THE RESISTANCE OF *Pseudomonas aeruginosa* ISOLATED FROM CLINICAL CASES TO ANTIMICROBIALS

A THESIS

SUBMITTED TO THE COUNCIL OF THE COLLEGE OF MEDICINE IN PARTIAL FULFILLMENTS OF THE REQUIREMENTS FOR THE MASTER DEGREE OF SCIENCE IN MICROBIOLOGY

BY/

Lena Fadhil Hamza AL-Gibouri

B.V.M.S.

September /٢٠٠٦ Sha'ban /١٤٢٧

بِسمِ اللَّهِ الرَّحمنِ الرَّحِيمُ

اَللّهُ لآاِلَه اَلآ هُوَ الحَيُّ الَقَيُوّمُ لا تَأْخُذَهُ سُنَةٌ وَلا نَومْ لَهُ مَا فِي الّسَمَوُاتُ وَمَافِي الأرَضِ مَنُ ذَا الَذِْي يَشْفعُ عِنْدَهُ إلا بِأذْنِهِ يَعْلَمُ مَا بَيْنَ أْيْدِيهِمْ وَمَا خَلْفَهُمُ وَلاَ يُحِيطُونَ بِشَيءٍ مِنْ عِلْمِهِ إلاَ بِمَا شَاءَ وَسِعَ كُرْسِيُهُ الَسَمَواتِ وَالأرَضْ وَلاَيُوءَدَهُ حِفْظَهُمَا وَهُوَ الَعَلِيُ الَعَظِيمْ

صَدَقَ اللَّهُ الَعَلِيُ الّعَظِيمُ

سورة البقرة – الاية(٢٥٥)

مقاومة بكتريا الزوائف الزنجارية المعزولة من حالات سريرية للمضادات الحيوية

رسالة مقدمة من قبل الطالبة

لينا فاضل حمزة الجبوري

بكالوريوس طب وجراحة بيطرية

الى

مجلس كلية الطب/ جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير

في علم الأحياء المجهرية

شعبان /١٤٢٧ **أيلول /٢٠٠٦**

الخلاصة

أجريت هذه الدراسة لغرض عزل و تشخيص بكتريا *P.aeruginosa* من حالات سريرية مختلفة شملت التهاب المجاري البولية ، حالات التهاب الاذن الوسطى والحروق وبيان مدى حساسيتها ل(٢٣) مضادا حيويا. تم مزج بعض تلك المضادات مع بعضها بتراكيز محددة وتم اختيار فعاليتها تجاه تلك العزلات . اضيفت لبعض المضادات بعض المواد الكيميائية وتم اختبار فعاليتها تجاه العزلات ايضا و اجريت مقارنة للنتائج بهدف تحديد الأكثر كفاءة .

المجموع الكلي لعزلات *P.aeruginosa* ٣٠ من مجموع (٢٦٠) عينة بواقع ١٠من اصل (٣٢) عينة حروق ,١٠ من اصل (٥٨) عينة قيح اذن , ١٠ من اصل (١٧٠) عينة إدرار. من المرضى الراقدين في مستشفى الحلة التعليمي والمراجعين إلى نفس المستشفى خلال الفترة من تشرين الأول /٢٠٠٤ إلى أيار/٢٠٠٥ . تم تشخيص البكتريا من خلال نتائج الاختبارات التشخيصية التي تضمنت الصفات الخلوية ، الصفات الزرعية و الصفات البايوكيميائية. تم اختبار فعالية (٢٣) مضادا حيويا تجاه العزلات البكتيرية لتحديد مدى مقاومتها لها وذلك بطريقة الانتشار بالأقراص(Disk diffusion) وتبين أن العزلات البكتيرية أبدت مقاومة نسبية لمعظم المضادات تحت التجربة وعلى الترتيب :-

(١٠٠٪)penicillin,ampicillin,carbencillin, cefixime, tetracycline, doxycycline, chloramphenicol,erythromycin and rifampin,(٩٠٪)cefotaxime and co-trimoxazole,(٨٦.٧٪)gentamicin and ceftizoxime,(٨٠٪)ticarcillin,(٧٦.٧٪) piperacillin,(٧٠٪)cefepime,(٦٣.٣٪)colistin,(٦٠٪)aztreonam,(٥٦.٧٪)tobromy-cin,(٢٦.٧٪) ciprofloxacin and norfloxacin, (٢٠٪) amikacin and azithromycin .

وعلى ضوء تلك النتائج خلصت الدراسة الى ان العزلات التي تمت دراستها من النوع المقاوم للعديد من المضادات(MDRPA)(Multi-drug-Resistant *P.aeruginosa*) وان اغلبها متواجد في بيئة المستشفيات الأمر الذي يجعل هذه البيئة بؤرة للإصابة. اوضحت النتائج ان الجنس والعمر وطبيعة

تعاطي المضادات وفترة الرقود في المستشفى جميعها عوامل خطورة (Risk Factors) قد تساعد على الإصابة . ومن خلال تحليل النتائج تبين ان الحروق أكثر انواع الإصابات عرضة للتلوث بهذه البكتريا.

تم خلال الدراسة الكشف عن التركيز المثبط الأدنى (MIC) بطريقة (Macro-broth Dilution) ل(١٢) مضادا حيويا وتبين وجود تزايدا في التراكيز المثبطة الدنيا للمضادات الحيوية من قبل العزلات البكتيرية مما يدلل على إنها من نوع (MDRPA) . كما تم خلال هذا البحث دراسة مزج المضادات بطريقة (Half-chess board) للكشف عن مدى تأثيرها على العزلات البكتيرية قيد الدراسة وقد اختير لهذا الغرض (١٠) مضادات هي

Penicillin, ampicillin, cefotaxime, ceftriaxone, ciprofloxacin,norfloxacin, amikacin, gentamicin,co-trimoxazole,rifampin.

وأظهرت النتائج تأثير تآزري بمستويات متفاوتة مقارنة بتأثيرها فرادا. تم اختبار مضادين من هذه المضادات وهما (gentamicin , ceftriaxone) للتعرف على درجة التآزر بينهما و بطريقة(Checker Board) ولوحظ ان الفعالية التأزرية القاتلة كانت كاملة (complete cidal) بالنسبة ل(٥) عزلات هي ($O_{٢٥}$, $B_{٣٠}$, $B_{١٢}$, $U_{٢٥}$, $U_{٢٤}$) وتأزرية جزئية (partial synergy) بالنسبة لعزلة واحدة هي ($O_{٥٥}$) . وتم خلال البحث الكشف عن أنتاج أنزيمات(Extended-spectrum-beta-lactamases) من قبل هذه البكتريا باستعمال مادة (EDTA) وكانت النتيجة موجبة ل(٩) عزلات باستعمال (ampicillin) و(١٢) عزلة باستعمال(cefixime) . وتم ايضا التعرف على التآزر مابين بعض المضادات وفيتامين (B-complex) وكانت النتيجة موجبة ل(٤)(٨٠٪) عزلات من اصل (٥).

Decision of Discussion Committee

We, the examining committee certify that we have read this thesis and have examined the student in it’s contents, and that in our

opinion, it is adequate as a thesis for the Master degree in science-microbiology

Signature :

Name : Prof. Dr. Hussein S. AL-Janabi

Address: Department of Medicine

College of Medicine

University of Kufa

Chairman

Signature :

Name: Asst. Prof. Dr. Mohammad Abdel Ekhwa AL-Faham

Address: Department of Microbiology

College of Medicine

University of Baghdad

Member

Signature :

Name: Asst. Prof. Dr. Kassim Najim

Address: Department of Biology

College of Sciences

University of Babylon

Member

Signature :

Name : Prof. Dr. Habeeb S. Naher

Address: Department of Microbiology

College of Medicine

University of Babylon

Member & Supervisor

Approve for the College Committee on Graduate Studies

Signature:

Name : Dr.Ali Khair Allah

The Dean.

DECLARATION

I certify that this work was carried out under my supervision at the College of Medicine, University of Babylon as partial requirements for the master degree of science in Microbiology

Advisor

Signature:

Name : Prof. Dr. Habeeb Sahib Naher

Department of Microbiology

College of Medicine

University of Babylon

Date: / /٢٠٠٦

Recommendation of Head of Microbiology Department

-

In view of the available recommendations , I forward this thesis for debate by the Examining Committee

Signature :

Name : Assit. Prof. Dr. Mohammad Sabri A.Razzak Al-Saeed

Head of Microbiology Department

College of Medicine

University of Babylon

Date: / /٢٠٠٦

Summary

This study was carried out through the October/٢٠٠٤ to May/٢٠٠٥ for isolation and identification of *P.aeruginosa* from different clinical cases which include ٢٦٠ subject of inpatients and outpatients who have suffering from : burns ,chronic otitis media and urinary tract infections (UTI) and detection of their susceptibility for twenty-three antibiotics. Some of these antibiotics were combined with each other by different concentrations and tested for their activities against those isolates. Moreover, chemical substances were added to some antibiotics in attempt to enhance their effects. The obtained results were compared with each other for determination of the highly active compound.

The total number of *P.aeruginosa* isolates accounted for ٣٠ of ٢٦٠ samples, the distribution of those isolates were: ١٠ from ٣٢ burn samples,١٠ from ٥٨ otitis samples and ١٠ from ١٧٠ UTI samples. The bacterial isolates were identified by routine diagnostic tests which included cellular, cultural and biochemical characteristics. The activity of ٢٣ antibiotics were detected by disk diffusion method and the results showed high degree of resistance for most antibiotics represented by: penicillin, ampicillin, carbenicillin, cefixime, tetracycline, doxycycline, chloramphenicol, erythromycin, rifampin (١٠٠٪), cefotaxime and Co-trimoxazole(٩٠٪),gentamicin and ceftizoxime(٨٦.٧٪),ticarcillin(٨٠٪) ,piperacillin (٧٦.٧٪), cefepime(٧٠%), colistin(٦٣.٣٪), aztreonam(٦٠٪), tobromycin (٥٦.٧٪), ciprofloxacin and norfloxacin (٢٦.٧٪), amikacin and azithromycin (٢٠٪). Accordingly, the results were consistent the what is called multi-drug-resistant *P.aeruginosa* (MDRPA).

The results of this study showed that the sex, age, long hospitalization and prolonged antimicrobial use were regarders as risk factors increased the

chance of infection. Results analysis showed that burns were more accessible for contamination by this organism. The minimum inhibitory concentrations(MIC) for (١٢) antibiotics by Macro-Broth dilution method were carried out. The results of this experiment showed a high level of resistance which confidently these isolates (MDRPA).

Half-Chess Board method was used to assess the potency of (١٠)antibiotics in combination with each other.Those antibiotics included: penicillin, ampicillin, cefotaxime, ceftriaxone, ciprofloxacin ,norfloxacin, amikacin, gentamicin, Co-trimoxazole and rifampin. The results showed synergetic effect at different levels compared with their effect singly. Gentamicin and ceftriaxone were tested by Checker- Board method and showed cidal synergetic activity against the isolates ($U_{٢٤}$,$U_{٢٥}$,$B_{١٢}$,$B_{٣٠}$,$O_{٢٥}$) but partial synergy against one ($O_{٥٥}$)isolate. Finally, the results indicated for extended-spectrum beta-lactamases production by this bacteria by using EDTA substrate and the positive result for ٩(٣٠٪) isolates with ampicillin and ١٢(٤٠٪) isolates with cefixime. The study showed synergistic effect between B-complex vitamin and antibiotics, positive result for ٤(٨٠٪) isolates of (٥) tested isolates.

