed on ice for min, then centrifuged at ٦٠٠٠rpm /١٥min.

٦- Supernatant was discarded, and the cells were resuspended in ١ml of ice cold ٠.١M $CaCl_٢$ (Sambrook *et al*., ١٩٨٩).

٣- ٢-١٢-٢ DNA uptake

١- The transformation tube which contains ٠.١µl of competent cells, ١٠µl of a DNA solution was added and incubated on ice bath for ٣٠min.

٢- Heat-shocked cell DNA mixture by placing the tube in a ٤٢°C water bath for ٩٠ Sec. and then ١٠min in ice bath temperature.

٣- Aliquot of ١ml of pre wormed (٣٧°C) SOC medium was added to the suspension and Incubated at ٣٧°C with moderate agitation for ٦٠min.

٤- The suspension was then ٠.١ml cells should be spread quickly but gently on selective plated, SOC agar plates by spreading.

٥- The plates were incubated upside down over night at ٣٧°C, (Sambrook *et al*., ١٩٨٩).

٣-٢-١٣ Curing of Plasmid DNA

٣-٢-١٣-١ Spontaneous Curing

This method for plasmid curing in *P. aeruginosa* was described by (Meyer, ١٩٧٤) as follows :

١- Nutrient broth was inoculated by ١٠ ml of single colony of *P. aeruginosa* isolate,

then incubated with shaking ١٠٠ rpm at ٣٧°C for ٢٤hrs.

٢- Several dilutions were prepared, and ٠.١ ml of last three dilutions were spread on to nutrient agar plates, after that the plates were incubated at ٣٧°C for ٢٤hrs.

٣- A master plate containing ١٠٠ colonies was made, then these colonies transferred to different antibiotic agar plates and the results were recorded.

The curing frequency was calculated according to the following equation (Atlas *et al*., ١٩٩٥)

Frequency of Curing= No. of Cured cells / No. of plated cells

٣-٢-١٣-٢ Plasmid curing by chemical agents

٣-٢-١٣-٢-١ Plasmid curing by Ethidium Bromide

This method was described by (Trevors, ١٩٨٦), elimination of antibiotic resistance plasmid DNA from *P. aeruginosa* isolates was done by Ethidium Bromide, as follow:

Ten ml of nutrient broth containing ٧٠٠ µg/ml Ethidium Bromide was inoculated with ٠.٣ ml of overnight culture of *P. aeruginosa* isolates, incubated at ٣٧°C for ٢٤, ٤٨, and ٧٢ hrs.

As long as serial dilution was performed up to $١٠^{-٧}$ by ٠.١ ml of interval incubated samples, and ٠.١ ml of last three dilutions were plated on nutrient agar plates, then all plates were incubated at ٣٧°C for ٢٤ hrs.

٣-٢-١٣-٢-٢ Plasmid curing by Sodium Dodecyl Sulfate

Plasmid curing by SDS was done by the method described by Tomoeda *et al*. (١٩٧٤), as follows:

١- Test tube containing ٥ ml of nutrient broth was prepared by adding appropriate antibiotic at final concentration, then incubated with single colony of *P. aeruginosa* isolate, and incubated at ٣٧°C for ٢٤ hrs.

٢- Serial dilution was prepared up to $١٠^{-٣}$ dilution by nutrient broth containing (٠.٠٥٪, ٠.١٪, ٠.٢٥٪, ٠.٥٪, ١٪, ٢٪, and ٢.٥٪) (W/V) (SDS), then third dilution incubated at ٣٧°C for ٢٤hrs, serial dilutions were prepared up to $١٠^{-٦}$, then ٠.١ ml of last three dilutions were spreaded on nutrient agar plates containing appropriate antibiotic and incubated at ٣٧°C for ٢٤hrs.

٣-٢-١٣-٣ Plasmid curing by physical agents

٣-٢-١٣-٣-١ Plasmid curing by elevated temperature

A single colony of *P. aeruginosa* isolate was inoculated into ١٠ ml of nutrient broth, after incubation at ٣٧°C for ٢٤hrs, then ٠.٢ ml of bacterial culture was inoculated to ١٠ ml of fresh nutrient broth, and incubated at ٤٦°C for ٢٤hrs with shaking ١٠٠ rpm, after incubation time several dilutions were performed up to $١٠^{-٧}$, then ٠.١ ml of last three dilutions were spread on plates of nutrient agar which contain different antibiotics at final concentration and incubated at ٣٧°C for ٢٤ hrs. Next day, the results were recorded by the loss of ability of the tested bacteria to survive on the medium which contains the antibacterial agents (Kheder, ٢٠٠٢).

٣-٢-١٣-٤ Selection of the cured bacterial cells

In all curing agent treatments, master plates were prepared containing ١٠٠ bacterial treated colonies. Pick and patch technique was used, in order to determine the cured cells on to the nutrient agar plates containing the antibiotics separately for the isolates, and untreated cells used as control. Observation of growth of inoculation region on the plates, if there are no growths on plates containing antibiotics, but growth observed on plates without antibiotics it means that the cells lost their property of resistance to certain antibiotics, then selected and stored for next tests, to be sure that the plasmid was cured through comparing with the original strains and plasmid DNA extracted from cured cells for Agarose gel electrophoresis study (Kheder, ٢٠٠٢).

٣-٢-١٤ Infections of the laboratory animal (Albino's rat) with *P. aeruginosa* isolates

٣-٢-١٤-١ Rat burn infection model

The rat burn infection model was described by (Pavlovskis and Wretlind , ١٩٧٩). Rats weighing ٢٠٠ to ٢٢٠ g, were selected for this study because of their high susceptibility.

The backs of the rats were shaved, and the rats were anesthetized with methoxyflurane. A template with a ٢.٥ by ٢.٥ cm opening (corresponding to approximately ١٥٪ of total body surface) was placed on the shaved area, covered with ٠.٥ ml of ٩٥٪ ethanol, and flamed for ١٥ seconds. The burn was nonlethal and did penetrate the keratin layer slightly, but did not reach the musculature layer Pavlovskis and Wretlind (١٩٧٩). Bacteria in logarithmic phase of growth were suspended shortly before use in phosphate-buffered saline (٢٣°C) at a desired concentration and placed in an ice bath, and ١ ml volumes were injected subcutaneously in the burned area immediately after the trauma.

٣-٢-١٤-٢ Challenge inoculum

The inoculum was prepared as previously described by Pavlovskis and Wretlind (١٩٧٩).

٣-٢-١٥ Histological study

The preparation of histological sections of rat skin and lungs depended on standard methods of (Baker *et al*., ١٩٧٥; Leeson & Leeson, ١٩٨١) as follows :

٣-٢-١٥-١ Fixation

The dissected skins with the dissected lungs organ were cut into small pieces from regions with morphological abnormalities and were put in a small container containing the Carnoy's fluid fixative for ١-٢ hours for fixation.

The fixative Carnoy's fluid was prepared by mixing ٦٠ml of absolute ethanol, ٣٠ml of chloroform and ١٠ml of glacial acetic acid. Then the specimens were transferred into ethanol ٨٠٪, for removing and washing the excessive fixative material.

٣-٢-١٥-٢ Dehydration and Embedding

• Specimens passed through a series of gradual concentration of ethanol starting from ٣٠٪, ٧٠٪, ٩٠٪, for ٢ hours in each concentration.

• In ١٠٠٪ for ٢ hours twice.

٣-٢-١٥-٣ Clearing or (De-alcoholization)

The specimens were placed in a Xylene (most rapid clearing agent) for ٢ hours.

٣-٢-١٥-٤ Infiltration (Impregnation with paraffin wax)

Specimens were thoroughly placed in the mixture of melted paraffin and xylene for ٢ hours at ٦٠°C, and placed in melted paraffin wax for ١ hour at ٦٠°C. The specimens were placed in new melted paraffin and transported into Vacuum cylinder at ٦٠^{o}C for one hour in order to withdraw the air bubbles.

٣-٢-١٥-٥ Moulds for Embedding

The specimens were placed singly in a concave glass block filled with melted paraffin after being covered by thin film of glycerol to prevent adhesion of the paraffin to the glass. The paraffin blocks were exposed to the cooling temperature for hardening and left in cool water or at ٤^{o}C for at least overnight.

٣-٢-١٥-٦ Trimming the block

The individual paraffin blocks were trimmed with a hand razor for preparing block with parallel sides.

٣-٢-١٥-٧ Sectioning (Cutting the section)

This process was done by rotary microtome; the thickness was gauged at four microns, and then microtome was operated until complete sections were again being cut and then maintained a regular cutting rhythm, ribbons of cross sections were obtained, and then the sections were affixed on glass slides.

٣-٢-١٥-٨ Mounting the sections on slides

Several slides should be cleaned, smeared with a drop of Mayer's egg albumin. Fixing the section on the slide by using a hot plate.

• Slide was flooded with distilled water ٥٥°C.

• The sections were placed on the slide, and ribbons of sections were arranged on the slide.

• The slides were placed on hot plate at a temperature ٤٥°C in order to stretch the ribbon and remove all creases.

• When the sections were fully extended, the slides were removed from the hot plate and drain off excess water.

• The slides were dried by transferring them to an incubator and leaving them at room temperature for ٤-٥ hours to complete the affixing process.

٣-٢-١٥-٩ Dewaxation

The sections were dewaxed by warming the section over the Bunsen burner until the wax just became melted, then immersed in the Xylene for two minutes.

٣-٢-١٥-١٠ Staining procedures

• The sections were hydrated by lower graded ethanol ١٠٠٪, ٩٠٪, ٧٠٪, and then distilled water, three minutes for each

• The sections were transferred to Haematoxylin stain for ٦ minutes.

• The sections were washed by alkaline water

• The sections were rinsed for few seconds with acid alcohol in order to differentiate the stain.

• The sections were blued by running tap water.

• The sections were transferred to counter stain (Eosin) for ٤ minutes.

• The sections were washed by running water, and then dehydrated via gradual ethanol ٧٠٪, ٩٠٪, and ١٠٠٪ for ٣ minutes for each concentration.

• The sections were cleared by Xylene for ٣ minutes.

• sections were mounted by Canada balsam and Xylene ١:١ then covered by cover slides and put in room temperature for drying.

The sections were examined by light microscope under magnification power ١٠٠ X and ٤٠٠ X. Photographs were taken by a digital-camera.

RESULTS AND DISCUSSION

٤-١ Collection, isolation and identification of *Pseudomonas aeruginosa* isolates

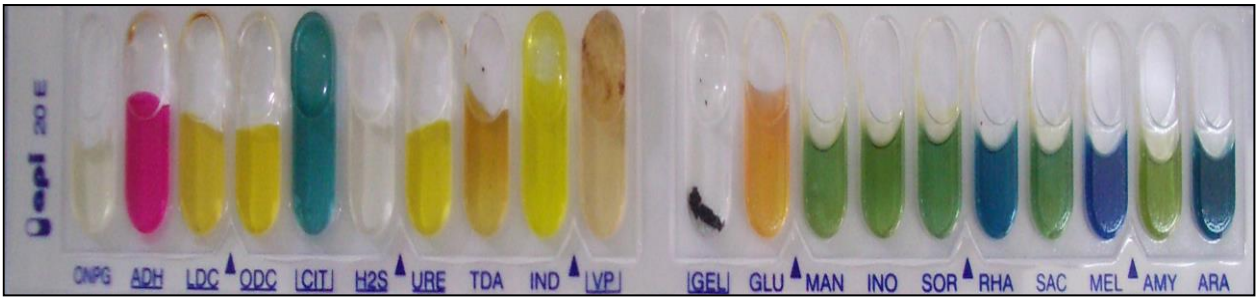
A total of ninety four (٩٤) samples were collected from different human infections (ear, urine, wounds, and burns), from the General, Urology, Teaching, and Emergency Hospitals in Sulaimaniya City.

All bacterial isolates were characterized selectively using cetrimide medium, cultural and morphological characteristics but only thirty two (٣٢) isolates were indicated as *P. aeruginosa*. The colonies of *P. aeruginosa* isolates were studied using nutrient agar plates and macConkey agar plates. They are small in size, fried-egg appearance, smooth with flat edge and an elevated appearance, while the others which are isolated from the secretions of urinary tract infections have a mucoid appearance on nutrient agar. The smooth and mucoid colonies are presumed to play a role in colonization and virulence.

Most of these isolates produce pyocyanin (blue green pigment), which is in accordance with that mentioned by Todar (٢٠٠٤). *P. aeruginosa* does not ferment lactose and is differentiated from lactose fermenting bacteria (Enterobacteriaceae). Culture is the specific test for diagnosis of *P. aeruginosa* infection. The bacterial cells from smear preparation are gram negative, rod-shaped, and occur as single, in pairs, or in short chains, presumptively regards *P. aeruginosa,* which in accordance with previous observation (Holt *et al*., ١٩٩٤; Jawetz *et al*., ٢٠٠١; and Todar, ٢٠٠٤).

The bacterial colonies were able to grow at ٤١°C but not at ٤°C; These criteria were used for the identification of *P. aeruginosa* from other species; this is in agreement with (Jawetz *et al*., ٢٠٠١), who found that *P. aeruginosa* have the ability to grow at ٤١°C and produce pyocyanin after growing on cetrimide medium.

Furthermore, biochemical tests were performed to support the results above, using api ٢٠E test which is a rapid accurate technique for the identification of the family Enterobacteriaceae (Kurlandsky and Fader, ٢٠٠٠).



***Pseudomonas aeruginosa* profile number (٢٢٠٤٠٠٤),**

[٩٨٪] accuracy of identification.

Figure (٤-١) results of api ٢٠ E test used for the identification of *P. aeruginosa* isolates.

All the isolates were oxidase positive, which was regarded an important characteristics for these bacteria as mentioned by (Bingen *et al*., ١٩٩٢); identification of *P. aeruginosa* strains usually based on clinical morphology, oxidase positive, the presence of characteristics pigments, and growth at ٤٢C as described by (Jawetz *et al*., ٢٠٠١). In general, according to the analytical profile index, (١٩٩٧), the synonyms number obtained from tested samples ranged between (٢٢٠٤٠٠٤), which is (٩٨٪) indicate that all isolates were *P. aeruginosa*. The bacterial isolate takes the letter (P) and the number of samples that isolated from (١ to ٣٢).

٤-٢ Distribution of the bacterial isolates according to their source of infections

Thirty two clinical isolates classified according to their source of infection as shown in Figure (٤-٢)

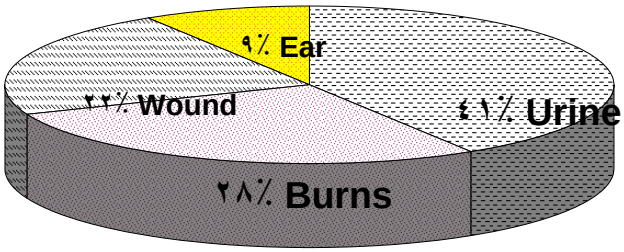


Figure (٤-٢): distribution of clinical isolates according to the source of infection.