Acknowledgements

I would like to thank my supervisor professor Dr. Habeeb Sahib Naher ,the Dean Dr. Ali Khair-Allah and the head of Microbiology department Dr. Mohammad Sabri .

My gratefulness to Prof. Dr. Sabah Neama (Dept. Pharmacology, College of Medicine, University of Babylon), Mr. Hattem A. Lattef , Mr. Zuher Saddek (Dept. Microbiology, College of Dentistry, University of Babylon), Dr. Amal Merza, Dr. Safa' Sahib and all staff of the Hilla Teaching Hospital for helping me.

Finally, my thanks is due to All-Mohmmod Clinic and Laboratory, Dr. Haider, my brother (the engineer Ahmad) and to all of my colleagues in the Dept. Microbiology, College of Medicine .

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LIST OF ABBREVIATIONS

Abbreviations	Meaning
B	Burn sample
BHIb	Brain-Heart Infusion broth
CAP	Community-acquired pneumonia
CF	Cystic Fibrosis
Co-trimoxazole	Sulfonamide-Trimethoprim
D.W.	Distiller Water
DRF	Drug Resistance Factor
EAC	External Auditory Canal
EDTA	Ethylene Diamine Tetra Acetic Acid
ICU	Intensive Care Unit
LPS	Lipopolysaccharied
MBLs	Metallo-beta-lactamases
MDRPA	Multi-drug Resistant *P.aeruginosa*
MIC	Minimum Inhibitory Concentration
NCCLs	National Committee for Clinical Laboratory Standards
O	Otitis media sample
P.aeruginosa	*Pseudomonas aeruginosa*
PBPs	Penicillin-Binding Proteins
PPPA	Pigments Producing *P.aeruginosa*
U	UTI sample
UTIs	Urinary Tract Infections

Conclusions

١. Burns are the most susceptible for infection by *P.aeruginosa*.

٢. *P.aeruginosa* is relatively difficult to eradicate from infected sites by traditional antimicrobial therapy.

٣. The structural and anatomical nature of *P.aeruginosa* confer it multi-mechanisms for an increasing multi-drugs resistance.

٤. Azithromycin, amikacin, ciprofloxacin and norfloxacin were the most effective antimicrobials against *P.aeruginosa* and confidently can be suggested for treatment to overcome this bacteria.

٥. The MIC method seems to be more reliable and more acceptable compared with disk diffusion method.

٦. Management of pseudomonal infections may require to combine more than single antibiotic precisely.

٧. Gentamicin and Ceftriaxone were composed effective couple for treatment MDRPA.

٨. Using antimicrobials in combination with active compounds such as EDTA and B-complex vitamin decrease the resistance of *P.aeruginosa* to antimicrobials.

Recommendations

١. Precisely prescribe the antimicrobials for treatment of pseudomonal infections.

٢. Genetic analysis to the local strains to study the mechanisms of the resistant of antimicrobials.

٣. Further studies should be achieved to detect the source of resistant of antimicrobials in hospital.
٤. Physicians, bacteriologists and clinical pharmacists all be aware the prevalence of rapid evolution of antimicrobial resistance and having the basic understanding of resistance mechanisms to antibiotics.
٥. Further studies should be achieved to detect new effective antipseudomonal antibiotics .
٦. Foundation a Committee of physicians, bacteriologists and substaff concerns the hospital environment regarding microbial flora (types ,sources and control). The Committee periodically checks the disinfection conditions (validity of disinfectant ,effectiveness,concentration at in use disinfectants.

Chapter One

Introduction And Literatures Review

١– Introduction :

The major member of the genus *Pseudomonas* associated with human infections is *Pseudomonas aeruginosa.* It is found sporadically in moist areas of the skin and in the intestinal tract of about ١٠٪ of healthy individuals as part of the resident microflora(Hugh and Gilardi ,١٩٧٤). In recent years it has become the major causative agent of nosocomial infections resulting in severe and complicated diseases with a high mortality rate. *P.aeruginosa* is an opportunistic organism that is able to cause infections mainly in compromised hosts (i.e. with impaired local or systemic defense mechanisms). The medical

problem resulted by this organism is it's ability to resist almost all antibacterial agents, leading to predominance it when sensitive organisms are suppressed by those agents (Lindberg,١٩٧٤; Cohn and Bronside,١٩٨٤; Cunha,٢٠٠٠).

P.aeruginosa is widely spread in nature and can survive and even multiply in moist environments that contain minimal amounts of organic substances. In hospitals environment, variety of reservoirs may serve as sources of infection and transmission. It may spread from a reservoir to a person or among persons, most often via the hands of hospital personnel (Ayoub,١٩٨١;Nordbring,١٩٨٢; Murray,*et al*.,٢٠٠٣).

The medically importance of this organism may be lies in it's ability to produce a variety of toxins, extracellular enzymes including elastases, proteases and hemolysins (Washington,١٩٨٤;Brooks,*et al*.,٢٠٠١). Furthermore, it has high intrinsic resistance to antibiotics at levels attainable in body tissues (Roberts and Douglas,١٩٧٨;Collee,*et al*.,١٩٩٦). Different mechanisms behind this resistance have been reported and will be discussed later on in this chapter. Until the

(١٩٦٠s), *Pseudomonas* infections were usually treated with polymyxins, agents that exhibit considerable toxicity, leading to develop new antipseudomonal beta–lactam compounds, including(piperacillin,ticarcillin,ceftazidime and meropenem and aminoglycosides such as gentamicin and tobromycin). Treatment with a combination of an aminoglycoside and a beta–lactam antibiotic was commonly adopted.There is little clinical evidence of the superiority of combined therapy although it has the potential for antibacterial synergy and reduced antibiotic resistance. Monotherapy with broad–spectrum beta–lactam agents such as (ceftazidime or imipenem)was used, but resistant

strains of *Pseudomonas* against these agents emerged again (Greenwood, *et al.*,٢٠٠٢).

At the present time there are many intensive efforts all around the world in attempt to discover new compounds to overcome the resistance phenomenon of *Pseudomonas* to antibiotics. This is, in part the hypothesis of this research. It was planned to fulfill the following main goals:

١. Isolation and identification of *P.aeruginosa* from different clinical cases (burns, chronic suppurative otitis media and urinary tract infections UTIs) in Hilla.
٢. Determination of the susceptibility of *P.aeruginosa* isolates to different antibiotics from different families by using two standard methods : (a) Disk Diffusion Method (b) Minimum Inhibitory Concentration (MIC).
٣. Detection the synergistic efficiency of selected antibiotics in combination status at different concentrations against *P.aeruginosa* isolates.
٤. Detection of the synergistic action of selected compounds being added to selected antibiotics against *P.aeruginosa* isolates.

٢- Literatures Review :

٢-١- General Description of *P.aeruginosa* :

In this chapter the general description represented by classification, culturing morphology and cell structure will be presented in brief.

P.aeruginosa belongs to the *Pseudomonadaceae*, which is one of families of *Eubacteriales* order which belongs to the class of *Schizomycetes* (Holt,*et*

al.,١٩٩٤).*P.aeruginosa* was first isolated from pus of wound and named *Bacterium aeruginosa* by Schroeter in(١٨٧٢). It was originally described as the causative agent of (blue pus)by Gessard in (١٨٨٢s) which named it *Bacillus pyocyaneas* and showed that this organism produced two characterized pigments, pyocyanin pigment, which is a bluish–green, non fluorescent, chloroform and water soluble, and fluorescein pigment, which is a fluorescent greenish–yellow and insoluble in chloroform but soluble in water (Gilligan,١٩٩٥). However, this property specifically characterizes *Pseudomonas aeruginosa,* and it will be more mentioned later in this chapter.

P.aeruginosa is a gram–negative, the cells are short rod–shaped straight or slightly curved. In liquid culture the cells occur singly, in solid culture pairs or occasionally in short chains.The organism is motile with one polar flagellum, non–spore forming.The cells of some strains are surrounded by a slime substance usually surround the capsule (alginate) (Murray,*et al*.,٢٠٠٣).

P.aeruginosa is an obligate aerobe but can grow unaerobically if nitrate is available.It is found frequently as part of the microbial flora of healthy individuals.In those individuals, the gastrointestinal tract is the most frequent site of colonization.Other sites such as throat, nasal mucosa, axillae and perineum may be colonized as well (Morrison and Wenzel,١٩٨٤; Mayhall,١٩٩٧).The organism grows readily on a wide variety of culture media in a wide range of temperature ranging from ٥–٤٢°c but the optimum temperature is ٣٧°c.The optimum pH is ٧.٤. It emits a sweet grape–like odor that is easily recognized (Pollack,٢٠٠٠).

During the diagnosis of this organism, there are some cultural variations must be considered.After aerobic incubation on nutrient agar for ٢٤ hours at

٣٧°c,six distinct colonial types of *P.aeruginosa* may be observed (Phillips, ١٩٦٩).Type ١ is the most common.The colonies produced by this type are relatively large, low convex, rough in appearance and oval with long axis in the line of the inoculums streak surrounded by a serrated strict of growth.Type ٢ colonies are small and smooth.Colonies type ٣ and ٤ are small, rough and rugose respectively.Type ٥ colonies are mucoid and alginate–produce is very striking, the copious exopolysaccaride produced may result in merging colonial growth.The dwarf colony type ٦ is the smallest coloy form *P.aeruginosa* (Collee,*et al.*,١٩٩٦).

Colonial dissociation from one colony type to another is frequently observed both on subculture and in primary diagnostic plates and does not necessarily indicate the presence of more than one strain (Greenwood,*et al.*,٢٠٠٢).

Some strains hemolyze blood of beta–hemolytic type and can grow on MacConkey's agar as lactose non–fermented but the characteristic pigments are often poorly observed on this medium (Baron,*etal.*,١٩٩٤). Most strains of *P.aeruginosa* are specifically characterized by producing pyocyanin, a water soluble turquoise blue or blue phenazine pigment visible, on uncolored media such as nutrient agar and no other bacteria produce such pigment, so it's detection is quite enough for identification of an isolate of *P.aeruginosa* (Baron, *et al.*,١٩٩٤;Holt,*et al.*,١٩٩٤;Gilligan,١٩٩٥;Collee,*et al.*,١٩٩٦). Some strains of *P.aeruginosa* produce other pigments pyoverdin (yellow–green) fluorescent

pigment, pyorubrin (red)and pyomelanin (brown) which may mask the pyocyanin (Brooks, *et al.,*٢٠٠١).

Clinical isolates are catalase and oxidase positive,*P.aeruginosa* differes from members of the *Enterobacteriac*eae by deriving energy from carbohydrates by an oxidation rather than fermentation, and only glucose is utilized. However all strains give a rapid positive oxidase reaction within ٣٠ seconds and this is a useful preliminary test for non–pigmented strains (Sundstrom,*et al.*,١٩٩٦).

Moreover,*P.aeruginosa* is able to dehydrolyze arginine, unable to decarboxylate lysine or ornithine, unable to produce indole and H٢S,Voges–Proskauer and Methyl–Red reactions are negative, can liquefy gelatin and utilize citrate (Macfaddin,٢٠٠٠). Furthermore, isolates from different colony types may reveal different biochemical and enzymatic activities and different antimicrobial susceptibility as well, but Brooks and his colleagues (٢٠٠١) pointed out that identification of this organism is principally based on colonial morphology including the presence of characteristic pigments, oxidase positivity, and growth at ٤٢°c.

٢-٢- The Antigenic Structures :

P.aeruginosa possesses some appendages mainly pili (fimbriae) flagellum, capsule, lipopolysaccharide (LPS) in addition to the pyocin production. Those structures will be summarized as they are closely associated with the pathogenicity of this organism.

Pili extend from the cell surface and promote attachment to host epithelial cells.Flagellum yields heat–labile antigens (H–antigen).Consequently it is non specific(Liu,١٩٧٤).Polysaccharide capsules are responsible for the mucoid

colonies seen in cultures from patients with cystic fibrosis(CF). The lipopolysaccharide (O–antigen), which exists in multiple immunotypes,is responsible for many of the endotoxic properties of the organism (Oliver,*et al*.,٢٠٠٠).Also *P.aeruginosa* produce pyocin, these substances resemble antibiotics and have active effect on different strains from the same species or other species. These substances used in typing of *P.aeruginosa* and diagnosis of the species (Aboud,٢٠٠١).

٢-٣- Typing Methods and Epidemiological Studies :

In addition to the biochemical tests which used in the diagnosis of the bacteria, typing or fingerprinting of individual strains is a major factor in the diagnosis of the bacteria and epidemiological investigations. Epidemiological studies of *P.aeruginosa* strains depend on the Pyocin typing, Phage typing and Serotyping. Serotyping based on (LPS) antigens by agglutination forms the basis of O serotyping (Pitt,١٩٨٨).

The disadvantage of this method it's limited discriminating power. Only nine serotypes account for over (٩٠٪) of isolates, this makes epidemiological investigation difficult unless the epidemic strain belongs to unusual serotype (Hancock,*et al*.,١٩٨٣),but this technique is unsatisfactory for typing mucoid variants since O–antigens are masked. It is also unsuitable for typing colonial dissociants, because antigenic changes are observed within a single culture, and for typing polyagglutinable *P.aeruginosa*, i.e. strains that are agglutinated by more than one serum in patterns that are outside the established cross-reactions of the serotype strains (Mutharia,*et al*.,١٩٨٢; Muller and

Gubina,٢٠٠٠). Pyocin typing offers greater discriminating power than Serotyping whilst retaining (simplicity and reliability),it is also suitable for polyagglutinating and mucoid strains (Collee,*et al*.,١٩٩٦).Recently, a number of genotyping methods have been developed for typing *P.aeruginosa*. These include restricition enzymes analysis (Olge,*et al*.,١٩٨٧;Greenwood, *et al*.,٢٠٠٢).

٢-٤- Pathogenicity :

P.aeruginosa is the species most commonly associated with human infections.Greenwood and his colleagues (٢٠٠٢) described this association for the high adaptability of the organism with the environmental conditions,it's innate resistance to antibiotics and disinfectants and it's armoury of putative virulence factors are summarized below and illustrated in table (١-١).

٢-٤-١- The Exteracellular Slime layer Substance :

It is a mucoid substance composed of glycolipoprotein surrounds the bacterial cell and protects the cell from–degrading enzymes and involved in cell adhesion to host epidermal surfaces and it responsible to give a large degree of antibiotic resistance for *P.aeruginosa* (Brooks *et al*.,٢٠٠١).

٢-٤-٢- The Capsule (Alginate) :

P.aeruginosa isolated from respiratory tracts of patients with cystic fibrosis or other obstructive airways disease produces large amounts of a substance usually referred to as alginate, an exopolysaccharide consisting of mannuronic and guluronic acids which gives rise to strikingly mucoid colonies (Collee,*et*

al.,١٩٩٦).Alginate is a heterogeneous mixture of hexoses produced by all *P. aeruginosa* strains on prolonged incubation in media with high carbon and low nitrogen content.The mucoid polysaccharide alginate represents the capsule which seems to enable the bacteria to resist natural defenses and to protect it from phagocytosis and antibiotics (Baron, *et al.*,١٩٩٤).

Table (١-١) Some of virulence factors of *P.aeruginosa* and their proposed role in pathogenicity

Factor	Proposed Role
Endotoxin	terminal shock
Exoenzyme S	Local and systemic toxicity
Heat-stable hemolysin	Toxic to alveolar macrophages
Leukocidin	Depression of host defenses
Phospholipase C	Hydrolysis of lecithin
Pigments(pyocyanin and fluorescein)	Antibacterial agent
Proteases(elastase and alkaline protease)	Local tissue necrosis and spreading factor
Toxin A	Lethality and inhibition of host defenses

(Hauser and Sriram, ٢٠٠٥)

٢-٤-٣- The Flagella :

Flagella are thread–like appendage composed of entirely of protein subunit called flagellin. The flagellum in *P.aeruginosa* is responsible for the motility of the bacteria (Brooks,*et al.*,٢٠٠١).

٢-٤-٤- The Pili (Fimbriae) :

Fimbriae are surface appendages sometimes called pili which are shorter and finer than flagella and they are composed of structural protein subunits termed pillius.Minor proteins located at the tips of pili are responsible for the attachment properties(Owlia*,et al*.,٢٠٠١).There are two classes of pili: ordinary pili which play a role in the adherence to host cells and sex pili which responsible for the attachment of bacterial cells to each other during conjugation (Brooks,*et al*, ٢٠٠١).The virulence of the bacteria depends on the production of toxins and colonization antigens which represented by the ordinary pili that provide the cells with adherent properties (Brooks,*et al.*,٢٠٠١).

٢-٤-٥- Enzymes and Toxins :

P.aeruginosa liberates some toxic products including catalase,lipase, lecithinase,elastases,proteases and two hemolysins, a heat-labile phospholipase C and a heat-stable glycolipid(Kolmos,*et al.,*١٩٩٣).All of these are toxic and enable the organism to invasive and destroy the tissues (Kolmos,*et al.*,١٩٩٣). Moreover, toxin A causes tissue necrosis and blocks protein synthesis by a mechanism of action identical to that of *Corynebacterium diphtheriae* toxin which affects the elongation of peptide chain, although the structures of the two toxins are not identical. Antitoxins to toxins A may be found in some human sera, including those patients who have

recovered from serious *P.aeruginosa* infections (Owlia, *et al.,*٢٠٠١). These toxins range from potent toxins that enter and kill host cells at or near the site of colonization to degradation enzymes that permanently disrupt the cell membranes and connective tissue in various organs (Owlia,*et al*.,٢٠٠١).

Endotoxins are composed from lipopolysaccharide which one of the components of the cell wall, release during autolysis or during microorganism dead. These Endotoxins are very toxic to the host's tissues because the found of lipid portion for lipopolysaccharide which called lipid A (Mousa ,١٩٩٧).These Endotoxins are resistant to heat and cause shock with hypotension especially in elderly persons. Also cause leucopenia, hypoglycemia and cause defect in the kidneys, heart and brain (Mousa,١٩٩٧).

٢-٤-٦- The Extracellular Substances :

٢-٤-٦-١- The Pyocin :

Pyocin is a substance produced by over ٩٠٪of *P.aeruginosa* strains, protein in nature and has effect on strains belong to the same family or to the *Enterobacteriaceae*.The pyocin different from other antibiotics which has limit effect on the other species from the same genus or which closed with it.The production of pyocin and the sensitivity to it be consider the dominant genetic

properties and it has an ability to depress the normal and cancer cells together (Aboud,٢٠٠١).

٢-٤-٦-٢- The Pyocyanin :

The pyocyanin is a blue phenazine pigment, soluble in water,release outside the cell produce from most strains of *P.aeruginosa* when grow on culture media with temperature ٢٨-٣٠°c(Collee,*et al.,*١٩٩٦).

Low and Emmerich were mentioned at the first one the activity of pyocyanin as antibiotic when they found the old culture to *P.aeruginosa* prevent the growth of some gram negative and positive bacteria. Pyocyanin causes broad-spectrum inhibition to the microorganism but doesn't have any benefit because it is toxic for all animals (Aboud,٢٠٠١).

٢-٥- Pathogenesis of *Pseudomonas aeruginosa* :

P.aeruginosa can infect almost any external site or organ. Most community infections are mild and superficial, but in hospitalized patients, infections are more common, more severe and more varied (Maniatis,*et al.,*١٩٩٥).

P.aeruginosa is pathogenic only when introduced into areas devoid of normal defenses, e.g. when mucous membranes and skin are disrupted by direct tissue damage; when intravenous or urinary catheters are used; or when neutropenia is present, as in cancer chemotherapy.The bacterium attaches to, and colonizes the mucous membranes or skin, invades locally and produces systemic disease (Maniatis,*et al.*,١٩٩٥).These processes are promoted by the

Pili, enzymes and toxins.Lipopolysaccharide plays a direct role in causing fever, shock, oliguria, leukocytosis and leucopenia, disseminated intravascular coagulation and adult respiratory distress syndrome (Pollack ,٢٠٠٠).

The most superficial infection associated with this organism is folliculitis (Bottone and Perez,١٩٩٣).Superficial infections of the ear canal due to *P.aeruginosa* frequently develop in individuals involved in aquatic environment (Rubin and Yu,١٩٩٠).The organism can invade the underlying tissues, damaging cranial nerves and causing osteomyelitis and meningitis it is may result (Rubin and Yu,١٩٩٠).

Eye infection caused by this organism usually follows minor trauma to the cornea (Holland,*et al.,*١٩٩٣).*P.aeruginosa* is a cause of community-acquired pneumonia (CAP) and most of nosocomial infections are ascribed to this organisms,as most of the hospital environments are colonized by it (Hatchette, *et al.*,٢٠٠٠).

Bacteremia and septic shock due to *P.aeruginosa* continue to be the major problems in hospitalized patients with underlying malignancies,cardiopulmonary disease, renal failure or diabetes. Bacteremia due to *P.aeruginosa* in intravenous drug users is usually associated with bacterial endocarditis and may also develop osteomyelitis of a variety bones (Boffi, *et al*.,٢٠٠١).

The organism is the leading cause of nosocomial respiratory tract infection (Bernardini,*et al*.,١٩٨٧). Also causes nosocomial urinary tract infections, wound infections, and peritonitis in patients with chronic ambulatory peritoneal dialysis (Anonymous,١٩٩٧). Wounds infections due to *P.aeruginosa*

are particularly big trouble in burn patients which mostly leads to significant mortality rates (Mousa, ١٩٩٧).

٢-٦- Resistance to Antimicrobial Agents :

Antibiotics resistance with some microorganisms has become a worldwide concern. It is either natural or acquired resistance. If organisms previously sensitive to an antibiotic become resistant to it this is referred to as acquired resistance, such as resistance to (ceftazidime,ciprofloxacin and imipenem).This type of resistance is either relative or absolute (Mingeot,*et al*.,١٩٩٩).Relative acquired resistance refers to the gradual increase over time of the (MIC) of an organism to a particular antibiotic,whereas absolute resistance occurs when there is a single-step mutation that occurs during or after therapy and increases the(MIC) of a previously susceptible isolate to extremely high levels unachievable using the therapeutic doses (e.g. the development of gentamicin–resistant *P.aeruginosa* (Erst, *et al*.,١٩٩٩).