Results showed that the urine isolates were the most frequent abundance that encountered ٤٠.٦٢٥%, while for burns; wounds and ear were ٢٨.١٢٥%, ٢١.٨٧٥%, and ٩.٣٧٥% respectively. This may be due to the fact that these samples were not taken regularly, but can therefore be considered as reflections of the actual situation of *Pseudomonas aeruginosa* of the patients in these hospitals.

Isolation of *Pseudomonas aeruginosa* was carried out from Burns, Wounds, Urine and Ear units as well, table (٤-١).

Table (٤-١): Distribution of *Pseudomonas aeruginosa* according to source of isolation:

Source of isolation	Isolate No.	No. of bacterial isolate	% of isolate
Urine	٤,٥,٦,٧,٨,٩,١٠,١١,١٢,١٣,١٤,١٥,١٦	١٣	٤٠.٦٢٥
Burns	٢٢,٢٤,٢٥,٢٧,٢٨,٢٩,٣٠,٣١,٣٢	٩	٢٨.١٢٥
Wound	١٧,١٨,١٩,٢٠,٢١,٢٣,٢٦	٧	٢١.٨٧٥
Ear	١,٢,٣	٣	٩.٣٧٥

In Tunisian, Pallilo and Salleh (١٩٩٢) found that the percent of *P. aeruginosa* was ٦٥٪ for respiratory tract, ١٣.٥٪ for urine, ٨.٥٪ for wounds and ١٣٪ for others among ٢١٣ isolates.

In another study in India, Puri *et al.* (١٩٩٦) obtained ٣٠٪ from wound, ٣٠.٥٪ from urine and ١٥.٢٪ from stool specimens. Tassion *et al.* (١٩٩٨) indicated ٣٠٪ for urine, ١٤٪ in pus, ٩٪ in sputum and wound among ٨٨ isolates in ١١ Greek hospitals. Also Delden and Iglwesky (١٩٩٨) reported that *P. aeruginosa* is responsible for ١٦٪ of nosocomial pneumonia cases, ١٢٪ of hospital acquired urinary tract infections, ٨٪ of surgical wound infections, and ١٠٪ of blood stream infections.

Although there are differences in the percent of infection between our results and others, these results still agree with that which says *P. aeruginosa* is an opportunistic pathogen that

causes human infections and can be isolated from soil, water and disinfectants (Marques *et al.*, ١٩٧٩; Tassois *et al*., ١٩٩٨; Abd Al-Amir, ١٩٩٩ and Todar, ٢٠٠٤).

٤-٣ Antibiotic resistance pattern of *P. aeruginosa* isolates

P. aeruginosa is currently one of the most frequent nosocomial pathogen and the infection due to this organism is often difficult to treat due to antibiotic resistance (Emori and Gayner, ١٩٩٣). It is a common phenomenon in most general hospitals that the frequency of occurrence of infections due to *P. aeruginosa* is increasing. The reasons for the increase are not completely understood, but undoubtedly they bear some relationship to the widespread use of antibiotic therapy and the resistance of *P. aeruginosa* to most of the widely used antibiotics. It is not unusual for strains of this organism isolated from infections to be resistant to three or more of the following antibiotics: sulphadiazine, ampicillin, kanamycin, streptomycin, chloramphenicol, neomycin, or tetracycline. The only antibiotic to which *P. aeruginosa* is usually sensitive is polymyxin, although strains resistant to this agent are common.

The mechanism of resistance to antibiotics includes, reduced cell wall permeability, production of chromosomal and plasmid mediated ß-lactamase (Livermore, ١٩٨٩), aminoglucoside-modifying enzymes (Prince, ١٩٨٩), and an active multi drug efflux mechanism (Li *et al*., ١٩٩٤).

Thirty two (٣٢) *P. aeruginosa* isolates were screened for their resistance to sixteen widely used antibiotics in medicine which are (ampicillin, amikacin, amoxicillin, carbencillin, ciprofloxacin, chloramphenicol, cefotaxime, erythromycin, gentamycin, lincomycin, neomycin, penicillin, rifampicin, streptomycin, tetracycline, and trimethoprim). See Table (٤-٢).

Table (٤-٢): The resistance of *Pseudomonas aeruginosa* isolates to antibiotics:

Antibiotic Sensitivity Tests																	
No. of Isolates	**Source of Isolation**	**Ap**	**AK**	**Ax**	**Car**	**Cip**	**Cm**	**CTX**	**Ery**	**Gm**	**Lin**	**N**	**Pi**	**Rif**	**Sm**	**Tc**	**Tri**
P. aeruginosa ١	**Ear**	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
P. aeruginosa ٢	**Ear**	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
P. aeruginosa ٣	**Ear**	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
P. aeruginosa ٤	**Urine**	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P. aeruginosa ٥	**Urine**	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+
P. aeruginosa ٦	**Urine**	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P. aeruginosa ٧	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
P. aeruginosa ٨	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
P. aeruginosa ٩	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
P. aeruginosa ١٠	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
P. aeruginosa ١١	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
P. aeruginosa ١٢	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
P. aeruginosa ١٣	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
P. aeruginosa ١٤	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
P. aeruginosa ١٥	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
P. aeruginosa ١٦	**Urine**	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
P. aeruginosa ١٧	**Wound**	+	+	+	+	-	+	+	+	(I)	+	+	+	+	+	+	+
P. aeruginosa ١٨	**Wound**	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+
P. aeruginosa ١٩	**Wound**	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P. aeruginosa ٢٠	**Wound**	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P. aeruginosa ٢١	**Wound**	+	(I)	+	+	-	+	+	+	-	+	+	+	+	+	+	+

Antibiotic Sensitivity Tests																	
No. of Isolates	**Source of Isolation**	**Ap**	**AK**	**Ax**	**Car**	**Cip**	**Cm**	**CTX**	**Ery**	**Gm**	**Lin**	**N**	**Pi**	**Rif**	**Sm**	**Tc**	**Tri**
P. aeruginosa **٢٢**	**Burn**	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
P. aeruginosa **٢٣**	**Wound**	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P. aeruginosa **٢٤**	**Burn**	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+
P. aeruginosa **٢٥**	**Burn**	+	+	+	+	**(I)**	+	+	+	+	+	+	+	**(I)**	+	+	+
P. aeruginosa **٢٦**	**Wound**	+	+	+	+	**(I)**	+	+	+	+	+	+	+	-	+	+	+
P. aeruginosa **٢٧**	**Burn**	+	-	+	+	-	+	+	+	-	+	-	+	+	-	+	+
P. aeruginosa **٢٨**	**Burn**	+	+	+	+	**(I)**	+	+	+	+	+	+	+	-	+	+	+
P. aeruginosa **٢٩**	**Burn**	+	**(I)**	+	+	+	+	+	+	+	+	-	+	+	-	+	+
P. aeruginosa **٣٠**	**Burn**	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+
P. aeruginosa **٣١**	**Burn**	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
P. aeruginosa **٣٢**	**Burn**	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+

Ap: ampicillin, Ak: amikacin, Ax: amoxicillin, Car: carbencillin, Cip: ciprofloxacin, Cm: chloramphenicol, Ctx: cefotaxime, Ery: erythromycin, Gm: gentamycin, Lin: lincomycin, N: neomycin, Pi: penicillin, Rif: rifampicin, Sm: streptomycin, Tc: tetracycline, and tri: trimethoprim.

* The symbols (+): Resistance to Antibiotics, (-): Sensitive to Antibiotics, and (I): intermediate.

All isolates show resistance to ampicillin, amoxicillin, carbencillin, chloramphenicol, cefotaxime, erythromycin, lincomycin, penicillin, tetracycline and trimethoprim), while they show variable resistance to amikacin, ciprofloxacin, gentamycin, neomycin, rifampicin and streptomycin.

Table (٤-٢) revealed that *P. aeruginosa* isolates revealed high resistance to most widely used antibiotics in medical treatment, results in an increased frequently of resistance in microbial flora including *P. aeruginosa*. The reasons for this pattern of resistance to such a wide variety of drugs are not understood at the biochemical level. This also could be a ruitful avenue of future research, with both academic and practical implications.

In recent years it has been emphasized that there is a remarkable increase in the incidence of infection by antibiotic resistance microorganisms in different parts of the world, for example Flick and Cluff (١٩٧٦) found that *P. aeruginosa* isolated from patients demonstrated resistance to carbencillin, while Marques *etal.* (١٩٧٩) observed that some of *P. aeruginosa* isolates from soil and water resistant to gentamycin, where as all isolates resistant to ampicillin, chloramphenicol, nalidixic acid, tetracycline and streptomycin.

The epidemiology of drug resistance in Enterobacteriaceae and some of the gram-positive cocci undergo a remarkable change in character with the widespread occurrence of resistance transfer factors (RTF). RTF may transfer to drug-sensitive strains by conjugation in much the same way and with much the same type of kinetics as F transfer in *E. coli*. Furthermore, RTF can act as sex factors in promoting conjugation and transfer of chromosome (Small *et al*., ١٩٩٣). Jibran (١٩٨٦) found that among (٨٣) *P. aeruginosa* isolates ٢٩٪ were resistant to carbencillin, while all isolates were resistant to ampicillin, cefotaxime, chloramphenicol, tetracycline, trimethoprim, and streptomycin. These results were relatively the same as results achieved in our work, and we can say that our results were in agreement with Jibran (١٩٨٦).

Tassios *et al*. (١٩٩٨) found that ١٠٪ out of ٨٨ isolates of *P. aeruginosa* were resistant to all antibiotics used. Quadri *et al.* (١٩٩٤) reported that the pattern of antibiotic resistance of bacterial pathogen usually varied from one geographic location to another and outbreak of disease caused by multiple resistant bacteria occurs more frequently in developing countries.

Antibiotic resistance is now generally accepted as a major public health issue, and these problem should be solved. Shahid (٢٠٠٤) reported that antimicrobial susceptibility of the multi drug resistant *P. aeruginosa* isolates were for AK ١٠٠٪, Tb. ٨٠٪, Gt ٣٠٪, Nt. ٧٠٪, Cr. ٤٠٪, Cp. ٢٠٪ C٢. ٢٠٪, and ٤٠٪, ٣٠٪, ٨٠٪, for Ct., Ce, Cl. Respectively.

The fluorinated quinolones, in particular ciprofloxacin, are still active against *P. aeruginosa*. Resistance may nevertheless, emerge during long term treatment of chronic infections. Resistance to other antibiotics including cephalosporin's and antipseudomonal antibiotics may also occur in future (Shahid, ٢٠٠٤). Given this drug-resistant nature of *P. aeruginosa*, it is important from a public health viewpoint to know whether RTF can either occur in this species or be transferred to it from the enterobacteria.

Therefore, to combat this problem, effort showed be made to isolate and characterize plasmids responsible for resistance in multi drug resistance (MDR) *P. aeruginosa* strains from all over country and a nation wide antibiotic policy should be defined after evaluating

the effectiveness of the regime so that the misuse of antibiotics is minimized and also the emergency of multi drug resistant organism can be restricted. This is a preliminary study on plasmid mediated ١٦ antibiotic resistance in *P. aeruginosa* isolates; however, there is a need for a large scale study to find out the plasmid mediated drug resistance in *P. aeruginosa* along with isolation and characterization of plasmids.

٤-٤ The plasmid profile of *P. aeruginosa* isolates

Electrophoresis characterization of total DNA and plasmid DNA content of *P. aeruginosa* isolates obtained from different human infections were extracted by salting out and alkaline lysis respectively and carried out for migration using ٠.٧ % agarose gel, at ٥٠ volt for ٥ hours.

Figure (٤-٣) shows the plasmid profile of four Pseudomonal isolates which represent two groups, the first one extracellular protease non producers (P١٢ and p١٦ isolates) and the second group had the ability to produce extracellular protease (P٢٢ and P٢٤ isolates). Results revealed two plasmid profiles: one of them indicated the presence of one small plasmid (P٢٤ and P١٢ isolates) and two small plasmids (P٢٢ and P١٦ isolates), while the other plasmid profiles revealed large single plasmids (P٢٢ and P٢٤ isolates) in agarose gel electrophoresis. These two large plasmids are mega plasmids which were above the chromosomal DNA in migration distance, because they are large in size and super coiled (Hardy, ١٩٨٦),

The previous studies by Gabisoniia *et al*., (١٩٩٢) and Tsakris *et al.* (١٩٩٢) elucidated that plasmid size bearing antibiotic resistance characteristics in *P. aeruginosa* ranged between (٢٠-١٠٠) mega Dalton. Nordmann (١٩٩٣) found that the size of plasmid ranged between (١.٩-٤٥.٠) MD also reported that the size of plasmid in the bacteria ranged between (٤-٨٠) Kbp. It is demonstrated from this study that the high resistance of the tested isolates to antibiotics may be related to the large size plasmids.

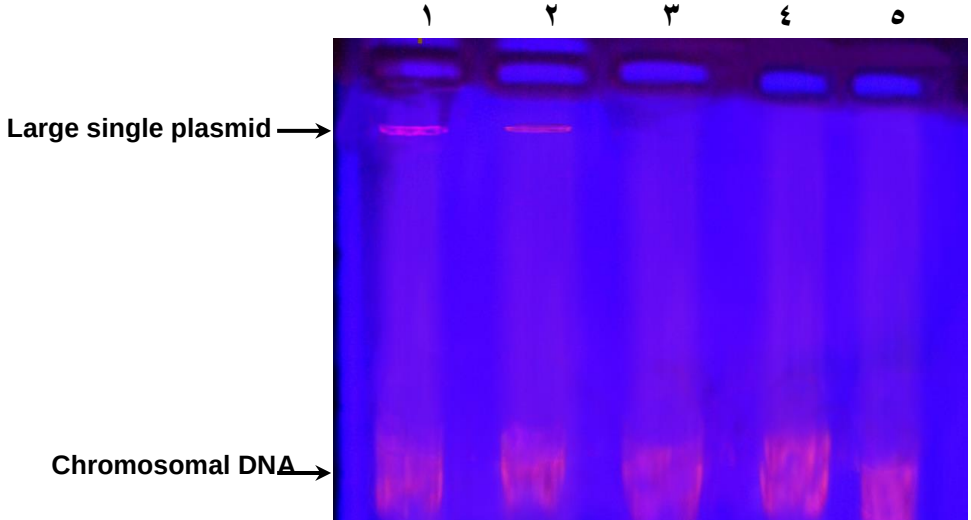

١
٢
٣
٤
٥
Large single plasmid
Chromosomal DNA

Figure (٤-٣): The plasmid profile of *Pseudomonas aeruginosa* isolates.

The total DNA extracted by salting out (Pospiech and Neuman, ١٩٩٥) and migrated on agarose gel ٠.٧٪, ٥٠ volt, for ٥ hr.

- Lane ١: DNA content of proteases producing *P. aeruginosa* P٢٢ isolate.
- Lane ٢: DNA content of proteases producing *P. aeruginosa* P٢٤ isolate.
- Lane ٣: DNA content of non-proteases producing *P. aeruginosa* P١٢ isolate.
- Lane ٤: DNA content of non-proteases producing *P. aeruginosa* P١٦ isolate.
- Lane ٥: DNA content of standard plasmidless strain *E.coli* MM٢٩٤.

٤-٥ Genetic transfer of *P. aerurginosa* plasmids by transformation

The transformation experiments were carried out according to Davis *et al.* (١٩٨٦), described by Shahid and Malik (٢٠٠٣), by using *P. aeruginosa* strain as the donor (P٤, P١٦, P٢٢, and P٢٧ isolates) and the plasmidless competent cells of *Escherichia coli* MM ٢٩٤ (Rif^r) as the recipient strain. The transformant cells appeared as follows :

Table (٤-٣): The transformation frequency and number of transformant colonies among four strains of *P. aeruginosa* isolates.

P. aeruginosa isolates and Transformant cells	Proteases	Growth on nutrient agar containing antibiotics in (µg/ml)															Frequency of transformation
		Ap	Ak	Ax	Car	Cip	Cm	Ctx	Ery	Gm	Lin	Pi	Rif	Sm	Tc	Tri	
P. aeruginosa P٤	-	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	
E. coli P٤	-	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	٢٥٥ x ١٠$^{-٥}$
P. aeruginosa P١٦	-	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	
E. coli P١٦	-	+	+	+	-	-	-	-	-	-	-	+	-	+	+	+	٥٦ x ١٠$^{-٤}$
P. aeruginosa P٢٢	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	
E. coli P٢٢	-	+	+	+	+	-	+	+	+	-	-	+	-	+	+	+	٤٩ x ١٠$^{-٤}$
P. aeruginosa P٢٧	+	+	-	+	+	-	+	+	+	-	+	+	+	-	+	+	
E. coli P٢٧	-	+	-	+	-	-	+	+	+	-	-	+	+	-	-	-	٤٢ x ١٠$^{-٤}$

*The *E. coli* MM٢٩٤ Rif^r recipient cell/ml concentration was (٢×١٠^{٦}) cell/ml.

* The symbols (+): Resistance to Antibiotics, (-): Sensitive to Antibiotics.

* Ap: ampicillin, Ak: amikacin, Ax: amoxicillin, Car: carbencillin, Cip: ciprofloxacin, Cm: chloramphenicol, Ctx: cefotaxime, Ery: erythromycin, Gm: gentamycin, Lin: lincomycin, Pi: penicillin, Rif: rifampicin, Sm: streptomycin, Tc: tetracycline, and Tri: trimethoprim.

The transformation results revealed that the transformant *E.coli* P٤ acquired multiresistance to ampicillin, amikacin, amoxicillin, carbencillin, chloramphenicol, cefotaxime, erythromycin, penicillin, rifampicin, streptomycin, tetracycline, and trimethoprim, while they were sensitive to ciprofloxacin, gentamycin, and lincomycin. These results indicated that the plasmid carrying multiple resistance genes coding ١٢ antibiotic resistances in transformant cells and *P. aeruginosa* P٤. The sensitivity to ciprofloxacin and gentamycin may mean that these antibiotic resistance genes are located on large plasmids, while the antibiotic resistance genes of Lincomycin are encoded by chromosomal DNA and this agrees with that result obtained by (Kheder,٢٠٠٢).

The transformation results indicated that the transformant *E.coli* P١٦ acquired multiresistance to ampicillin, amikacin, amoxicillin, penicillin, streptomycin, tetracycline, and trimethoprim, while they were sensitive to each of carbencillin, ciprofloxacin, chloramphenicol, cefotaxime, erythromycin, gentamycin, lincomycin, and rifampicin. These results found that the plasmid carrying multiple resistance genes encoding (٧) antibiotic resistances in transformant cells and *P. aeruginosa* P١٦. These may be due to that only one small plasmid among two plasmids was successfully transferred to *E.coli* MM٢٩٤ host, which may cause the differences in resistancy or sensitivity when cultured on different antibiotics. The results observed that the some antibiotic resistance genes located on another small plasmid which was failed to enter *E.coli* MM٢٩٤ as showed in figure (٤-٤), while the sensitivity to that ٨ antibiotics may be refer to that the antibiotic resistance genes located on large plasmid or chromosomal DNA rather than small plasmid.

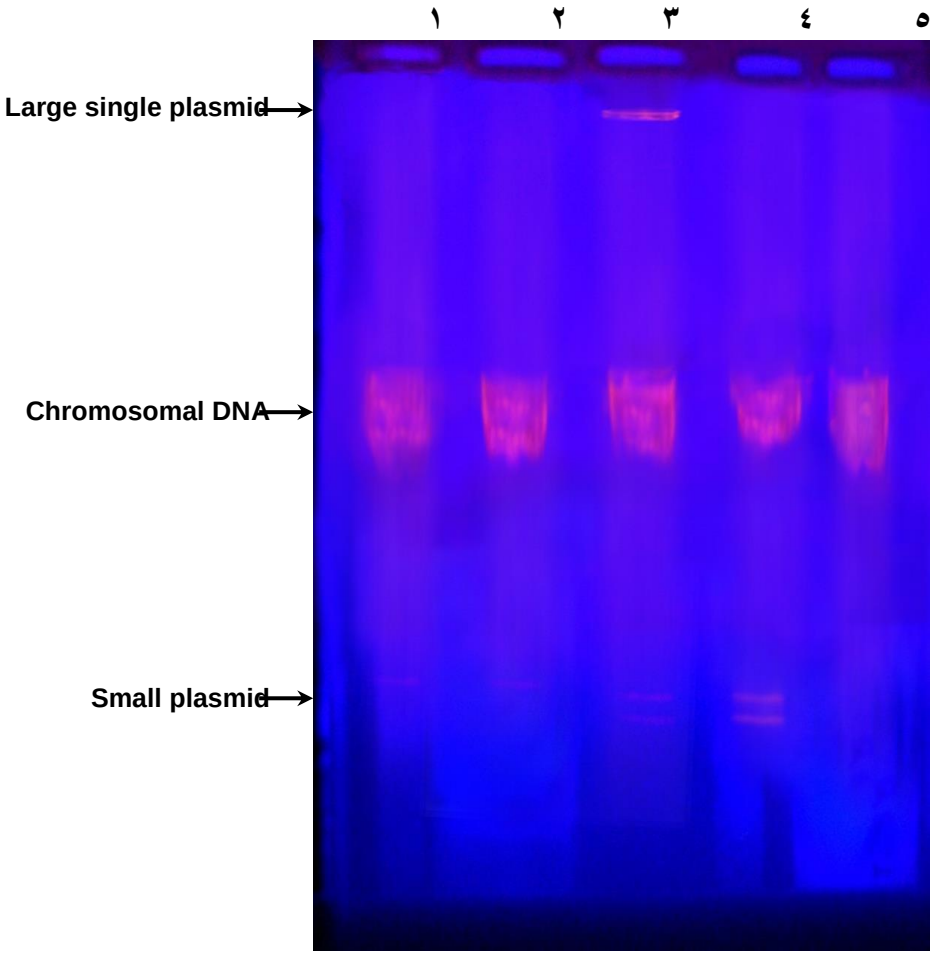


Figure (٤-٤): The plasmid profile of the transformed bacterial cells of *E. coli* MM٢٩٤.

The total DNA extracted by salting out (Pospiech and Neuman, ١٩٩٥) and migrated on agarose gel ٠.٧٪, ٥٠ volt, for ٦hr.

- Lane ١: DNA content of non-proteases producing *P. aeruginosa* P١٦ isolate, which have one small plasmid.
- Lane ٢: DNA content of transformant *E. coli* P١٦ isolate that contain small transformant plasmids.

- Lane ٣: DNA content of proteases producing *P. aeruginosa* P٢٢ isolate, which have two small plasmids with single large plasmid.
- Lane ٤: DNA content of transformant *E. coli* P٢٢ isolate that contains two small transformant plasmids.
- Lane ٥: DNA content of standard plasmidless strain *E.coli* MM٢٩٤.

The transformation results also indicated that the transformant *E.coli* P٢٢ acquired multiresistance to each of ampicillin, amikacin, amoxicillin, carbencillin, chloramphenicol, cefotaxime, erythromycin, penicillin, streptomycin, tetracycline, and trimethoprim while they were sensitive to each of ciprofloxacin, gentamycin, rifampicin, and lincomycin. These result indicated that the plasmid carrying multiple resistance genes encoding ١١ antibiotic resistances in transformant cells and *P. aeruginosa* P٢٢, the successful of ١١ transformant antibiotic resistance genes may be due to the fact that two small plasmid DNA were successfully transformed to *E.coli* MM٢٩٤ as shown in Figure (٤-٤), about the untransformant antibiotic resistance genes i.e ciprofloxacin, gentamycin, and rifampicin refer to that the wild strain was also sensitive to each antibiotics after the previous antibiotic susceptibility tests, while the sensitivity to lincomycin means that the responsible gene of Lincomycin encoded by chromosomal DNA and this agree with that obtained by (Kheder, ٢٠٠٢).

The transformation results showed that the transformant *E.coli* P٢٧ acquired multiresistance to ampicillin, amoxicillin, chloramphenicol, cefotaxime, erythromycin, penicillin, and rifampicin, while they were sensitive to amikacin, carbencillin, ciprofloxacin, gentamycin, lincomycin, streptomycin, tetracycline, and trimethoprim. These results indicated that the plasmid carrying the multiresistance genes which encoded ٧ antibiotic resistances were transformed successfully to *E.coli* P٢٧, while the fail of transformation by (٨) antibiotic resistance genes may be because of that the wild strain *P. aeruginosa* P٢٧ was sensitive to each of amikacin, ciprofloxacin, and streptomycin after screening of antibiotic susceptibility test, and antibiotic resistance genes of lincomycin was previously indicated that they encoded by chromosomal DNA, while the sensitivity among each of gentamycin, tetracycline, and trimethoprim may be due to the fact that they encoded by large single plasmid or chromosomal DNA.

The important note here was that the *P. aeruginosa* P٢٢ and P٢٧ isolates when they were transformed to *E. coli* MM ٢٩٤ the ability to protease production was lost i.e (they was unable to produce proteases enzyme). This drives us to conclude that the genes responsible for protease production may be located on the chromosomal DNA or encoded by

megaplasmids, which was previously proved by (Michael and Iglewski, ١٩٩١), protease (elastase) gene was located on chromosomal DNA rather than on plasmid DNA.

Table (٤-٣) shows also that there are differences among isolates regarding the transformation frequencies, the highest recording for *P. aeruginosa* P١٦ (٥٦ x ١٠$^{-٤}$), P٢٧ transferred at low frequency (٤٢ x ١٠$^{-٤}$), and (٢٥٥ x ١٠$^{-٥}$), (٤٩ x ١٠$^{-٤}$) were recorded for P٤ and P٢٢ respectively. These plasmids may be due to the size of transferred plasmid; small circle plasmids are transferred much more efficiently than large circle (Hardy, ١٩٨٦). The transformation frequency for each isolate is not too high. This may be related to nicking of the prepared plasmid DNA, but it is still active. Figure (٤-٤) shows the plasmid profile of *E. coli* P١٦ and *E.coli* P٢٢, and it appears that these plasmid have approximately the same molecular weight (Similar size), which supports the foundation above. We can also conclude that antibiotic resistance plasmids of *P. aeruginosa* had been transformed successfully to *E. coli* MM ٢٩٤ strain.

The plasmid DNA of *P. aeruginosa* P١٥ was not successfully transferred even after repeating the transformation process several times. The large size of the plasmid may have been exposed to breakage during their preparation. This could be considered as a reason for the failure of transformation of those isolates (Hardy, ١٩٨٦). We can conclude that the laboratory *E. coli* MM ٢٩٤ strain treated with $CaCl_٢$ can represent an efficient host for a commendation of the plasmid DNA transfer of *P. aeruginosa* (Kheder, ٢٠٠٢). In addition, the preparation of plasmid DNA by cesium chloride-Ethidium bromide centrifugation can increase the transformation frequency because the plasmid in *P. aeruginosa* exists in a super coil state (Hardy, ١٩٨٦), and these facilities are not available in our laboratories to perform this technique.

Transformant MM ٢٩٤ colonies were screened for pyocyanin production (the main characteristics of this species). After incubation for several days at ٣٧°C, no pigment was observed to produce. This demonstrated that the genes responsible for pigmentation in *P. aeruginosa* are located on chromosome, and this agrees with previous reports of (Laird *et al*., ١٩٨٠; and Bindereif and Neil ands, ١٩٨٣).

٤-٦ The conjugative ability of plasmid DNA in *P. aerurginosa* isolates

Conjugation of plasmid is an unconventional mode of sexual mating which involves direct cell to cell contact in which plasmids or other genetic materials are transferred from donor to a recipient cell via a specialized appendage (Talaro and Talaro, ١٩٩٦). It occurs primarily in gram-negative bacteria. The donor cell possesses a plasmid (fertility, or F factor) allowing it to synthesize a set of pili or conjugative pilus (Moat *et al.,* ٢٠٠٢*).*

The conjugation process was done in order to study the plasmid content profile of each *P. aeruginosa* (P٩, P١٥, P١٨, P٢٢, and P٢٦ isolates), and to find whether the plasmids encoded of drug resistance is conjugative or non-conjugative, up on mating of *P. aeruginosa* (P٩, P١٥, P١٨, P٢٢, and P٢٦ isolates) with the *E.coli* MM٢٩٤ the frequency of transconjugant colonies were (٨٨x١٠$^{-٤}$, ٤٥٥x١٠$^{-٥}$, ١٢٢x١٠$^{-٤}$, and ١٥٤x١٠$^{-٤}$) respectively selected on the selection media. The isolates of *P.aeruginosa* used were sensitive to rifampicin, and resistant to ampicillin and streptomycin which are used as a genetic marker.