Given the number of compounds active against *P.aeruginosa*,it might be expected that treatment of infections caused by this bacterium would be straight forward.However,the situation is complicated by the predilection *P.aeruginosa* to develop resistance to nearly any antibacterial agent. During the chemotherapy era, there was a remarkable progress in resistance of *P.aeruginosa* to antibiotics occurred. Previously this organism was referred to as strongly drug resistant, then multi-drugs resistant, at the present time it is described as all drugs resistant (Weinstein, *et al.,*١٩٨٠ ;Gotoh, ٢٠٠١).

Resistance is problematic at three levels: intrinsic resistance, acquired resistance and emergences of resistance during therapy. Each of these must be considered when choosing an antibiotic regimen for patients infected with *P.aeruginosa* (Hauser and Sriram,٢٠٠٥). These types of resistance will be summarized below.

In general *P.aeruginosa* is naturally less susceptible than other gram-negative bacilli to many antibiotics such as(ampicillin,most cephalosporin,and the macrolides)(Bouza,*etal.,*١٩٩٩).This is because of its relative ability to transport some antibiotics out of the cell preventing accumulation of antibiotic molecules(Friedrich,*etal.*,١٩٩٩).*P.aeruginosa* also harbors an inducible chromosomally encoded beta-lactamase,referred to as AmpC beta-lactams even through it's naturally expressed at very low levels (Mingeot,*et al.,*١٩٩٩).The genome of *P.aeruginosa* consists of ٦.٣ million base pairs-the largest bacterium sequenced to date. This information has already provided insights into the organism's versatility and inherent resistance (Greenwood ,*et al.*,٢٠٠٢).

The intrinsic resistance is mainly a result of the diffusion barrier of the bacterial outer membrane, amino–acid substitution in the target molecules, via point mutation in each genetic determinant, and antimicrobial inactivating enzymes (Grayson and Eliopoulos,١٩٩٠).

Biofilm formation by *P.aeruginosa* may also contribute to antibiotics resistance. Bacteria grown as biofilms(which are an organized communities of bacterial cells that grow on surface) are much more resistance to antibiotics than those bacteria grown as disperssed growth (Erst, *et al.*,١٩٩٩).

In addition to its intrinsic resistance *P.aeruginosa* has the ability, through mutational changes or acquisition of exogenous genetic elements (plasmids and transposons) to develop resistance to each of the antipseudomonal antibiotics (Grayson and Eliopoulos,١٩٩٠).One type of mutation simultaneously comprises (penicillins, cephalosporins and the fluoroquinolons and enhances resistance to choromphenicol and tetracyclines) is by accelerating multi-drug efflux (Poole, *et al.*,١٩٩٣). Furthermore, the chromosomally encoded AmpC beta-lactamase is capable of degrading beta-lactam antibiotics, such as (piperacillin and ceftazidime),when mutations result in production of large amounts of this enzyme.Some beta-lactamase inhibitors compounds such as (tazobactam, sulbactam and clavunic acid) were developed in attempt to overcome the action of these compounds were latterly detected to be not quite enough to inactivate this enzyme (Hauser and Sriram,٢٠٠٥). Other mutations involve loss of the D٢ porins that mediate carbapenem resistance has been reported as well (Buscher, *et al.,*١٩٨٦; Livermore,١٩٩٢).

Resistance to aminoglycosides group may occur by mechanisms that differentially affect members of this group.Thus, resistance to gentamicin and tobromycin is often not accompanied with resistance to amikacin (Weinstein, *et al*.,١٩٨٠).Resistance to aminoglycosides is created by reduction uptake of these antibiotics across the outer or cytoplasmic membrane and inactivation by aminoglycosides modifying enzymes (Shannon and Phillips,١٩٨٢;Miller,*et al*., ١٩٩٧).

Recently, strains of *P.aeruginosa* resistant all antimicrobials except polymyxin B have been reported. This is not surprising, since there are many and varied mechanisms by which *P.aeruginosa* can resist the actions of

antibiotics (Spencer,١٩٩٦;Toleman,*et al.,*٢٠٠٣). Susceptibility rates appear to be decreasing with the time, especially with regard to (piperacillin,ceftazidime,imipenem and ciprofloxacin). In a study on *P.aeruginosa* isolated from United States intensive care units ciprofloxacin susceptibility rates decreased from(٨٩٪) in ١٩٩٣ to (٦٨%) in ٢٠٠٠ (Neuhauser,*et al.,*٢٠٠٣).

Even when antimicrobial agents to which an isolate is susceptible are chosen, a successful therapeutic outcome is not ensured. One reason for this uncertainty is that *P.aeruginosa* has shown a regrettable propensity to develop resistance to antibiotics during therapy (Neuhauser,*et al*.,٢٠٠٣).Resistance develops because of the natural occurrence of mutations essential for antibiotic penetration or activity.Within infected tissue,selection for individual bacteria that harbor these mutations occurs because the antibiotic is present, and eventually these organisms compose the majority of the bacterial population (Hauser and Sriram, ٢٠٠٥).

As expected, antimicrobial agents for which a single mutation is sufficient to compromise activity are most prone to the emergence of resistance during therapy (Bhavnani, ١٩٩٨). These include imipenem and the fluoroquinolones, for example,resistance emerged in a range of ٢٥٪ to ٥٠٪ of *P.aeruginosa* infected patients who received imipenem monotherapy and (٣٣٪) to (٥٨٪) of such patients who received ciprofloxacin monotyerapy (Cunha ,٢٠٠٠).

P.aeruginosa resistance is not restricted to the hospital environment but has been reported in community–acquired infections,such as otitis externa, folliculitis and osteomyelitis (Maniatis,*et al*.,١٩٩٥).Outpatients with chronic indwelling urethral catheters and endocarditis in intravenous drug users

(Costerton,*et al.,*١٩٩٩).The prevalence of resistance among *P.aeruginosa* strains has been extensively reported, especially with the expansion in the number of beta–lactam agents with improved antipseudomonal activity. Resistance may not be as widespread as reported and there may be considerable geographic variation (Costerton ,*et al*.,١٩٩٩).

Nosocomially acquired *P.aeruginosa* isolates tend to be more resistant to antimicrobials than do community–acquired strains, frequently displaying resistance to multiple classes of antimicrobial agents. However,development of resistance may occur during antimicrobial therapy and is particularly well documented during monotherapy(Cunha and Gill,١٩٩٥).It has become increasingly clear that resistance development in *P.aeruginosa* is multifactorial with mutations in genes encoding porins, efflux pumps, penicillin–binding proteins,and chromosomal beta–lactamase (Hsueh,*et al*.,٢٠٠٢).All of these are contributing to resistance to beta–lactams,carbapenems,aminoglycosides, and fluoroquinolones (Hsueh,*etal.*,٢٠٠٢).In addition, *P.aeruginosa* strains may contain extended–spectrum beta–lactamases, which can degrade imipenem. Genes encoding these enzymes can be either chromosomal or located on plasmids or integrons (Lepper ,*et al.,*٢٠٠٢).

٢-٧- Antipseudomonal Antibiotics :

P.aeruginosa has been reported worldwide to be a common pathogen both in hospitals and community setting. It is always a difficult pathogen to eradicate. It also carries a higher mortality rate than other gram–negative bacteria. Furthermore, the past decade has witnessed a dramatic increase in

resistance of this organism to antimicrobial agents. Use of a single antimicrobial agent,even when *P.aeruginosa* is sensitive is associated with the development of resistance during therapy (Cunha,٢٠٠٠).The rapid evolution of antimicrobial resistance towards traditional antibiotics leads to develop alternative antibiotics which are relatively more effective against *P.aeruginosa*. These antibiotics which are usually referred to as antipseudomonal antibiotics will be described below.

Antipseudomonal antibiotics include the third-generation cephalosporins (cefoperazone, cefsulodin, ceftazidime), fourth-generation cephalosporins (cefepime, cefpirome, cefclidin), extended–spectrum penicillins (ticarcillin, piperacillin, azlocillin), monobactams (aztreonam), carbapenems (imipenem, meropenem) and Quiolones (ciprofloxacin, enoxacin, ofloxacin). The addition of beta–lactamase inhibitors to extended–spectrum penicillins and cephalosporins has expanded the antibacterial spectra of these agents against most of gram–positive and gram–negative organisms (Wateret and Wunderink,٢٠٠١). Generally, resistance of microorganisms to antibiotics depends on the type of microorganism and type of the antibiotic as well. An explanation is given below regarding behavior of *P.aeruginosa* towards some of these antibiotics.

٢-٧-١- Extended–Spectrum Penicillins :

Although the exact mechanism of action of pencillins is not well understood yet, researches have shown that they bind reversibly to several enzymes outside the bacterial cytoplasmic membrane.These enzymes which known as penicillin–binding proteins (PBPs), are involved in cell–wall synthesis

and cell division. Interference of antibiotic with these processes increases internal osmotic pressure and ruptures the cell (Baer and Williams,١٩٩٦).

The antibacterial activity of penicillins depends partly on their ability to bind to the target enzymes.Gram–negative bacteria posse an outer membrane around the cell wall that decreases accessibility to the PBPs (Obritsch, *et al*., ٢٠٠٥). The production of penicillinases (enzymes that convert penicillin to inactive penicilloic acid) by the bacteria also contributes to bacterial resistance.

Aminopenicillins and extended–spectrum penicillins penetrate the outer membrane of gram–negative bacteria more readily than the natural penicillins. The greater ability of the penicillin derivatives to gain access to the PBPs may relate to their increased antibacterial activity against these gram–negative organisms (Graig and Ebert, ١٩٩٤).

٢-٧-٢- Cephalosporins :

Cephalosporins inhibit cell–wall synthesis by binding to bacterial enzymes located on the cell membrane. The antibacterial action of cephalosporins depends on their ability to penetrate the bacterial cell wall and bind with proteins in the cytoplasmic membrane. Once the drug damages the cell wall by binding with the PBPs, the body's natural defense mechanisms destroy the bacteria (Kaye,*et al.*,٢٠٠٠).To produce a bactericidal effect on the bacterial cell,the cephalosporins must reach the PBPs in sufficient concentrations to inhibit cell–wall synthesis and must resist the destructive action of the beta–lactamases produced by some bacteria which inactivate the drug by hydrolyzing its beta–lactam ring (Abrams,٢٠٠٠).

The major mechanism of resistance to beta–lactam antibiotics is beta-lactamase production(Graig and Ebert,١٩٩٤).Both plasmid-mediated and chromosomally–mediated beta–lactamase production can occur.Plasmid–mediated beta–lactamases are responsible for resistance to penicillins but not to antipseudomonal cephalosporins or carbapenems (Graig and Ebert,١٩٩٤).

Monotherapy, with either aztreonam or antipseudomonal cephalosporins or penicillins, can result in selection of stably derepressed mutants an induction mutation that produce massive amounts of chromosomally–mediated beta–lactamases (Sanders, ١٩٨٧). Although there are rare exceptions, the vast majority of beta–lactamases produced by *P.aeruginosa* are not affected by the beta– lactamase inhibitors such sulbactam, clavulanate or tazobactam (Godrey,*et al*.,١٩٨١).

Other mechanisms of resistance to beta–lactams are rare. A part from the loss of protein, diminished permeability of the bacterial outer membrane to beta–lactams accounts for some of the relative resistance of *P.aeruginosa* to beta–lactams (Angus,*et al*.,١٩٨٢).