All attempts failed to select transconjugant up on mating of *P. aeruginosa* P١٨ isolate with *E.coli* MM٢٩٤, no transconjugant colonies were obtained on Muller hinton agar plates containing ampicillin, streptomycin, and rifampicin; that means the plasmid contents of *P. aeruginosa* P١٨ isolate is non conjugative plasmid. Failure of obtaining transconjugant colonies may be due to their lack to one of the conjugation requirements in the donor strain (*mob* genes, *bom* sequence and formation of conjugation bridge). These results were confirmed by (Sagias *et al*., ١٩٧٥), who found that among eleven clinical isolates of *P. aeruginosa* the plasmid contents of three of them were untransmissible to *E. coli* .Also Pallilo and Salleh (١٩٩٢) demonstrated that the efficiency of transfer was far lower than in similar types of transfer by *E. coil* strains. No attempts were made in this study to transfer the supposed Pseudomonas RTF to sensitive strains of *P. aeruginosa*.

Similarly, Grinsted *et al*. (١٩٧٤) showed that an *E. coil* strain could transfer its RTF to drug-sensitive *P. aeruginosa* strains at low frequency.

The results shown in Table (٤-٤) indicate that mating has occurred between *P. aeruginosa* P٩, P١٥, P٢٢, and P٢٦ isolates and *E.coli* MM٢٩٤ and the frequency of transconjugant colonies obtained was ٨٨x١٠$^{-٤}$, ٤٥٥x١٠$^{-٥}$, ١٢٢x١٠$^{-٤}$, and ١٥٤x١٠$^{-٤}$ respectively.

The growth of transconjugant colonies was tested on a Muller hinton agar containing carbencillin, chloramphenicol, erythromycin, gentamycin, lincomycin, tetracycline, and trimethoprim separately. The transconjugant colonies for *P. aeruginosa* P٢٢ and P٢٦ isolates with *E. coil* MM٢٩٤ appeared sensitive to carbencillin and erythromycin and to erythromycin respectively. Sensitivity of all tested transconjugant colonies to the above antibiotics used may be due to the location of the antibiotic resistance genes either on the non-transmissible plasmid or on the chromosome of *P. aeruginosa* isolates.

Table (٤-٤): Conjugation processes between *P. aeruginosa* proteases producing and non proteases producing strains, and *E.coli* MM٢٩٤.

		Screening for antibiotic susceptibility test			Proteases production	Selective medium for transconjuagnt (Ap+Sm+Rif)	Frequency of conjugation
		Ap	Sm	Rif			
Donor	*P.aeruginosa* P٩	+	+	-	-		
Recipient	*E.coli* MM٢٩٤	-	-	+	-		
Transconjugant	*E.coli* MM٢٩٤ P٩	+	+	+	-	١٧٦	٨٨x١٠$^{-٤}$
Donor	*P.aeruginosa* P١٥	+	+	-	-		
Recipient	*E.coli* MM٢٩٤	-	-	+	-		
Transconjugant	*E.coli* MM٢٩٤ P١٥	+	+	+	-	٩١	٤٥٥x١٠$^{-٥}$
Donor	*P.aeruginosa* P١٨	+	+	-	+		
Recipient	*E.coli* MM٢٩٤	-	-	+	-		
Transconjugant	*E.coli* MM٢٩٤ P١٨	No transconjugant colonies obtained			-		
Donor	*P.aeruginosa* P٢٢	+	+	-	+		
Recipient	*E.coli* MM٢٩٤	-	-	+	-		
Transconjugant	*E.coli* MM٢٩٤ P٢٢	+	+	+	-	٢٤٤	١٢٢x١٠$^{-٤}$
Donor	*P.aeruginosa* P٢٦	+	+	-	+		
Recipient	*E.coli* MM٢٩٤	-	-	+	-		
Transconjugant	*E.coli* MM٢٩٤						

	P٢٦	+	+	+	-	٣٠٨	١٥٤x١٠$^{-٤}$

* The *E.coli* MM٢٩٤ Rif^{r} recipient cell concentration was ٢ x ١٠^{٦} cell/ml.

* The symbols (+): Resistance to Antibiotics & proteases producer, (-): Sensitive to Antibiotics & non proteases producer.

* Ap: ampicillin, Rif: rifampicin, Sm: streptomycin.

From these studies, it is concluded that the transfer of drug resistance between enterobacteria and *P. aeruginosa* may take place in nature. However, it should be pointed out that there is as yet insufficient evidence to prove that RTF is involved in this transfer, although the circumstantial evidence suggests this to be likely.

Chakrabarty, (١٩٧٦) classified plasmids in *Pseudomonas* to P١, P٢, P٣ and other groups. Plasmid belonging to group (P١) can be transferred between varieties of gram negative including *E.coli*, while P٢ and P٣ are transmissible among *Pseudomonas* species but not *E.coli* constitute the P٢ compatibility group.

On the other hand, all the transconjugant bacterial colonies obtained from a crosses with *E.coli* MM٢٩٤ strain show resistance to the antibiotic lincomycin; the interpretation for this foundation involves that the *P. aeruginosa* isolates (act as a donor) contain plasmid called R٦٨.٤٥ (a derivative of R٦٨). This plasmid is able to mobilize the bacterial chromosome from many origins (Haas and Holloway, ١٩٧٦), and may be the Lin resistance gene included in this transfer. In addition, also all the transconjugant bacterial colonies obtained in this process show inability to produce the proteases, by those results we indicate that the genes responsible for proteases may be located on the chromosome or on the non-transmissible plasmids; this agrees with the results demonstrated by (Michael and Iglewski,١٩٩١), who observed the protease genes encoded by chromosomal DNA.

Figure (٤-٥) shows the plasmid profile of transconjugant cells of *E.coli* MM٢٩٤ acting as recipient through conjugation processes with *P. aeruginosa* (P٩, P١٥, P٢٢, and P٢٦ isolates). These plasmid may be considered as self-transmissible and of different size, and they support the results above. Jacoby (١٩٧٧) determined resistance to Amp, Gm, Sul, Tri and $HgCl_{٢}$) which were non-conjugative in *P. aeruginosa* and found that a relatively small amount of chromosome are transferred, while Hardy (١٩٨٦) elucidated that R١٠٠ plasmid in *E.coli* which conferred resistance to antibiotic including Tetracycline comprises all the genes necessary for conjugation.

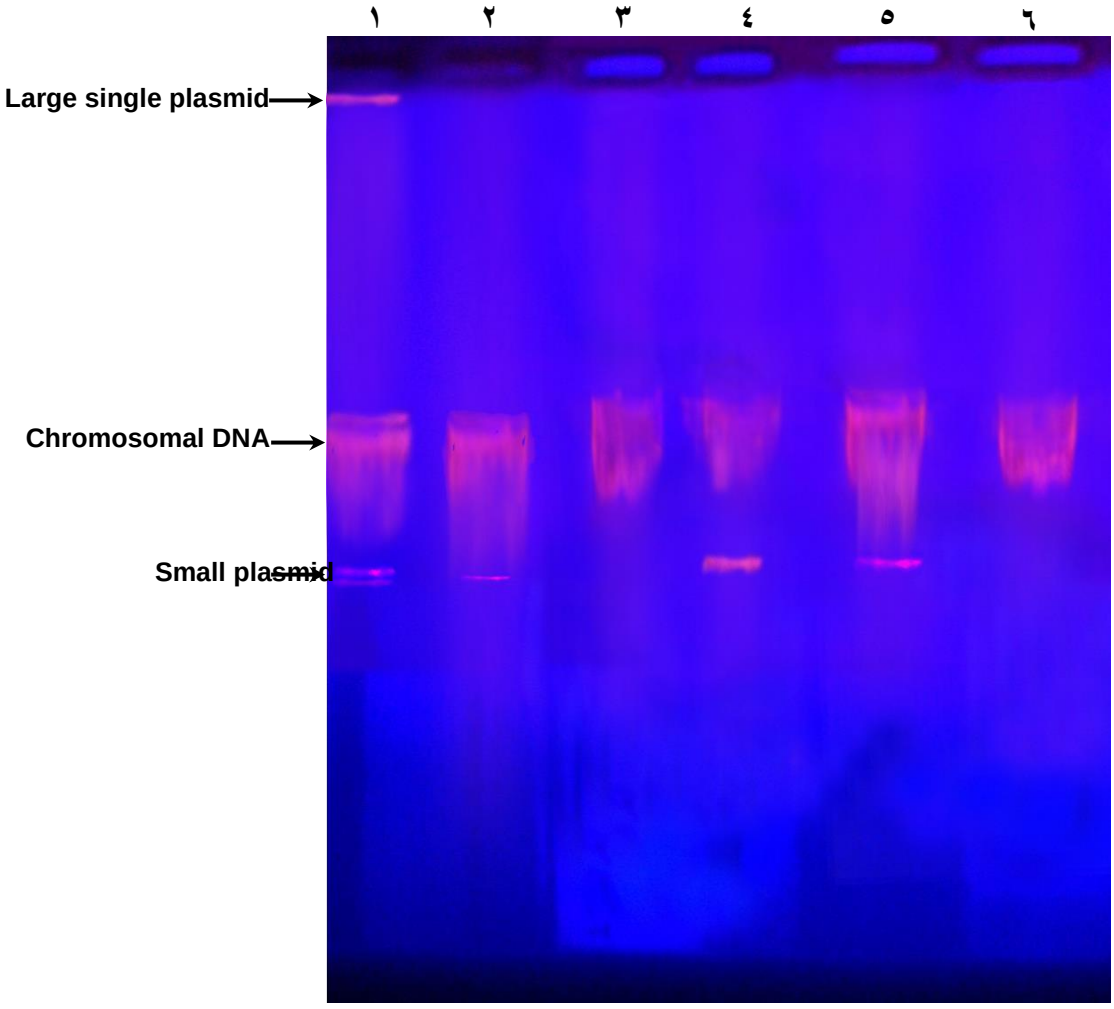


Figure (٤-٥): The plasmid profile of the transconjugant bacterial cells of *E. coli* MM٢٩٤.

The total DNA extracted by salting out (Pospiech and Neuman, ١٩٩٥) and migrated on agarose gel ٠.٧٪, ٥٠ volt, for ٦ hr.

- Lane ١: DNA content of proteases producing *P. aeruginosa* P٢٢ isolate
- Lane ٢: DNA content of transconjugant *E.coli* P٢٢ isolate that contain conjugative plasmid.
- Lane ٣: DNA content of standard plasmidless strain *E.coli* MM٢٩٤.
- Lane ٤: DNA content of non-proteases producing *P. aeruginosa* P٩ isolate.
- Lane ٥: DNA content of transconjugant *E.coli* P٩ isolate that contain conjugative plasmids.
- Lane ٦: DNA content of standard plasmidless strain *E.coli* MM٢٩٤.

Pigment production was not observed in transconjuagnt colonies when plated on nutrient agar supplemented with (Rif and Sm) or (Am and Sm) as a genetic marker. Therefore the transconjugant colonies were screened for pyocyanin pigment production by transferring some colonies of transconjugant colonies approximately ٢٠ colonies, belonging to each conjugation cross on minimal agar medium supplemented with glucose or glucose and final concentration of Amp incubated at ٣٧°C for ٢٤ hours.

We observed the pigment production by all tested transconjugants either in the presence of Amp or without Amp. The production of pyocyanin in the transconjugant colonies could be related to the presence of the plasmid R٦٨.٤٥ carrying the genes encoding Amp, Gm and Tc resistances. This plasmid is available in the *P. aeruginosa* isolates which can be transmissible to the genes responsible for pigment production together with Lin resistance gene which may be adjacent to the position of integration of this plasmid in the chromosome, and transferred to the MM٢٩٤ strain (Hardy, ١٩٨٦). After screening and plating all transconjugant colonies on skimmed milk agar the results revealed that all transconjugant colonies were unable to produce proteases; these observations may be due to the protease producing genes encoded by chromosomal DNA and this finding agrees with that indicated by (Michael and Iglewski ١٩٩١).

٤-٧ Curing experiments among different *P. aeruginosa* isolates

An alarming increase in resistance of *Pseudomonas* spp. to various antimicrobial agents has been reported by many workers (Paul *et al*., ١٩٩٢), but studies demonstrating the relation of plasmid and drug resistance in clinical isolates of *P. aeruginosa* by curing experiments is scanty in our country. On the other hand, we carried out this experiment to know the source of proteases productions gene that may be encoded chromosomally or

located on the plasmid DNA. In general curing experiments were achieved by two ways as follows:

٤-٧-١ Spontaneous curing.

Spontaneous curing of the plasmid DNA content for *P. aeruginosa* P٣, P١٩, and P٢٧ isolates were performed according to Meyer (١٩٧٤). No spontaneously losses of antibiotic resistance genes were obtained for any of the tested isolates.

These results of resistance may be due to the ability of antibiotics resistance plasmids in these isolates to segregate regularly and are stable within them whether antibiotics present or absent. Hardy (١٩٨٦) reported that the plasmid appeared to have evolved particularly in a genious way of increasing its stability, through decreasing cell division. On the other hand, after testing the isolates we noted that the ١٢ colonies of *P. aeruginosa* P١٩ isolates were lost after subculturing on different antibiotics; this may be due to spontaneous curing of plasmids among the colonies during different subculturing; this agrees with the results obtained by Davis and Smith (١٩٧٨), who found that many resistance plasmids are unstable and may be rapidly lost by most members of bacterial population in the absence of antibiotics.

Also Barkay and Colwell (١٩٨٣) demonstrated that spontaneous curing resulted in loss of plasmid resistance to mercury in *P. fluorescense*. Finally Snyder and Champness (١٩٩٧) explained that since cells are seldom cured of even low-copy number plasmids, some mechanism must ensure that plasmids, especially those with low copy numbers, will be partitioned faithfully into the daughter cells each time the cell divides.

٤-٧-٢ Curing with chemical agents.

Three plasmid curing agents, Sodium dodecyle sulphate (SDS) and Ethidium bromide (EB) were used to cure plasmid DNA that confer the antibiotic resistance in the *P. aeruginosa* isolates.

٤-٧-٢-١ Curing by Sodium dodecyle sulphate (SDS).

Curing experiments with different concentrations of SDS were performed on the Pseudomonal isolates to determine changes in plasmid content associated with antibiotic resistance pattern.

Table (٤-٥): Curing of plasmid DNA of *P. aeruginosa* isolates by ١٪ SDS with different incubation times.

Antibiotics	Antibiotic susceptibility test of *P.aeruginosa* isolates			Screening of cured isolates by antibiotic susceptibility test								
				٢٤ hours			٤٨ hours			٧٢ hours		
	P٣	P١٩	P٢٧	P٣	P١٩	P٢٧	P٣	P١٩	P٢٧	P٣	P١٩	P٢٧
Ap	+	+	+	+	+	-	+	-	-	+	-	-
Ak	+	+	-	-	-	-	-	-	-	-	-	-
Car	+	+	+	-	-	-	-	-	+	-	-	-
Cm	+	+	+	-	+	+	-	+	-	-	+	+
Ery	+	+	+	+	+	-	+	+	+	+	+	+
Gm	-	-	-	-	-	-	-	-	-	-	-	-
Lin	+	+	+	+	+	+	+	+	+	+	+	+
Sm	+	+	-	-	-	-	-	+	-	+	+	-
Tc	+	+	+	-	+	+	-	+	+	-	+	-
Tri	+	+	+	+	+	-	+	+	-	+	+	-
Proteases	-	+	+	-	+	+	-	+	+	-	+	+

* The symbols (+): Resistance to Antibiotics & proteases producer, (-): Sensitive to Antibiotics & non proteases producer.

* Ap: ampicillin, Ak: amikacin, Car: carbencillin, Cm: chloramphenicol, Ery: erythromycin, Gm: gentamycin, Lin: lincomycin, Sm: streptomycin, Tc: tetracycline, and tri: trimethoprim.

Table (٤-٥) shows the effect of SDS ١٪ (w/v) as curing agent on the plasmid DNA of *P. aeruginosa* P٣, P١٩, P٢٧ isolates with three incubation times ٢٤, ٤٨, and ٧٢ hours. Table (٤-٥) demonstrates that the effect of SDS on antibiotic resistance plasmid DNA differed with different isolates and the incubation times used.

The results demonstrated that the resistance to Lincomycin was remaining for all isolates and during three times of incubation, and Trimethoprim resistance in *P. aeruginosa* P٣ and P١٩ isolates was not affected by ١٪ SDS during all times of incubation. In addition, Ampicillin resistance in *P. aeruginosa* P٣ isolates also was not affected by ١٪ SDS during different incubation times. However, *P. aeruginosa* P٣ and P١٩ isolates were not cured by ١٪ SDS and showed their still resistance to erythromycin during all incubation times. Elsewhere, the ability to protease production remains active among P١٩ and P٢٧ during (٢٤, ٤٨, and ٧٢) hours of incubation period, after selection on skimmed milk agar pates the results indicate that the genes responsible for protease production may be located on the chromosomal DNA of *P. aeruginosa* isolates under study, and this results agree with these obtained by Guzzo *et al*. (١٩٩٠). In addition, Michael and Iglewski (١٩٩١) documented that proteases gene encoded by the chromosome in *P. aeruginosa*.

On the other hand, bacterial colonies appeared to be sensitive to each of Amikacin and Gentamycin for all tested isolates and during all incubation times. It was revealed that the isolates cured for amikacin and gentamycin resistances. The SDS treated colonies were tested on carbencillin and streptomycin separately, and the results appeared that all isolates were sensitive with different rates; in addition, SDS affected other antibiotic resistance genes from all isolates with different rate including ampicillin, chloramphenicol, tetracycline, and trimethoprim.

The results achieved by SDS treatment revealed that the *P. aeruginosa* isolates respond in different rate to ١٪ SDS, and this may be related to the permeability through outer membrane, and to the location of antibiotic resistance genes. Sonstein and Baldwin (١٩٧٢) elucidate that the effectiveness of SDS may be related to plasmid copy number, or amount of enzyme which inactivate antibiotics. This conclusion is supported by Ingram *et al*. (١٩٧٢) who mentioned that amount of β-lactamase produced by cured strain was only ٤٠٪ - ٦٠٪ of the β-lactamase produced by fully resistance strain.

Agarose gel electrophoresis in Figure (٤-٦) of the cured isolates shows that loosing small plasmid is performed in *P. aeruginosa* P٢٧ isolates after being treating with ١٪ SDS; small

plasmid DNA was cured among *P. aeruginosa* P١٩ isolates by ١٪ concentration of SDS. This result documented that two megaplasmids remain after curing in both *P. aeruginosa* P٢٧ and P١٩ isolates. Furthermore, one small plasmids among two plasmid DNA were cured by using ١٪ SDS among strain *P. aeruginosa* P٣ isolates during all incubation times. The cured plasmid may be the R-plasmid which harbors most of antibiotics resistance genes.

Adachi *et al*., (١٩٧٢) founded that SDS was only effective in elimination of sex (F) and R-plasmids in *E. coli* in a high frequency at concentration higher than ١٪, and reported that the longer incubation times (٢٤ to ٧٢hr), higher the frequency of sensitive

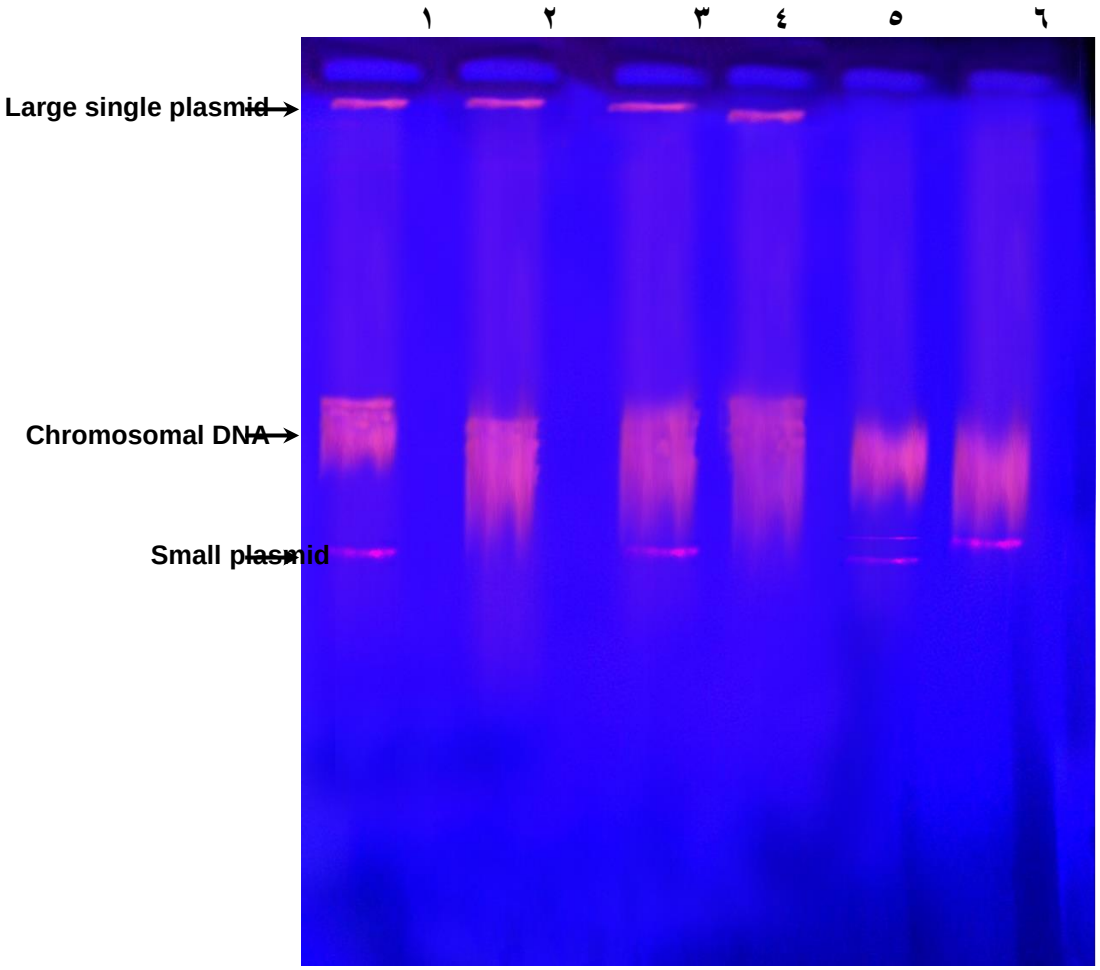


Figure (٤-٦): The plasmid profile of *P. aeruginosa* isolates by curing with Sodium Dodecyle Sulfate (SDS).

The total DNA plasmid extracted by salting out (Pospiech and Neuman, ١٩٩٥) and migrated on agarose gel ٠.٧٪, ٥٠ volt, for ٦ hr.

- Lane ١: DNA content of proteases producing *P. aeruginosa* P٢٧ isolate
- Lane ٢: DNA content of cured *P.aeruginosa* P٢٧ isolate, showed that small plasmid was cured by SDS.
- Lane ٣: DNA content of proteases producing *P. aeruginosa* P١٩ isolate.
- Lane ٤: DNA content of cured *P.aeruginosa* P١٩ isolate, showed that small plasmid was cured by SDS.
- Lane ٥: DNA content of non-proteases producing *P. aeruginosa* P٣ isolate.
- Lane ٦: DNA content of cured *P.aeruginosa* P٣ isolate**,** showed that only one plasmid among two small plasmids were cured by SDS.

isolates. Moreover, Trevors (١٩٨٦) demonstrated that mucoid cells are less permeable than unmucoid, and since SDS is known to cause the disruption of biological membrane, so that possibility exists that SDS act as curing agent for plasmid conferring antibiotic resistance, by disrupting the membrane sites of plasmid attachment. Al-Saffawi (٢٠٠١) reported that the SDS affected the antibiotic resistance genes in *Staphylococcus aureus* isolates from different environments; and these genes, encoding to ampicillin, gentamycin, and streptomycin resistances with ١٠٠٪ and penicillin genes with ٦٥٪ when used at concentration of ٠.٠٠٢٪ with elevated temperature , while some concentration fail as curing agent for most tested isolates.

Kheder (٢٠٠٢), obtained that the SDS of ١٪ concentrations affected the antibiotic resistance genes in *P. aeruginosa* isolated from the human infections, curing occurred among four isolates for eleven tested antibiotics for ٢٤, ٤٨, and ٧٢ hours of incubation and by different rates.

٤-٧-٢-٢ Curing by Ethidium bromide.

Ethidium bromide (EB) was used as a curing agent; the method described by (Shahid, ٢٠٠٤) was used. The minimal inhibitory concentration of ethidium bromide was determined for the bacterial isolates in trypticase soy broth (TSB) and the highest concentration permitting growth was used for plasmid curing.

Table (٤-٦): Curing of plasmid DNA of *P. aeruginosa* isolates by ٧٠٠µg/ml of Ethidium bromide in different incubation times.

Antibiotics	Antibiotic susceptibility test of *P.aeruginosa* isolates			Resistance to antibiotics					
				٢٤ hours			٤٨ hours		
	P٣	P١٩	P٢٧	P٣	P١٩	P٢٧	P٣	P١٩	P٢٧
Ap	+	+	+	+	+	+	+	+	+
Ak	+	+	-	-	+	-	-	+	-
Car	+	+	+	-	-	-	-	-	-
Cm	+	+	+	+	+	+	+	+	+
Ery	+	+	+	+	+	+	+	+	+
Gm	-	-	-	-	-	-	-	-	-
Lin	+	+	+	+	+	+	+	+	+
Sm	+	+	-	+	+	-	+	+	-
Tc	+	+	+	+	-	-	+	-	-
Tri	+	+	+	+	+	+	+	+	+
Proteases	-	+	+	-	+	+	-	+	+

* The symbols (+): Resistance to Antibiotics & proteases producer, (-): Sensitive to Antibiotics & non proteases producer.

* Ap: ampicillin, Ak: amikacin, Car: carbencillin, Cm: chloramphenicol, Ery: erythromycin, Gm: gentamycin, Lin: lincomycin, Sm: streptomycin, Tc: tetracycline, and tri: trimethoprim.

Table (٤-٦) shows the effect of EB at concentration of ٧٠٠µg/ml as a curing agent against *P. aeruginosa* P٣, P١٩, and P٢٧ isolates at different incubation times (٢٤, and ٤٨ hours). The result shows that the ethidium bromide has no effect on curing of plasmid DNA carrying the ampicillin, chloramphenicol, erythromycin, lincomycin, and trimethoprim resistance genes, for all tested isolates, and at different incubation times used.

The results demonstrated the EB effect the plasmid DNA carrying each of Carbencillin and Gentamycin resistance genes from all isolates at all incubation times, while the effect of EB on plasmids DNA that confer resistance to Amikacin, Streptomycin, and Tetracycline appeared in different rate, so that in Amikacin only plasmid DNA of *P. aeruginosa* P٣ and P٢٧ isolates respectively were cured (sensitive) during incubation times. Elsewhere, ethidium bromide was unable to affect the protease production genes during the two incubation times and by different concentrations; from that result, again we think that the proteases gene may be located on chromosome of *P. aeruginosa* under studies, and our results re-confirmed (agree) with that obtained by Guzzo *et al*. (١٩٩٠).

It is worth mentioning, that the selection occurred on (skimmed milk agar) plates during (٢٤ and ٤٨) hours of incubation. In general, EB affect the plasmid DNA containing genes encoding to amikacin, carbencillin, gentamycin, streptomycin, and tetracycline resistances with various rates, the antibiotic resistance genes may be located on low copy number plasmid; this agrees with Keyser *et al*. (١٩٨٢) who reported that low copy number plasmid was efficiently cured by EB.

Additionally, the effects of time increasing on cell exposures to EB, result in decreasing the sensitivity against the antibiotic used. Hohn and Korn, (١٩٦٩) indicated that the agents causing complete inhibition of plasmid replication like Acridine orange and Ethidium bromide, intercalate between base pairs in DNA. EB also cause plasmid loss by inhibiting plasmids replication, but in the more EB sensitive cells, by banding of EB to DNA and RNA and inhibition of DNA polymerase. Furthermore, Waring (١٩٦٦) and, Hudson and Vinograd (١٩٦٧) suggested that differences in DNA polymerase and RNA polymerase are responsible for differences in EB sensitivity to bacterial strains due to differences in the rate of agent's penetration in different strain of Enterobacteriaceae.

Rubins and Rosenblum, (١٩٧١) speculated that further exposure to EB the rate of elimination decreased and resistance to EB increased, and resistance levels tended to increase slightly after ٢٤ hours of growth in EB. This finding agrees with our obtained results. The previous table showed those plasmids that contain some antibiotic resistance genes in the isolates were not eliminated with EB such as ampicillin, chloramphenicol, erythromycin, and trimethoprim resistances for all tested isolates, and amikacin, streptomycin, and tetracycline resistances for some isolates. This could be due to high copy number of these plasmids in the isolates; this agree with that documented by Pallida *et al*. (١٩٩٢) who demonstrated that in *P. aeruginosa* the percent of cured plasmid DNA is not more than ٢٠٪ in optimal conditions. Al-Saffawi (٢٠٠١) explained that the effect of EB on the antibiotic resistance plasmids in *S. aureus* isolated from the different environments, differed with different isolates, while some isolates were not affected by the EB treatment.

٤-٧-٣ Curing by physical agents.