٢-٧-٣- Carbapenems :

Carbapenems are broad–spectrum,bactericidal,beta–lactam antimicrobial agents. Like other beta–lactam drugs, they inhibit synthesis of bacterial cell walls by binding with PBPs (Abrams,٢٠٠٠).The group consists of two drugs; imipenem and meropenem.

The resistance of *P.aeruginosa* to the carbapenems has become an important issue in some hospitals in which resistance rates have reached greater than (٤٠٪).This resistance is mediated by a plasmid mediated metallo beta–lactamase (imipenemase) or unaltered outer membrane protein, and can be selected by heavy–imipenem use (Quim,*et al*.,١٩٨٨;Trias*,et al*.,١٩٨٩; Watanabe,*et al.,*١٩٩١ and Senda,*et al.,*١٩٩٦). Strains of *P.aeruginosa* that are resistant to carbapenems may also demonstrate reduced permeability of the bacterial outer membrane by loss of a porin protein (Lepper,*et al*.,٢٠٠٢).

Imipenem, the first drug of this type, has good activity against many gram-negative rods, gram-positive organisms, and anaerobes. It is resistant to B-lactamases but is inactivated by dihydropeptidases in renal tubules. Consequently, it is administered together with a peptidase inhibitor, cilastatin. Imipenem may be indicated for infections due to organisms resistant to other drugs, *Pseudomonas* species rapidly develop resistance, and the concomitant use of an aminoglycoside is therefore required; however, this does not delay the development of resistance. Such a combination may be effective treatment for febrile neutropenic patients. Meropenem is similar to in pharmacology and antimicrobial spectrum of activity. However, it is not inactivated by dihydropeptidases (Hugo and Russell,١٩٩٨).

٢-٧-٤- Aminoglycosides :

Aminoglycosides are a group of drugs sharing chemical antimicrobial pharmacologic and toxic characteristic (Baer and Williams,١٩٩٦). The group

mainly includes (streptomycin, neomycin, kanamycin, amikacin, gentamicin, tobromycin, sisomicin and netilmicin). All inhibit protein synthesis of bacteria by attaching to and inhibiting the function of the ٣٠ S subunit of the bacterial ribosome (Brooks *,et al.,*٢٠٠١).

Resistance to aminoglycosides includes two main mechanisms, enzymatic inactivation and decreased uptake. Aminoglycosides resistance in *P.aeruginosa* is most commonly due to aminoglycoside–inactivating (modifying) enzymes coded by genes on plasmids or on chromosome (Kaye,*et al*.,٢٠٠٠). Modification of the aminoglycosides by these enzymes results in weak binding to the ribosome the site of action of aminoglycosides. *P.aeruginosa* is active in producing such enzyme since it can produce at least (١٤) different aminoglycosides–modifying enzymes (Sabath,١٩٨٤). Reduced uptake of the aminoglycoside into the bacterium is, in some cases, a mechanism of emergence of resistance during therapy for *P.aeruginosa* infection. Cross–resistance for all aminoglycosides generally results, but the level of resistance is less than that resulting from enzymatic modification (Giamarellou and Antoniadou,٢٠٠١).

٢-٧-٥- Quinolones :

Quinolones exhibit concentration–dependent bactericidal activity and exert their activity by binding to bacterial topoisomerases (DNA gyrase) and by binding to bacterial target sites, quinolones interfere with DNA replication, DNA repair and transcription as well as other cellular functions, rapidly leading to bacterial death (Hooper and Walfson,١٩٩٣).

Resistance of *P.aeruginosa* to quinolones is a chromosomally mediated process. No clinical isolates exhibiting plasmid–mediated resistance have been

reported. Resistance to quinolones can occur by mutation of the chromosomal genes that code for the gyrases (Hooper and Walfson,١٩٩٣). Another mechanism of resistance is impaired penetration of the outer membrane of the organism.

٢-٧-٦- Monobactams :

Monobactams have a unique monocyclic beta–lactam ring. The naturally occurring monobactams are produced by bacteria found in soil and have weak antibacterial activity. Aztreonam is a synthetic monobactam with a narrow spectrum of activity that includes many gram-negative aerobic bacteria and has little or no activity against gram–positive aerobic and anaerobic bacteria (Abrams,٢٠٠٠).Aztreonam is the only available monobactam that possesses antipseudomonal activity and resembles aminoglycosides in it's activity, but ceftazidime is twice as active. Compared with ceftazidime, aztreonam is a poor inducer of the chromosomally mediated beta-lactamases,but plasmid-mediated beta-lactamases cause a moderate hydrolysis to aztreonam (Giamarellou and Antoniadou, ٢٠٠١).

Chapter Two

Materials And Methods

١- Materials :

١-١-The Instruments and apparatuses: **the necessary instruments and apparatuses available in the laboratory were worthily used for preparing the appropriate experiments in this study.**

١-٢- Culture Media :

The culture media shown in table (٢-١) below have been used for isolation and identification of bacterial isolates.

Table (٢–١) The Culture Media

Culture media	The manufacturing company
١. Nutrient agar	**Mast(UK)**
٢. Nutrient broth	**Mast(UK)**
٣. MacConkey's agar	**Mast(UK)**
٤. Blood agar	**Oxoid(UK)**
٥. Brain-Heart Infusion agar	**Mast(UK)**
٦. Brain-Heart Infusion broth	**Oxoid(UK)**
٧. Mueller-Hinton agar	**Difco(USA)**
٨. Simmon's citrate agar	**Oxoid(UK)**
٩. Kligler's iron agar	**Oxoid(UK)**
١٠.MR-VP medium	**Oxoid(UK)**
١١.Indole test medium	**Oxoid(UK)**

١-٣- Chemical : **Table (٢-٢) below shows the general chemical substances used in this study.**

Table (٢–٢) The Chemical Substances used in this Study

The chemical substances	The manufacturing company
١.stains and reagents	
Crystal violet	**Fluka(UK)**
Safranine	**Fluka(UK)**
Iodine	**BDH(UK)**
Hydrogen peroxide (H_2O_2)	**Oxoid(UK)**
Tetramethyl-p-phenylene-diamine dihydrochloride (oxidase test)	**Fluka(UK)**
Dimethylamine benzylaldehyde	**Fluka(UK)**
Methyl red reagent	**BDH(UK)**
٢.Acids, alkalines and alcohols	
Absolute ethanol	**BDH(UK)**
Amyle-alcohol	**BDH(UK)**
Acetone	**BDH(UK)**
Oleic acid	**Fluka(UK)**
Hydrochloric acid (HCL)	**BDH(UK)**
Sulfuric acid (H_2SO_4)	**Fluka(UK)**
Sodium chloride (NaCL)	**BDH(UK)**
Barium chloride ($BaCL_2.2H_2O$)	**BDH(UK)**
Calcium chloride ($CaCL_2$)	**BDH(UK)**
Potassium hydroxide (KOH)	**BDH(UK)**
Mercuric chloride ($HgCL_2$)	**BDH(UK)**
Glycerol	**Fluka(UK)**
α –naphthol	**BDH(UK)**
Ethylene diamine tetracetic acid (EDTA)	**Rideal(UK)**
B-complex vitamin	**MOW(India)**

١–٤- Antimicrobial Disks :

The antimicrobial disks shown in table (٢–٣) were used for detection the susceptibility of the *P.aeruginosa* isolates to these antibiotics. The potency of these antibiotics was checked first towards, gram–positive and gram–negative isolates represented by *Staphylococcus aureus* and *E.coli* respectively. The results of this experiment were recorded according to the standard guidelines recommended by National Committee for Clinical Laboratory Standards (NCCLs,٢٠٠٣).

Table (٢-٣) Antimicrobial Disks used in this Study

Antimicrobic agent *	Symbol	Concentration µg/disk	Diameters of inhibition zones (mm)		
			resistant	intermediate	susceptible
Penicillins					
Penicillin	**P**	١٠ **	≤١١	١٢-٢١	≥٢٢
Ampicillin	**Am**	١٠	≤١١	١٢-١٣	≥١٤
Piperacillin	**PRI**	١٠٠	≤١٧	-	≥١٨
Carbencillin	**PY**	١٠٠	≤١٣	١٤-١٦	≥١٧
Ticarcillin	**Tc**	٧٥	≤١٤	-	≥١٥
Cephalosporins					
Ceftizoxime	**ZOX**	٣٠	≤١٤	١٥-١٩	≥٢٠
Cefixime	**CFM**	٥	≤١٥	١٦-١٨	≥١٩
Cefotaxime	**CTX**	٣٠	≤١٤	١٥-٢٢	≥٢٣
Cefepime	**FEP**	٣٠	≤١٤	١٥-١٧	≥١٨
Aminoglycosides					
Amikacin	**AK**	٣٠	≤١٤	١٥-١٦	≥١٧
Gentamicin	**CN**	١٠	≤١٢	١٣-١٤	≥١٥
Tobromycin	**TOB**	١٠	≤١٢	١٣-١٤	≥١٥
Fluoroquinolone					
Ciprofloxacin	**CIP**	٥	≤١٥	١٦-٢٠	≥٢١
Norfloxacin	**NOR**	١٠	≤١٣	١٤-١٦	≥١٧

Tetracyclines					
Tetracycline	**TE**	٣٠	≤١٤	١٥-١٨	≥١٩
Doxycycline	**DO**	٣٠	≤١٢	١٣-١٥	≥١٦
Macrolide					
Erythromycin	**E**	١٥	≤١٣	١٤-٢٢	≥٢٣
Azithromycin	**AZM**	١٥	≤١٣	١٤-١٧	≥١٨
Dichloroacetic acid derivative					
Chloramphenicol	**C**	٣٠	≤١٢	١٣-١٧	≥١٨
Polymyxins					
Colistin	**CT**	١٠	≤١٨	٩-١٠	≥١١
RifamycinB derivative					
Rifampin	**RA**	٥	≤١٦	١٧-١٩	≥٢٠
Monobactams					
Aztreonam	**ATM**	٣٠	≤١٥	١٦-٢١	≥٢٢
Sulfonamide derivative					
Co-trimoxazole	**SXT**	٢٥	≤١٠	١١-١٥	≥١٦

*** All disks above from Bioanalyse company (Turk.).**

**** Express concentration by International Unit (IU)/ disk.**

١-٥- Antimicrobial Powders :

The following antimicrobial powders were used to determine the Minimum Inhibitory Concentration (MIC). The data concern with these antimicrobial are shown in table (٢-٤) together with the (MIC) standard values for *P.aeruginosa* as recommended by (NCCLs ,٢٠٠٣).