٤-٧-٣-١ Curing by elevated temperature.

Elevated temperature (٤٦°C) was used to achieve curing among the plasmid DNA that confer resistance to antibiotics from *P. aeruginosa* P٣, P١٩, and P٢٧ isolates.

The results showed that all treated isolates appear sensitive to all antibiotics except lincomycin. On the other hand, the results in Table (٤-٧) speculated that some resistance which appeared among few antibiotics by different isolates; *P. aeruginosa* P٣ isolates were resistant to carbencillin, while for *P. aeruginosa* P١٩ isolates were resistance to chloramphenicol, and trimethoprim. Elsewhere, the production of proteases by the *P. aeruginosa* P١٩ and P٢٧ isolates remain active after expositing to elevated temperature (٤٦°C), by those results we conclude that the genes of proteases production may be encoded by chromosome in *P. aeruginosa* isolates; this agrees with that observed by (Michael and Iglewski, ١٩٩١).

In Figure (٤-٧) agarose gel electrophoresis of the curried isolates showed that loosing all small plasmids was performed among three tested *P. aeruginosa* P٣, P١٩ and P٢٧ isolates after being treated with elevated temperature (٤٦°C) and during all incubation times. The cured plasmids may be the R-plasmid which harbors most of antibiotic resistance genes. Furthermore, this result documented that two megaplasmids also were cured after curing with elevated temperature (٤٦°C) in both *P. aeruginosa* P٢٧ and P١٩ isolates.

From the obtained results, a conclusion can be made that curing by elevated temperature is the most efficient method among others. This may be due to the fact that the enzymes which contribute in the DNA replication processes are more affected by this high temperature (Trevors, ١٩٨٦). Our interpretation involves changing the shape (folding of the polypeptide) of the enzymes responsible for the DNA replication of plasmids DNA which are available in the *P. aeruginosa* isolates including the plasmids that confer resistance to the antibiotics. This change in the folding of DNA polypeptide may convert the enzymes into the inactive form at this temperature, i.e. the enzymes are sensitive to elevated temperature (Kheder, ٢٠٠٢).

Table (٤-٧): Curing by elevated temperature (٤٦°C) among plasmid DNA of *P. aeruginosa* isolates.

Antibiotics	Antibiotic susceptibility test of *P.aeruginosa* isolates (**before** exposing to **٤٦°C**)			Screening of cured isolates by antibiotic susceptibility test(**after** exposing to **٤٦°C**)		
	P٣	P١٩	P٢٧	P٣	P١٩	P٢٧
Ap	+	+	+	-	-	-
Ak	+	+	-	-	-	-
Car	+	+	+	+	-	-
Cm	+	+	+	-	+	-
Ery	+	+	+	-	-	-
Gm	-	-	-	-	-	-
Lin	+	+	+	+	+	+
Sm	+	+	-	-	-	-
Tc	+	+	+	-	-	-
Tri	+	+	+	-	+	-
Proteases	-	+	+	-	+	+

* The symbols (+): Resistance to Antibiotics & proteases producer, (-): Sensitive to Antibiotics & non proteases producer.

* Ap: ampicillin, Ak: amikacin, Car: carbencillin, Cm: chloramphenicol, Ery: erythromycin, Gm: gentamycin, Lin: lincomycin, Sm: streptomycin, Tc: tetracycline, and Tri: trimethoprim.

Many researchers demonstrate the mechanism by which the elevated temperature creates curing of plasmids DNA, of these, Taylor (١٩٦٣) reported when the bacterial cells grow at temperature (٣٠°C) and shifted to elevated temperature, DNA synthesis occurs equal to approximately ٣٠٪ of the cellular contents of DNA. The effect of elevated temperature on plasmid curing may be due to decreasing the amount of DNA synthesized. Evans *et al*. (١٩٧٩) indicated that the blocking of protein synthesis at elevated temperature can affect the stability of previous synthesized DNA, and some strains appear to be more sensitive to these conditions than others.

Furthermore, enzymatic activity declines above the specific temperature that is characteristic of the heat stability of the particular enzyme (Hardy, ١٩٨٦). However, plasmids appear to be dependent on host enzymes for their replication, therefore, most of the proteins synthesized during changing (converting) of temperature might be utilized for cell division, by that, chance of plasmid replication decreases then curing occurred. The results obtained by elevated temperature indicate that the genes which are located on the chromosomal DNA of all tested isolates for example (proteases gene and Lincomycin resistance gene) were not affected by high temperature comparing with that encoded by plasmids DNA; our results agree with those obtained by Asheshor (١٩٦٦) who described that the chromosomal location of penicillin resistance gene was not eliminated by high temperature.

Previously few studies have been performed on the effect of temperature on the DNA synthesis and plasmid curing. May *et al*. (١٩٦٤) obtained high frequency of plasmid curing results after the growth of some strains at elevated temperature, and observed that tetracycline and penicillinase positive strain of *S. aureus* after growth at ٤٣ to ٤٤°C give rise to increasing proportion of tetracycline sensitive and penicillinase negative bacteria. Ike *et al*. (١٩٨٠) demonstrated more antibiotic sensitive cells after shifting the temperature from ٣٠ to ٤٠°C. Al-Amir (١٩٩٩) documented that there is a clear effect of elevated temperature on *P. aeruginosa* isolates plasmids, which agree with the results of the present study.

Al-Saffawi (٢٠٠٠) speculated that (٤٣°C) affects on genes are responsible for β-lactam antibiotic in *S. aureus* isolates, but in contrast some isolates remain their resistance to elevated temperature after curing experiments. Kheder (٢٠٠٢) found that the ٤٦°C affected on the antibiotic resistance plasmids DNA for four tested isolates and curing was obtained among them except for the genes responsible for Lin., because they are encoded chromosomally.

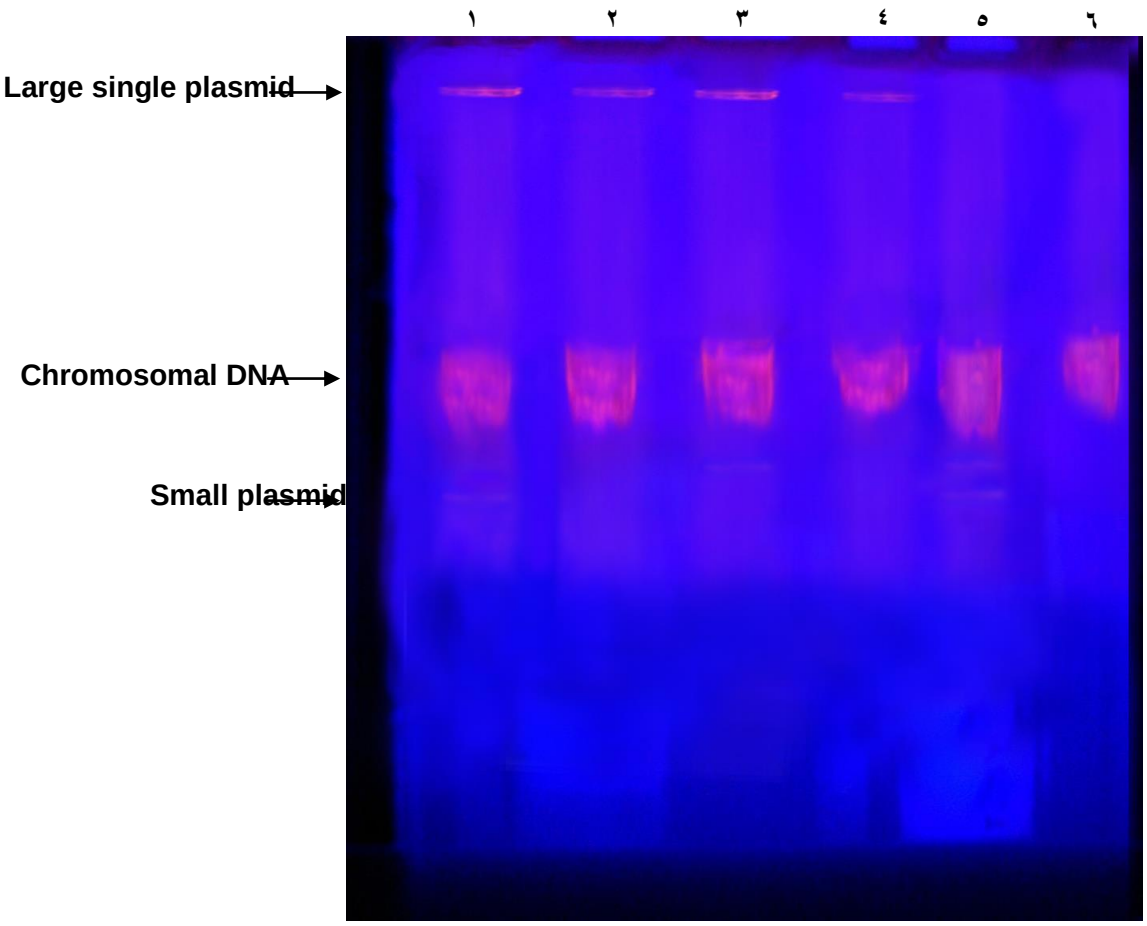


Figure (٤-٧): The plasmid profile of *P. aeruginosa* isolates by curing with Elevated temperature (٤٦°C).

The total DNA extracted by salting out (Pospiech and Neuman, ١٩٩٥) and migrated on agarose gel ٠.٧٪, ٥٠ volt, for ٦ hr.

- Lane ١: DNA content of proteases producing *P. aeruginosa* P٢٧ isolate
- Lane ٢: DNA content of cured *P.aeruginosa* P٢٧ isolate, showed that small plasmid was cured by SDS.
- Lane ٣: DNA content of proteases producing *P. aeruginosa* P١٩ isolate.
- Lane ٤: DNA content of cured *P.aeruginosa* P١٩ isolate, showed that small plasmid was cured by SDS.
- Lane ٥: DNA content of non-proteases producing *P. aeruginosa* P٣ isolate.
- Lane ٦: DNA content of cured *P.aeruginosa* P٣ isolate**,** showed that two small plasmids were cured by SDS.

٤-٨ Histopathological study of rat (skin and lung) s infected by *P. aeruginosa* and role of extracellular proteases.

In the present study, we examined the role of proteases enzyme in the pathogenesis and virulence of *P. aeruginosa* infection of burns. Several recent reports had demonstrated that proteases of *P. aeruginosa* play a great role in the virulence of this microorganism and are part of the pathogenic process in these infections.

Burned skin seems to be especially susceptible to infection by *P. aeruginosa* Nathan *et al*. (١٩٧٣). The pathogenesis of *P. aeruginosa* infections has been studied in experimentally produced burns in a burned mouse model Stieritz (١٩٧٥). From these studies it was demonstrated that the burned skin site was allowed for the initial colonization and proliferation of the organism in vivo. Haidaris and Michael, (١٩٧٨) who showed that mice immunized with elastase or its toxoid showed increased resistance to experimental *P. aeruginosa* infections, suggest that this protease contributes to the pathogenesis of the organism. Thus, it appears that protease is a virulence-enhancing factor rather than a major virulence factor. The data presented by Pavlovskis and Wretlind (١٩٧٩) found that and also indicated that to some extent these effects may be neutralized by antiprotease serum. These observations should provide a broader basis for studies leading to immunoprophylaxis and treatment of pseudomonas infections.


Papillary layer of the epidermis
Reticular layer

Figure (٤-٨): Histopathology of burned rat skin three days after subcutaneously injection with (١ml) of normal saline. (٤٠٠X) Skin sections:

- Both layers of skin (epidermis and dermis) are normal in morphology and structure.

٤-٨-١ Histopathological characterization of burned rat skin infected by protease producer & non-protease producer *P. aeruginosa* and *E. coli* MM٢٩٤.

The mechanism by which protease exerts its toxic action is not known. In septicemic infection, protease may contribute to tissue damage when it is produced locally by Pseudomonas. Iglewski *et al*. (١٩٧٨), for example, found protease in tissue homogenates from pseudomonas-infected mice (personal communication). Snell *et al*. (١٩٧٨) found that the subcutaneous injection of purified protease reduced elongation factor ٢ activity in mouse liver. However, previous work with nontoxigenic, protease-producing strains indicates that protein synthesis inhibition by protease during an infection is minimal Pavlovskis *et al*. (١٩٧٨) and that extremely high doses are required to kill mice as compared with toxigenic strains. It is unlikely; therefore, that protease by itself is responsible for the lethality of the organism.

In the present study, we have used the burned-rat models to examine the contribution of extracellular proteases to the pathogenesis of *P. aeruginosa* infections in burn wounds. Animals challenged subcutaneously with the proteases producing *P. aeruginosa* P٢٢ isolates, and incubation occurred at room temperature for ٧٢ hour (٣days), observed results showed that after histopathological examination of the rat skin lesions, demonstrated complete sloughing of epidermis, Sever congestion and thrombosis of dermal blood vessels and presence of large quantities of inflammatory exudates in the dermis. The papillary and reticular layer of the dermis can not be differentiated from each other, also we note damage or destruction of all of the skin appendages like (hair follicles, sebaceous gland……etc), Furthermore, destructions and folding of skeletal muscle , skin necrosis appeared in Figure (٤-٩) when compared with normal burned skin histology in Figure (٤-٨).

These events were documented by Meinke *et al*. (١٩٧٠) who found that studies consisting of the subcutaneous injection of purified protease (١.٤ to ١٤ units) resulted in the rapid destruction of skin and subcutaneous tissue at the injection site. Hemorrhagic lesions developed and became ulcerating and necrotic almost immediately when ١٤ units were administered, such lesions are characterized by rounded, ulcerated areas punctuated by black necrotic centers.