Table (٢-٤) The using Antimicrobial Powders for Minimum Inhibitory Concentration Test

Antimicrobic powder	The manufacturing company	Suggested ranges µg/ml *	Standard values µg/ml **		
			susceptible	intermediate	Resistant
Penicillin	Ajanta ,India	٠.٥-١٢٨	≤٨	-	≥١٦
Ampicillin	Ajanta ,India	٠.٥-١٢٨	≤٨	-	≥١٦
Cefixime	Anvaxx ,USA	٠.٥-١٢٨	≤١	٢	≥٤
Cefotaxime	Brown&burk,UK	٠.٥-٥١٢	≤٨	١٦-٣٢	≥٦٤
Ceftriaxone	Anvaxx ,USA	٠.٥-٥١٢	≤٨	١٦-٣٢	≥٦٤
Amikacin	Kee ,India	٠.٠٦-١٢٨	≤١٦	٣٢	≥٦٤
Gentamicin	Troge ,Germany	٠.٠٦-١٢٨	≤٤	٨	≥١٦
Ciprofloxacin	Brown&burk,India	٠.٠١٥-٨	≤١	٢	≥٤
Norfloxacin	Arbor ph., India	٠.٠١٥-٨	≤٢	٤	≥٨
Co-trimoxazole	Safa, Iraq	٠.٥-١٢٨	≤٣٢	-	≥٦٤
Doxycycline	Ajanta, India	٠.٥-١٢٨	≤٤	٨	≥١٦
Rifampin	Swone,India	٠.٥-١٢٨	≤١	٢	≥٤

***Suggested ranges from (Collee, *et al.*,١٩٩٦).**

****Standard values recommended by (NCCLs,٢٠٠٣)(Murray,*et al.*,٢٠٠٣).**

٢- Methods :

٢–١- Patients :

In this study ,٢٦٠ clinical swabs were collected from both sexes of different ages who referred to Surgical Teaching Hospital in Hilla, through October/ ٢٠٠٤ to May/ ٢٠٠٥.Thirty clinical swabs were positive for *P.aeruginosa*, ten of those swabs from burns, the other ten from chronic suppurative otitis media and the other last ten from UTI. Hence the specimens–swabs were burns exudates, ear exudates and urine respectively.

٢–٢- Collection of Specimens :

Those swabs were taken according to the methods suggested by (Collee, *et al.*,١٩٩٦). In ear infections: swabs moistened with Brain-Heart Infusion (BHI) broth or saline were taken after cleaning the external auditory canal (EAC) from cerumen and pus by ٧٠٪ ethyl alcohol. In burns: swabs were taken from the depth of burn or lesion, a dry swab must first be moistened with a little amount of (BHI)broth or saline. In UTIs: specimens of urine were generally collected in plastic universal sterile containers. All swabs and specimens must be transported to the laboratory without delay. The samples were immediately inoculated in, MacConkey's agar, blood agar and nutrient agar and incubated for overnight at ٣٧°c under aerobic conditions.

٢–٣- Sterilization :

The common culture media and solutions being used in this study were sterilized by the autoclaving.Furthermore,the sterility was checked by incubation the autoclaved media at ٣٧°c for overnight. The pH was adjusted at ٧.٢ for all culture media (Collee,*et al.*, ١٩٩٦). ____

٢–٤- Preparation the Culture Media :

The using culture media have been prepared according to the information which reported on the containers and sterilized by the autoclave and to ensure these media were not contaminated by put them in the incubator in the temperature ٣٧°c for ٢٤ hours and after that put them in the refrigerator.

The important culture media in the diagnosis have been prepared at the following method :

٢-٤-١- Blood Agar Medium (١٠٪) Human Blood :

This medium was used to cultivate bacterial strains and to study the type of blood hemolysis (Collee, *et al.* ,١٩٩٦).

٢–٤–٢- Gelatin Agar Medium :

It has used for detection of proteolytic activity or gelatin liquefaction. It was prepared by adding ٤.٤٪ of gelatin to nutrient agar medium (Macfaddin,١٩٧٩).

٢–٤–٣- Motility Medium :

This medium has been used to detect the motility of bacteria, prepared by dissolving ٤ gm agar with ١٠٠ ml of nutrient broth, and then dispensed into sterile test tubes (٥ ml in each) (Collee, *et al.*,١٩٩٦) .

٢–٤–٤- Lipolytic Agar Medium :

This medium was used to detect the ability of organism to produce lipase enzyme. It was prepared by dissolving ٠.١ gm of peptone, ٠.٢٥gm of sodium chloride, ٠.٠٠٥gm of calcium chloride and ٢ gm agar in ٥٠ ml of D.W. containing ٠.٥ ml of oleic acid according to (Chamberlain and Brueggemann, ١٩٩٧).

٢–٥- The Chemical Solutions and Reagents :

٢–٥ –١- Gram Stain Solutions :

Those were prepared and used according to the method recommended by (Macfaddin,١٩٧٩).

٢- ٥–٢- Catalase Test Reagent :

This reagent was prepared in ٣% using H_2O_2 as dilute, it was used to study bacterial ability to produce catalase enzyme (Baron,*et al.*,١٩٩٤).

٢– ٥–٣- Oxidase Test Reagent :

This reagent was prepared by dissolving ١gm (tetramethyl-paraphenylnene–diamine–dihydrochloride) in ١٠٠ ml D.W. and stored in dark bottle (Collee,*et al.*,١٩٩٦).

٢– ٥–٤- Frazier's reagent :

This reagent was prepared by dissolving ١٥ gm of mercuric chloride ($HgCl_2$) in ٢٠ ml of concentrated HCL ٩٨%, with the addition of ١٠٠ ml

D.W. It was used to detect the ability of bacteria to liquefy gelatin (Collee, *et al.*,١٩٩٦).

٢–٥–٥- Kovac's Reagent :

It was prepared by dissolving ٥gm of p–dimethylamine benzyladehyde in ٧٥ ml of amyle–alcohol and then ٣٥ ml of concentrated HCL was added. It was used to detect the Indol production (Macfaddin,٢٠٠٠).

٢–٥–٦- Methyl Red reagent :

It was prepared by dissolving ٠.١ gm of methyl red indicator in ٣٠٠ ml of ٩٥٪ ethanol and the volume was completed up to ٥٠٠ ml by using D.W. This reagent was used for detection complete glucose hydrolysis (Collee, *et al.*,١٩٩٦).

٢–٥–٧- Voges –Proskauer reagent :

It is composed of two solutions A and B, those solutions were prepared according to Macfaddin (٢٠٠٠) as follows :

A– α–naphthol, which was prepared by dissolving ٥ gm of α–naphthol in ١٠٠ ml of absolute ethylic alcohol.

B– Potassium hydroxide (KOH) solution which was prepared by dissolving ٤٠ gm of KOH in ١٠٠ ml of D.W. and was used to detect the partial glucose hydrolysis.

٢–٥–٨- McFarland Solution (tube No. ٠.٥) :

It is composed of two solutions, A and B and were prepared according to (Baron, *et al.*,١٩٩٤).

A–which was prepared by dissolving ١.١٧٥ gm of barium chloride ($BaCl_2.2H_2O$) in ١٠٠ ml D.W.

B–which was prepared by added ١ ml of concentrated sulfuric acid (H_2SO_4) in ١٠٠ ml D.W. After that add ٠.٥ ml from solution A to ٩٩.٥ ml of solution B. This solution used to compare the turbidity of bacterial suspension to $١٠^{٨}$ cell / ml.×obtain an approximate cell density of ١.٥

٢–٦- Identification :

The grown colonies on the nutrient agar with characterized diffusible pigments were selected for further diagnostic tests. The results of the following experiments regarding diagnosis at *P.aeruginosa* were according to (Baron and Finegold,١٩٩٠;Baron,*etal.*,١٩٩٤;Collee,*etal.*,١٩٩٦;Macfaddin,٢٠٠٠;Murray ,*etal.*,٢٠٠٣).

A.Cultural characteristics**: These characteristics include; colonial morphology (size of colony, it's color and the affect of it on the media such as blood hemolysis, diffusible pigments on nutrient agar and inability to ferment lactose on MacConkey's agar. The fluorescent characteristic water soluble pigment in broth was detected under UV-light (Jibran, ١٩٨٦).**

B.Microscopic Examination**: These include the examination of shape, gram–stain reaction, arrangement of cells with each other, motility and capsule presence.**

C. Biochemical Tests:

١. Oxidase Test:

A filter paper was moistened with several drops of freshly prepared oxidase reagent ١٪, then a small portion of the colony to be tested was picked up and rubbed on the moistened filter paper. Changing the color to blue or purple within ٣٠ seconds indicated for a positive test (Baron, *et al* ,١٩٩٤).

٢. Catalase Test:

Few drops of ٣% H_2O_2 were placed onto a portion of a pure colony on the clean, dry slide, the evolution of bubbles of gas, indicates for a positive test (Baron, *et al*.,١٩٩٤).

٣. Blood Hemolysis Test:

Blood agar medium was streaked with a pure colony of bacteria and incubated at ٣٧°c for ٢٤–٤٨ hours. The appearance of a clear zone around

the colony indicates for ß–hemolysis while the presence of green–color indicates for α–hemolysis (Baron, *et al.*,١٩٩٤).

٤. Gelatin Liquefication Test:

Gelatin agar medium was inoculated with the colony of tested organism and incubated for ٢٤ hours in ٣٧°c. Then, the plates were flooded by the solution of Frazier reagent for ٥–١٠ minutes. Gelatin–liquefying was identified by the present of clear zone around the tested colony (Collee, *et al.*,١٩٩٦).

٥. Motility Test:

Tubes containing motility medium were stabbed once at the center with on inoculating needle then, incubated at ٣٥°c for ٢٤–٤٨ hours. The motile bacteria spread out from the line of inoculating (Baron and finegold ,١٩٩٠).

٦. Citrate Utilization Test:

The surface of simmon's citrate slant medium was inoculated with colony of the tested bacteria and incubated at ٣٧°c for ١–٣ days. Conversion of the medium color from green to blue indicates for the ability of utilize citrate as a sole carbon source (Macfaddin,٢٠٠٠).

٧. Kligler's Iron Agar Test for H_2S Production:

Only the colonies grown on MacConkey's agar were touched by a straight wire and inoculated on to medium by stabbing the buttom of the tube and streaking the surface of slant. Fermentation was detected by a change in the indicator phenol red to yellow. The pH changes in the buttom

and the slant of medium were recorded after ١٨–٢٤ hours of incubation. Gas formation is usually visualized as bubbles in the medium caused by the gas formed in the agar. For reaction of black precipitate in the buttom indicates for the production of H٢S (Baron and Finegold,١٩٩٠).

٨. Growth at ٤٢°c:

P.aeruginosa **has a characteristic ability to grow at ٤٢°c in successive subculture. Tubes containing nutrient broth were inoculated with selected colonies and incubated at ٤٢°c for ٢٤ hours. The ability to grow was indicated for the positive result (Jibran,١٩٨٦)).**

٩. Indol Test:

Tubes containing peptone water were inoculated with the colony of tested bacteria and incubated at ٣٧°c for ٤٨ hours, then several drops of kovac's reagent were added to the broth medium with gentle. The appearance of red ring on the surface liquid medium was regarded as a positive result (Cruickshank, ***et al.,*** **١٩٧٥).**

١٠. Methyl Red Test:

Tubes containing MR–VP broth were inoculated with the colony of tested bacteria and incubated at ٣٧°c for ٤٨ hours. To test culture acidity ٠.٥ ml of methyl red reagent was added. A positive result was detected by the appearance of red color (Baron and finegold ,١٩٩٠).

١١. Voges – Proskauer Test:

MR–VP medium was inoculated with the colony of tested bacteria and incubated at ٣٧°c for ٤٨ hours, ٠.٠٦ ml of α–naphthol reagent and ٠.٢ ml of ٤٠٪ KOH solution were added. After ١٥ minutes, the formation of red color is indicative for the presence of aceton (acetyl methyl carbinol) (Baron and finegold,١٩٩٠).

١٢. Lipase Test:

Lipolytic agar medium was inoculated with the colony of tested organism and incubated for ٢٤ hours in ٣٧°c. Positive lypolysis is represented by the presence of halo of precipitate surrounding the colony (Symth and Alford,١٩٨٤).

٢–٧- Stock Culture :

The diagnosed bacterial isolates were stored for the daily use in BHI broth which contains ٥٪ glycerol and then freezed under -٢٠°c for long time (Fakhridden,٢٠٠١). The isolates were re-subculture at interval approximately one month.

٢–٨- Sensitivity Tests to Antimicrobials :

The susceptibility of *P.aeruginosa* isolates were determined by two methods: disk diffusion method and Minimal Inhibitory Concentration (MIC) as follows:

A. The Kirby–Bauer **standardized single disk method was carried out (Bauer,*et al.*,١٩٦٦).**

١. Preparation of Culture Media

-Mueller–Hinton medium was employed for this experiment. The medium was cooled to ٤٥–٥٠°c and autoclaved appropriate volume was poured in the Petri dishes.

-When the medium solidified, the Petri dishes were incubated at ٣٧°c for ٣٠ minutes to evaporate the excess moisture.

٢. Preparation of the inoculum

-With a sterile wire loop, the tops of ٤–٥ pure colonies were transferred to a tube containing ٥ ml of BHI broth.

-The broth was incubated at ٣٧°c until it's turbidity standard. This usually required at least ٤–٦ hours incubation. The cells density was estimated as ١.٥×١٠^{٨} cell /ml by comparison with McFarland standard tube No.٠.٥ .

٣. Inoculation

Within ١٥ minutes of adjusting the density of the inoculum, sterile cotton swab on a wooden applicator stick was dipped into the standardized bacterial suspension. The excess fluid was removed by rotating the swab with firm pressure against the inside of the tube above the fluid level. The swab was then used to streak the dried surface of a Mueller–Hinton plate in ٣ different planes(by rotating the plate approximately ٦٠ each time) to obtain an even distribution of the inoculum.

The plate lids were replaced and the inoculated plates were allowed to remain on a flat and level surface undisturbed for ٣ to ٥ minutes to allow absorption of excess moisture.

٤. Application of the Disks

With a sterile forceps, the selected disks were placed on the surface of medium and pressed firmly but gently into the agar with sterile forceps. Within ١٥ minutes the inoculated plates were incubated at ٣٧°c for ١٨ hours in an inverted position.

٥. Reading of Results

After incubation the diameters of the complete inhibition zones were noted measured by using a ruler. The end point, measured to the nearest millimeter was compared with zones of inhibition determined by NCCLs (٢٠٠٣) and to decide the susceptibility of bacteria to antimicrobial agent, whether being resistant or sensitive.

B. Determination of Minimum Inhibitory Concentrations (MIC)

The minimum inhibitory concentrations were measured for each antimicrobial agents toward the bacterial isolates according to the method recommended by Baron, *et al.*,(١٩٩٤) and Collee, *et al.*,(١٩٩٦) depending on the turbidity of the bacterial growth. The MIC was recorded as the lowest concentration prevents the bacteria under test to grow.

١. Preparation of the Culture Media

The brain–heart infusion broth was used prepared in the paragraph number (٢–٢–٤).

٢. Preparation of the bacterial Suspension

The bacterial suspension which specific for MIC tests were prepared by transport a pure bacterial growth from overnight growth on blood agar plate directly suspended in screw tube contain ٥ ml of normal saline.

The broth was incubated at ٣٧°c for ٤–٦ hours until it's turbidity compared to that of the recommended turbidity standard.

٣. Preparation Stock Solution

A suitable weight from antimicrobial powder was dissolved in volume of sterilized D.W. to obtain the required concentration. Serial dilutions from the initial concentration were prepared by the two fold dilution method in the liquid culture media (BHI).

٤. Procedure

In this study the macro-dilution method was used as follows :

- Nine sterile test tubes (for each antibiotic) were numbered from ١ to ٩ .

- ١ ml of BHI broth was dispensed in each tube.

-١ml of the antimicrobial solution was added to tube No.١ using a sterile pipette and mixed well. ١ ml of the mixture was transferred to tube No.٢ . The contents of tube ٢ were then mixed thoroughly and ١ ml was transferred to tube ٣ . This process was continued up to tube No.٩ from

which ١ ml was discarded, consequently, the final volume in each tube was ١ ml and the final concentrations of the antibiotic ranged from ٠.٥–١٢٨ µg/ml. The ١ ml of the bacterial suspension previously prepared was added for each tubes. In this experiment, two tubes were used as control. The first one contained ١ ml of BHI broth plus ١ ml of bacterial suspension, while the second control called negative growth control which contained only ٢ ml of BHI broth.

-All the test tubes were incubated at ٣٧°c for ١٨–٢٤ hours. the MIC was recorded as the lack of visual growth relatively to the controls.

٢–٩- The combined effect of some antimicrobials against *P.aeruginosa* in vitro :

Six identified isolates of *P.aeruginosa* were selected for this experiment according to their remarkable resistance to the tested antibiotics. The experiment included ten antibiotics table (٢-٥). The method of Half–Chess board recommended by Garrod (١٩٧٨).

The results were compared with control and they interpreted as follows: growth similar to that of control is static action. Reduced number of colonies is partial cidal action. Small number of colonies is complete cidal action and finally if there is no growth, it is total cidal action.

Table (٢–٥) Suitable Concentrations of Antibiotics for Tests of Cidal Activity (Garrod ,١٩٧٨)

Antimicrobial agent	Half-chess board final concentration in broth µg/ml
Penicillin	١٠
Ampicillin	١٠
Cefotaxime	١٠
Ceftriaxone	١٠
Amikacin	٥
Gentamicin	٥
Ciprofloxacin	٥
Norfloxacin	٥
Co-trimoxazole	١٠
Rifampin	٥

Table(٢-٦)Diagram of Half-Chess Board technique

antimicrobial	P	Am	CTX	CRO	AK	CN	CIP	NOR	SXT	RA
P	١ ml p	١ml p ١ml Am	١ml p ١ml CTX	١ml p ١ml CRO	١ml p ١ml AK	١ml p ١ml CN	١ml p ١ml CIP	١ml p ١ml NOR	١ml p ١ml SXT	١ml p ١ml RA
Am		١ml Am	١ml Am ١ml CTX	١ml Am ١ml CRO	١ml Am ١ml AK	١ml Am ١ml CN	١ml Am ١ml CIP	١ml Am ١ml NOR	١ml Am ١ml SXT	١ml Am ١ml RA
CTX			١ml CTX	١ml CTX ١ml CRO	١ml CTX ١ml AK	١ml CTX ١ml CN	١ml CTX ١ml CIP	١ml CTX ١ml NOR	١ml CTX ١ml SXT	١ml CTX ١ml RA
CRO				١ml CRO	١ml CRO ١ml AK	١ml CRO ١ml CN	١ml CRO ١ml CIP	١ml CRO ١ml NOR	١ml CRO ١ml SXT	١ml CRO ١ml RA
AK					١ml AK	١ml AK ١ml CN	١ml AK ١ml CIP	١ml AK ١ml NOR	١ml AK ١ml SXT	١ml AK ١ml RA
CN						١ml CN	١ml CN ١ml CIP	١ml CN ١ml NOR	١ml CN ١ml SXT	١ml CN ١ml RA
CIP							١ml CIP	١ml CIP ١ml NOR	١ml CIP ١ml SXT	١ml CIP ١ml RA
NOR								١ml NOR	١ml NOR ١ml SXT	١ml NOR ١ml RA

SXT									١ml SXT	١ml SXT ١ml RA
RA										١ml RA

P: penicillin, Am: ampicillin, CTX: cefotaxime, CRO: ceftriaxone, AK: amikacin, CN: gentamicin, CIP: ciprofloxacin, NOR :norfloxacin, SXT: co-trimoxazole, RA: rifampin.

٢–١٠- The Combined effect of Ceftriaxone plus Gentamicin on *P.aeruginosa* isolates :

Some selected antimicrobials were combined together in appropriate concentrations to determine the action against *P.aeruginosa* compared with their action singly. These antibiotics are presented now. The combined action of Ceftriaxone plus Gentamicin was tested against the same six isolates of *P.aeruginosa* which used in the experiment above. All susceptibility tests were performed in BHI broth. The concentrations of Ceftriaxone ranged from ٨,٤,٢,١,٠.٥,٠.٢٥ µg/ml and the concentrations of Gentamicin ranged from ٤,٢,١,٠.٥,٠.٢٥,٠.١٢ µg/ml. MICs of Ceftriaxone and Gentamicin, alone and in combination, were determined by a checker board technique which recommended by (Garrod,١٩٧٨) as shown in table (٢–٧).

Table (٢–٧) Diagram of Checker Board Technique with Different Concentrations of Drug A :Gentamicin and Drug B : Ceftriaxone

Drug A	٤	٢	١	٠.٥	٠.٢٥	٠.١٢	-
Drug B	٨	٨	٨	٨	٨	٨	٨
Drug A	٤	٢	١	٠.٥	٠.٢٥	٠.١٢	-
Drug B	٤	٤	٤	٤	٤	٤	٤
Drug A	٤	٢	١	٠.٥	٠.٢٥	٠.١٢	-
Drug B	٢	٢	٢	٢	٢	٢	٢
Drug	٤	٢	١	٠.٥	٠.٢٥	٠.١٢	-

A Drug B	١	١	١	١	١	١	١
Drug A Drug B	٤ ٠.٥	٢ ٠.٥	١ ٠.٥	٠.٥ ٠.٥	٠.٢٥ ٠.٥	٠.١٢ ٠.٥	- ٠.٥
Drug A Drug B	٤ ٠.٢٥	٢ ٠.٢٥	١ ٠.٢٥	٠.٥ ٠.٢٥	٠.٢٥ ٠.٢٥	٠.١٢ ٠.٢٥	- ٠.٢٥
Drug A Drug B	٤ -	٢ -	١ -	٠.٥ -	٠.٢٥ -	٠.١٢ -	**Nil ***

***Nil : control tube**

٢–١١- Synergistic Effect of EDTA with

Some Antibiotics on *P.aeruginosa* Isolates :

In this experiment two different techniques were used as follows:

A. The Minimum Inhibitory Concentration

Serial dilutions by two-fold dilution method were prepared from the initial concentration of ampicillin ranged ١٢٨,٦٤,٣٢,١٦,٨,٤ µg/ml and cefixime with concentrations ranged ٣٢,١٦,٨,٤,٢,١ µg/ml.

In this experiment the agar-dilution method was used which recommended by (Garrod,١٩٧٨; Baron,*et al.*,١٩٩٤; Butt, *et al.*,٢٠٠٣). Mueller-Hinton agar was used, concentrations of antimicrobial agents were incorporated into agar plates ,one plate for each concentration to be tested. The isolates to be tested were diluted to a slightly greater turbidity than that of a McFarland standard tube No.٠.٥. Each plate contain from ١ml of

diluent's antimicrobial agent and ١٤ml of agar. After the plates dried ,inoculated with a wire loop spread over a small area and incubated for ٢٤ hours at ٣٧°c.The lowest concentration of the antibiotic that allows a slight growth is the MIC.

B. The Agar–Well Diffusion Method

١.The culture media (Mueller–Hinton agar) plates were prepared by the routine method.