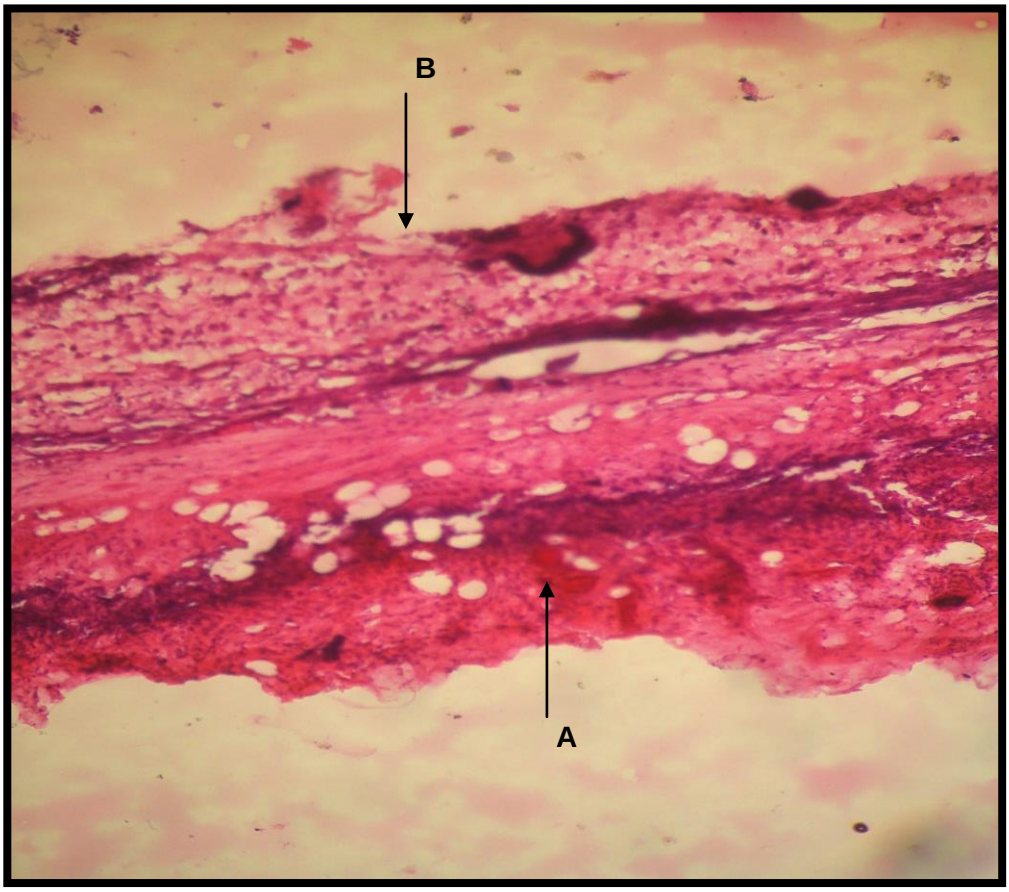


Figure (٤-٩): Histopathology of burned rat skin three days after subcutaneously injection with (١ml) of proteases producing *P. aeruginosa* P٢٢ isolate. (٤٠٠X)

Skin sections shows: Complete sloughing of epidermis, associated with severe congestion and thrombosis of dermal blood vessels (**A**), Presence of large quantities of inflammatory exudates in the dermis (**B**), and destruction of all of the skin appendages like (hair follicles, sebaceous gland……etc).

Since the subcutaneous route never elicited death or gross pathological changes of the internal organs, acute inflammation reactions were observed in skin sections. Also Pavlovskis and Wretlind (١٩٧٩) reported that proteases are the major cause of skin necrosis and destructions, they also suggested that protease may be important in overcoming the host's initial defense mechanisms. This may be accomplished either by proteolytic action which provides additional nutrients, or by destruction of anatomical barriers and more rapid invasion of the host. The latter possibility is indirectly supported by the work of Wretlind and Kronevi (١٩٧٨).

In the other hand, Cicmanec and Holder (١٩٧٩) mentioned that subcutaneous inoculation at the burned site of as few as ١٠ *P. aeruginosa* cells was ١٠٠٪ lethal to burned mice. Quantitative bacterial counts less than ١٠٥ cells per gram of tissue rarely indicate invasive infection. Counts exceeding ١٠٥ cells per gram are accurate in predicting invasive infection approximately ٥٠٪ of the time.

Snell *et al*. (١٩٧٨) showed that although infections with low inocula of *P. aeruginosa* PA-١٠٣ caused no reduction of EF-٢ levels in the livers of infected mice, significant reductions were observed when these inocula were supplemented with injections of small amounts of protease. Other studies using the same model have shown significant enhancement of mortality when strain PA-١٠٣ challenge was supplemented with protease or elastase compared with non-enzyme-supplemented challenge Holder and Haidaris (١٩٧٩). The studies cited above have established the role of protease as a virulence-associated factor in

P. aeruginosa infections. The mechanism by which proteases act as virulence factors remains unclear. We know, however, that proteases supply *P. aeruginosa* with small peptides of usable size which can be transported into the cell Sokatch (١٩٦٩). If we ask why *P. aeruginosa* is more virulent in a burned wound than other microorganisms is not completely known. It has been reported that unique products of *P. aeruginosa* such as exotoxin A Pavlovskis and Shakelford (١٩٧٤); Snell *et al*. (١٩٧٨) and proteases Holder and Haidaris (١٩٧٩); Pavlovskis and Wretlind (١٩٧٩); may contribute to its pathogenesis in burned skin.

To determine the capacity of non-proteases producing *P. aeruginosa* P٤ isolates to disseminate from skin and cause infection after subcutaneously inoculation in rat, we monitored animals for development of tissue necrosis, but after histopathological examination study of the rat skin lesions, in Figure (٤-١٠) we demonstrated that both layers of skin (epidermis and dermis) were normal in structure and morphology. There was no morphological effect by non-proteases producing *P. aeruginosa* P٤ isolates. In this study, we have used the burned rat model to examine the contribution of (proteases producing & non-proteases producing isolates) to the pathogenesis of *P. aeruginosa* infections in burned rat models. The event of low or no infection by non-proteases producing *P. aeruginosa* P٤ isolates may be because that these strains were unable to cause sever infection in burned rat model compared with the protease positive strains, and this confirmed the fact that proteases have been suggested as possible virulence factors for *P. aeruginosa* in the experimental burned rat models.

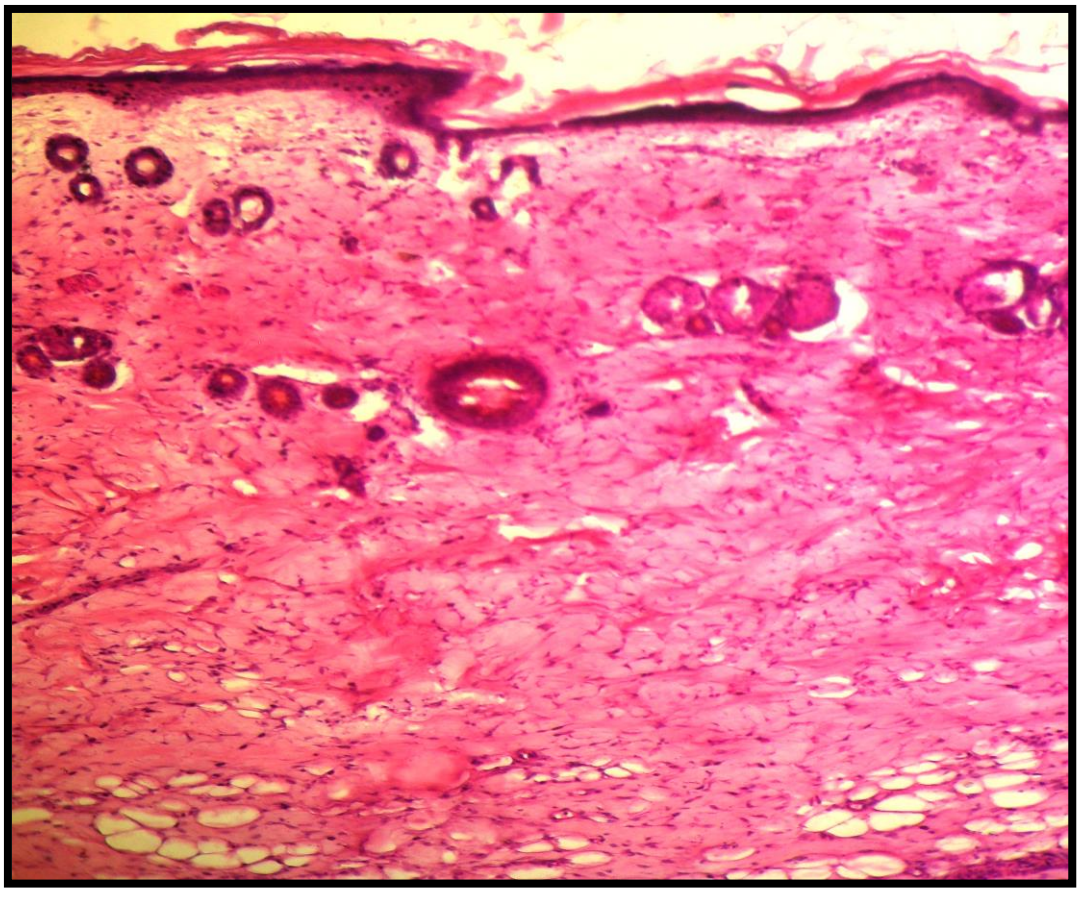


Figure (٤-١٠): Histopathology of burned rat skin three days after subcutaneously injection with (١ml) of non-proteases producing *P. aeruginosa* P٤ isolate. (٤٠٠X) Skin sections shows:

- Both layers of skin (epidermis and dermis) are normal in morphology and structure, which seen no affect by non-proteases producing *P. aeruginosa*.

This event was approximately consistent with that obtained by Rumbaugh *et al*. (١٩٩٩) who found that the defect in the spreading of *P. aeruginosa* within burned skin may contribute (directly or indirectly) to the observed general defect. The simplest explanation for this contribution is that a reduction in the horizontal spreading of *P. aeruginosa* within burned skin may cause a concomitant reduction in the numbers of the microorganisms that spread vertically (through the connective and lymphoid tissues underneath the burned skin). This may lead to a reduction in the number of microorganisms that are disseminated within the bodies of burned mice and an eventual reduction in invivo virulence. The pathogenesis of *P. aeruginosa* infection is attributed to the production of both cell-associated and extracellular virulence factors include exotoxin A, alkaline protease and elastase (Homma, ١٩٨٠; Berka *et*

al., ١٩٨١; Woods and Iglewski, ١٩٨٣; Nicas and Iglewski, ١٩٨٥; Gilligan, ١٩٩١). Burned skin wounds seem to be especially susceptible to infection by *P. aeruginosa* Nathan *et al*. (١٩٧٣).

The pathogenesis of *P. aeruginosa* infections have been studied in experimentally produced burns in a burned mouse model (Stiertz and Holder ١٩٧٥). Another study consistent with enhancement of virulence in proteases producing strains showed that in a burned mouse model fewer viable bacteria were found in the blood of animals infected with non-proteases producing *P. aeruginosa* than were found in the blood of animals infected with protease-producing strains Pavlovskis and Wretlind (١٩٧٩). In addition, antiprotease serum therapy enhanced survival of animals infected with protease-producing strains Cicmanec and Holder (١٩٧٩).

In the present study infection of rat skin by wild type of *E. coli* MM٢٩٤ achieved by subcutaneously challenge of rat skin with *E. coli* MM٢٩٤, and then histopathological study was examined, the result shown in Figure(٤-١١) revealed that both layers of skin (epidermis and dermis) were appeared normal in morphology and structures. These may be because of that this normal flora lack the virulence factors (the extracellular factor like proteases and elastases) contribute in the burn skin post infections or unable to lyses the tissue and then cause the necrosis, or the dosage was so low to cause the lethal infections. Therefore, the skin appeared in normal morphology and structures. This observation is consistent with those obtained by Cicmanec and Holder (١٩٧٩) who reported that infections by other organisms such as wild type of *E. coli* and *Klebsiella* spp. occurred only after subcutaneous ٩×١٠^{٧} organisms.>inoculation with

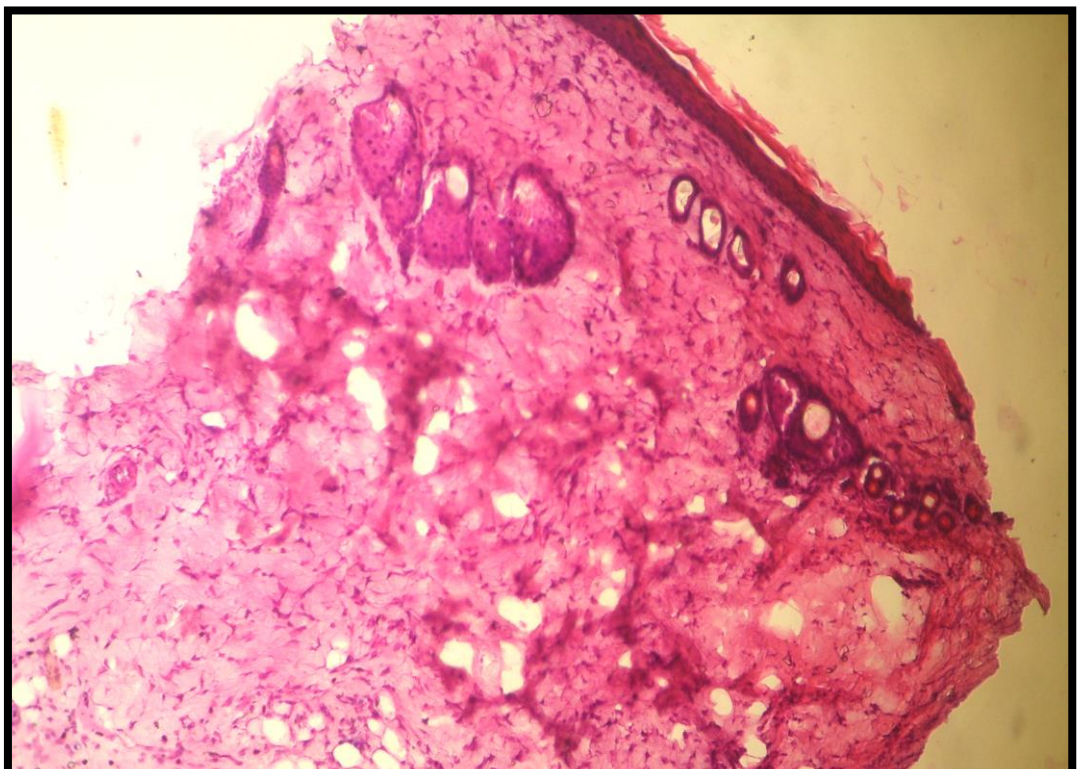


Figure (٤-١١): Histopathology of burned rat skin three days after subcutaneously injection with (١ml) of *E. coli* MM٢٩٤. (٤٠٠X)

- **Skin sections shows:** Both layers of skin (epidermis and dermis) are normal in morphology and structure, which seen no affect by *E. coli* MM٢٩٤.

٤-٨-٢ Histopathological characterization of rat lungs infected by proteases producing *P. aeruginosa* strains.

The process achieved was similar to that described previously; the animals were challenged subcutaneously by the proteases producing *P. aeruginosa* strains; the observed results showed that, macroscopically lungs analysis of the animals revealed the normal lungs in appearance and morphology, and histopathological examination of the rat lung lesions, we demonstrated sever congestion of blood vessels, inflammation by neutrophils, multifocal areas of emphysema and thickening of interstitial tissue due to infiltration of large numbers of inflammatory cells(polymorphonuclear neutrophils and mononuclear) leukocytes, occluding of many alveoli by inflammatory exudates and finally interstitial pneumoniaties figure (٤-١٣) when compared with normal histology of lungs (normal alveoli appearance) seen in figure (٤-١٢).

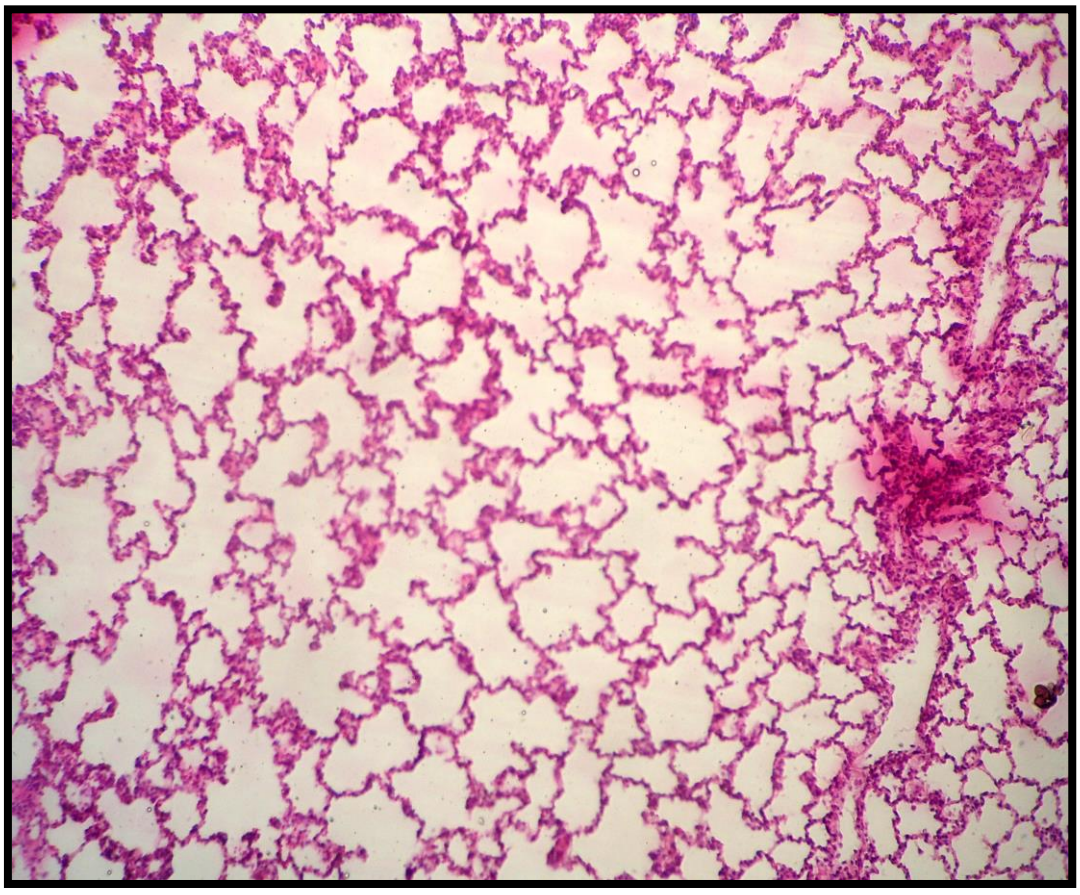
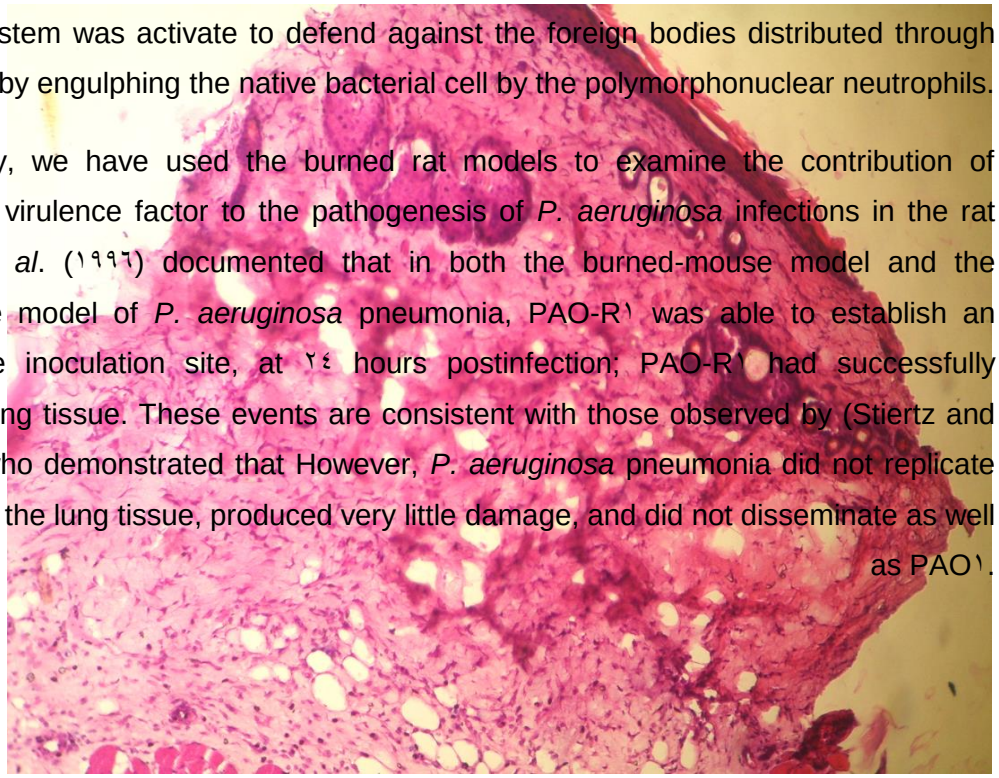


Figure (٤-١٢): Histopathology of rat lung three days after subcutaneously injection with (١ml) of normal saline. (٤٠٠X)

Lung sections shows:

- **Normal appearance of alveoli.**

These events may be due to the fact that pili play an a important role for the attachment to the respiratory tract and cause a moderate infections, but the degree of infection and pneumoniaties refers to that the dosage number of bacteria was not at the level to cause severe infections, also the ability of bacterial cells were few to attach the lung epithelial cells, and the duration of infection was limited to (٢-٣) days. Furthermore, this may be because that immune system was activate to defend against the foreign bodies distributed through lungs by engulphing the native bacterial cell by the polymorphonuclear neutrophils.



In this study, we have used the burned rat models to examine the contribution of proteases as a virulence factor to the pathogenesis of *P. aeruginosa* infections in the rat lungs. Tang *et al*. (١٩٩٦) documented that in both the burned-mouse model and the neonatal-mouse model of *P. aeruginosa* pneumonia, PAO-R١ was able to establish an infection at the inoculation site, at ٢٤ hours postinfection; PAO-R١ had successfully colonized the lung tissue. These events are consistent with those observed by (Stiertz and Holder, ١٩٧٥) who demonstrated that However, *P. aeruginosa* pneumonia did not replicate efficiently within the lung tissue, produced very little damage, and did not disseminate as well as PAO١.

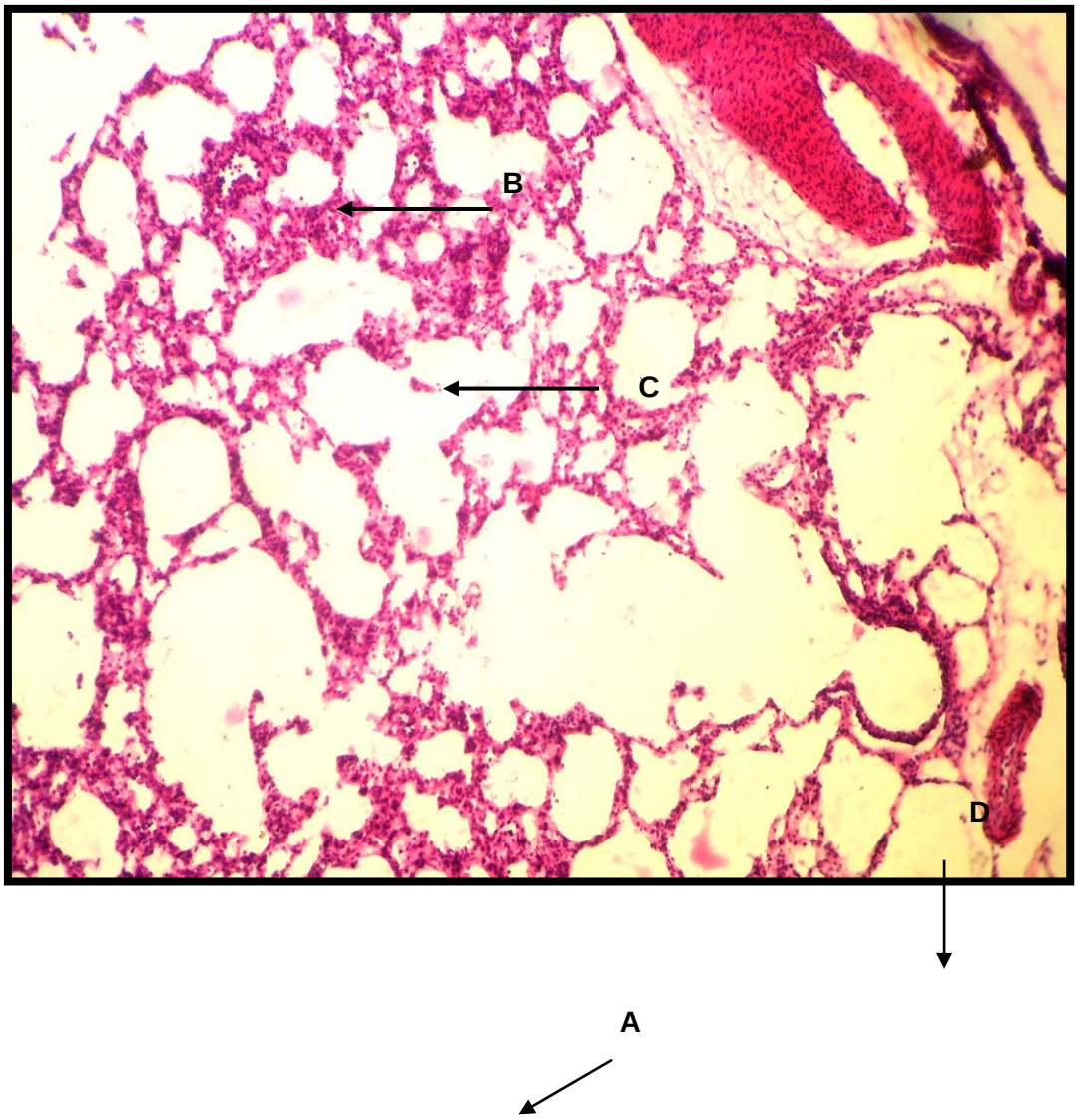

B
C
D
A

Figure (٤-١٣): Histopathology of rat lung three days after subcutaneously injection with (١ml) of proteases producing *P. aeruginosa* P٢٢ isolate. (٤٠٠X)

Lung sections show:

A. Multifocal areas of emphysema.

B. Thickening of interstitial tissue due to infiltration of large numbers of inflammatory cells (neutrophils macrophages and lymphocytes).

C. Occluding of many alveoli by inflammatory exudates.

D. Peribronchiolar infiltration of inflammatory cells (neutrophils macrophages and lymphocytes).

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RECOMMENDATIONS

١- Purification and characterization of proteases of both wild types (*P. aeruginosa*) and *E. coli* HB١٠١ clone to determine (enzymatic activity, protein characteristics and protein inhibitors).

٢- Determine DNA sequence of protease genes in both wild types (*P. aeruginosa*) and *E. coli* HB١٠١ clones, to indicate the real size of protease genes.

٣- Studying the primary structure of the protein and its role in the pathogenicity of the secreting microorganism and enzyme overproduction by the gene dosage effect.

٤- Protein engineering to locate the active-site residues and/or to alter the enzyme properties to suit its commercial applications.

٥- Further studies must be done to investigate and demonstrate the burned skin rat-models infected by both wild types (*P. aeruginosa*) and *E. coli* HB١٠١ clone by scanning and transition electron microscopes to indicate the histological abnormalities of cells and tissues.

٦- To protect the hospitalized patients, especially burned patients from the consequence infections caused by *P. aeruginosa* strains, all scientific efforts must be done to eliminate these bacterial infections by (curing of virulence plasmids, mutation of virulence genes, construction and development of new protease inhibitors).

General characteristics of *Pseudomonas aeruginosa*

rce of lation	Protease	Oxidase	Catalase	Lipase	Urease	Pyocyanin production	Starch hydrolysis	Gelatin liquefaction	Glucose utilization	Kligler (Slope)	Kligler (Bottom)	Kligler (H_2S) production	Motility (One polar flagella)	Capsule	Growth at ٤ °C	C a
Ear	-	+	+	-	+	+	-	+	+	Red Alkaline	Red Alkaline	-	+	+	-	
Ear	-	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
Ear	-	+	+	+	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	-	-	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	-	-	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	+	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	+	+	+	-	+	+	Red	Red	-	+	+	-	

General characteristics of *Pseudomonas aeruginosa*

rce of lation	Protease	Oxidase	Catalase	Lipase	Urease	Pyocyanin production	Starch hydrolysis	Gelatin liquefaction	Glucose utilization	Kligler (Slope)	Kligler (Bottom)	Kligler (H_2S) production	Motility (One polar flagella)	Capsule	Growth at ٤ °C	C a
										(Alkaline)	(Alkaline)					
rine	-	+	+	+	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	+	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	-	-	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
rine	-	+	+	+	+	+ +	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
ound	+	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
ound	+	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
ound	+	+	+	+	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
ound	+	+	+	-	-	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
ound	+	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
urn	+ +	+	+	-	+	+	-	-	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
ound	+	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
urn	+ +	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
urn	+	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	

General characteristics of *Pseudomonas aeruginosa*

rce of lation	Protease	Oxidase	Catalase	Lipase	Urease	Pyocyanin production	Starch hydrolysis	Gelatin liquefaction	Glucose utilization	Kligler (Slope)	Kligler (Bottom)	Kligler (H_2S) production	Motility (One polar flagella)	Capsule	Growth at ٤ °C	C a
ound	+	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
urn	+ +	+	+	+	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
urn	+	+	+	+	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
urn	+	+	+	-	+	+	-	-	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
urn	+ +	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
urn	+	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	
urn	+	+	+	-	+	+	-	+	+	Red (Alkaline)	Red (Alkaline)	-	+	+	-	