٢.The bacterial suspension was prepared as follows, with a sterile loop,٤-٥ isolated colonies from overnight pure culture were transferred to a tube containing ٥ ml of BHI broth. The broth was incubated at ٣٧°c for ٤-٦ hours. Then, the turbidity of bacterial suspension matching with the turbidity of the McFarland tube No.٠.٥ by using the normal saline.

٣. Sterile cotton swab was dipped into the bacterial suspension and streaked the dried surface of the Mueller–Hinton plates in ٣ different planes.

٤.The wells on the Mueller-Hinton plates were prepared by using Pasture pipette with a diameters of ٤ mm. The appropriates concentrations of the antibiotics ٦٤,٣٢,١٦,٨ µg/ml for ampicillin and ١٦,٨,٤,٢ µg/ml for cefixime were dropped in the wells (٠.١ml from each concentration)and the EDTA in a concentration of ٥٠ mg/١٠٠ ml with amount ٠.١ ml in the specific well.

٥.The plates were incubated at ٣٧°c for ٢٤ hours, then the activity of the mixture was detected by determine the inhibition zone around the wells as recommended by (Aulton,١٩٨٨ and Butt, *et al.*,٢٠٠٣) .

٢–١٢- Synergistic Effect of B–Complex with Some

Antibiotics on *P.aeruginosa* Isolates :

A. The Minimum Inhibitory Concentration

The combined action of B-complex plus amikacin, cefotaxime, gentamicin, ciprofloxacin and ceftriaxone against ٥ resistant isolates of *P.aeruginosa* was tested. Serial dilutions by two–fold dilutions method were prepared from the initial concentration of each antibiotic: ciprofloxacin by concentration ٢,١,٠.٥,٠.٢٥,٠.١٢,٠.٠٦ µg/ml,amikacin,cefotaxime, ceftriaxone by concentration ٦٤,٣٢,١٦,٨,٤,٢,١ µg/ml, gentamicin ٣٢,١٦,٨,٤,٢,١,٠.٥ µg/ml and B-complex by concentration ٠.٢ ml/١٠٠ ml sterilized D.W (Ichimiya, *et al.*١٩٩٤).

In this experiment the macro-broth dilution method was used. BHI broth was used plus diluent's antibiotic and inoculated the tubes with (١)ml of the bacterial suspension after matching with the turbidity of the McFarland tube No.(٠.٥). Incubated the test tubes for ٢٤ hours at ٣٧°c.

The lowest concentration of the antibiotic that inhibits the growth of the bacteria was detected as the lack of visual growth relatively to the controls.

B. The Agar-Well Dilution Method

This experiment was carried out exactly as (٢-٢-١١.B) ,but use the concentrations ١,٠.٥,٠.٢٥,٠.١٢ µg/ml for ciprofloxacin and the concentrations ٦٤,٣٢,١٦,٨ µg/ml for ceftriaxone as well as the concentration of B-complex also ٠.٢ ml/١٠٠ ml sterilized D.W.

Chapter Three

Results And Discussion

١- Diagnosis of *P.aeruginosa* :

The preliminary cultural diagnosis for bacterial isolates exhibited that all the thirty ٣٠ isolates characterized by circular rough colonies with large size relatively with long axis in the line of the inoculums streak surrounded by a serrated strict of growth, except some isolates of urinary tract infections (UTIs) characterized by circular smooth colonies with small size. All the identified isolates produced the diagnostic diffusible pigments on nutrient agar, although the pigments varied according to the isolates between bluish–green in burns and UTIs isolates to yellowish–green in color in otitis media isolates and all those isolates produced a sweet grape–like odor which was easily recognized (Morrison and Wenzel, ١٩٨٤; Gorbach, *et al.*,١٩٩٨ and Brooks, *et al.*,٢٠٠١).

All the isolates grew on ١٠٪ human blood agar. Some isolates which included: ٢ isolates of otitis media, ٣ isolates of burns and ٣ isolates of UTIs were hemolyzed blood with ß-hemolytic type and the remained isolates did not hemolyze blood. All those isolates grew on MacConkey's agar, but did not ferment lactose sugar(Baron,*etal.*,١٩٩٤;Collee,*etal.*,١٩٩٦).Under the microscope, the bacterial cells appeared as short bacilli arranged in single or short chain, negative for Gram–stain reaction, motile,non–spore forming and without capsule (Collee,*et al.*,١٩٩٦). In this study some biochemical tests, table (٣–١) were carried out and the results were compared with standard results documented by (Baron and Finegold,١٩٩٠;Baron,*etal.*,١٩٩٤;Collee,*etal.*,١٩٩٦;Macfaddin,٢٠٠٠). Accordingly the results revealed the characteristic closely related to the *P.aeruginosa* were inagreement with referential results. For the practical

purposes, isolates on blood agar that exhibited the characteristic colonial morphology and odor, that are ß–hemolytic and oxidase–positive can be presumptively identified as *P.aeruginosa* (Baron and Finegold,١٩٩٠). Since no other bacteria produce the water soluble or media diffusible pigments, so it's detection is sufficient for identification at on isolate as *P.aeruginosa* (Baron and Finegold,١٩٩٠). Moreover, all pigments producing *P.aeruginosa* (PPPA) are able to grow at (٤٢°c),an ability which regarded as confirmative character to (PPPA) (Baron and Finegold,١٩٩٠).All these observations were considered through the identification at *P.aeruginosa* isolates diagnosed in this study.

Table (٣–١) Biochemical Tests for Bacterial Isolates in this Study

Test	Result
Catalase test	+
Gelatin Liquefication	+
Gram-stain	-
Growth at ٤٢°c	+
Hemolysis(ß-hemolysis)	+/-
H_2S production	-
Indole test	-
Kligler's iron agar	K/no change
Lipase test	+
Methyl-red	-
Motility	+
Oxidase test	+
Pigments production	+
Simmon's citrate	+
Voges-Proskauer	-

+ : positive test, - : negative test, K : Alkaline.

٢- Risk Factors for Infection :

Table (٣–٢) shows the effect of some risk factors which related with the infection of *P.aeruginosa* strains. The results of the present study showed significant difference (p<٠.٠٥) between males and female, since that the number of infected male accounted ١٨(٦٠٪) versus ١٢(٤٠٪)from infected females. Such variation in pseudomonal infections were also reported by other studies (Anonymous,١٩٩٧;Suman,*et al.*,٢٠٠٥).However,Nihad and his colleagues (٢٠٠٢) stated that no obvious effect of the sex on distribution of *P.aeruginosa* except in case of UTIs, since it has been found that ٢٦ (٧٨.٧٨ %) of isolates were from infected female while only ٧(٢١.٢١%) were from infected male.There differences may be attributed to social and anatomical differences between males and females. These results belong to reasons related with male patients in this study such as smoking and swimming in some patients infected by otitis media ,use of catheters in some cases of (UTIs) causing catheters associated UTIs as well as the less care by hospital and prolonged hospitalization (Herfindal and Gourley, ٢٠٠٠;Anonymous ٢٠٠٢).

Regarding the age factor, the results showed significant difference (p<٠.٠٥)with respect to the infection of *P.aeruginosa.* Since the age group (١-١٥) years and (٤٦–٦٠)years were more susceptible for infections, that the percentages accounted for (٣٦.٧٪)and (٤٠٪) respectively. Herfindal and Gourley (٢٠٠٠) stated that children and older patients were found to be more susceptible to pseudomonal infections compared with other age groups because some disorders such as immunodeficiency, malnutrition as

well as the poor hygienic conditions may increase the chance of pseudomonal infections (Hauser and Sriram ,٢٠٠٥).

Table (٣–٢) Risk Factors for Infection

Risk factor	Infection of *P.aeruginosa* (n=٣٠)%
١.The sex : the males the females	١٨(٦٠٪) ١٢(٤٠٪)
X^2 Cal.=٥.٣٣٥	X^2 Tab.=٣.٨٤
٢.The age(year): (١-١٥) (١٦-٣٥) (٣٦-٤٥) (٤٦-٦٠)	١١(٣٦.٧٪) ٤(١٣.٣٪) ٣(١٠٪) ١٢(٤٠٪)
X^2 Cal.=١٧.٥٤	X^2 Tab.=٧.٨١
٣.Immunocompromised states	١٧(٥٦.٧٪)
٤.Prolonged antimicrobial use	٢٤(٨٠٪)

*S : Significant Difference (p<٠.٠٥)

The results of the present study were also indicated that infections among patients with immunocompromised states accounted for ١٧(٥٦.٧٪).Owlia and his coworkers(٢٠٠١) pointed out that the patients with cancer who have neutropenia resulting from chemotherapy or hematologic malignancies were more susceptible for infection by *P.aeruginosa*. This bacteria is one of the major opportunistic human pathogens that causes serious and sometimes fatal infection in

immunocompromised hosts. Examples of compromising conditions include disrupted physical barriers to bacterial invasion (e.g. burn injuries, IV lines, urinary catheters, dialysis catheters, endotracheal tubes) and dysfunctional immune mechanisms, such as those occurring in neonates, cystic fibrosis (CF), AIDs, neutropenia, complement deficiency, hypogammaglobulinemia, and iatrogenic immunosuppression (Herfindal and Gourley,٢٠٠٠).

Prolonged antimicrobial use as treatment for pseudomonic patient is seemed to be another risk factor for infection with *P.aeruginosa* especially multidrugs–resistant *P.aeruginosa* (MDRPA) (Obritsch,*et al.*,٢٠٠٥).The results obtained from this study were quite in accordance with such results,since most isolates were detected in a percentage of (٨٠٪) of patients who were already under different chemotherapy courses. *P.aeruginosa* seems to evolve fast against antibiotics,either physically adaptation and/or tolerance or genetically represented by intrinsic resistance (Murray,١٩٩٧;Rahal,*et al.*,٢٠٠٢). On the other hand Sorberg and his coworkers (٢٠٠٢) stated that in hospitalized patients who exposed to numerous antimicrobial agents, the intrinsic and acquired resistance enable the organism to survive, subsequently and the chance for infection will increase as well as the distribution of the organism around the environment particularly hospital environment will increase. Unproperly use of antibiotics may stimulate *P.aeruginosa* to resist antibiotics (Livermore, ٢٠٠٢).

٣- Site of Infection :

P.aeruginosa **causes a broad spectrum of infections which are associated with urinary,respiratory and gastrointestinal tract,burn, wound,eyes,ears,as well as with other sites(Herfindal and Gourley,٢٠٠٠).Table (٣–٣) shows the distribution of *P.aeruginosa* according to the site of infection. The results of the present study showed that burns are the most accessible site by *P.aeruginosa* ٣١.٣٪. This may be ascribe to the highly distribution of this organism around the hospital environment as previously mentioned and the exposure to the burns wounds to that environment. The results were in agreement with those results obtained by other similar studies of (Kolmos, *et al.*,١٩٩٣ ;Xu, *et al.* ٢٠٠٢).**

Table (٣–٣) Frequency of *P.aeruginosa* according to the Site of Infection

Type of sample	Total	*P.aeruginosa* isolates n(%)
Ear swabs	٥٨	١٠(١٧.٢٪)
Urine samples	١٧٠	١٠(٥.٩٪)
Burn swabs	٣٢	١٠(٣١٪)
Total	٢٦٠	٣٠(١١.٥٪)

(Kolmos,*etal.*,١٩٩٣) pointed out that *P.aeruginosa* is an important pathogen in burned patients,٢٥–٢٩٪ of burned patients become colonized during the course of their disease ,and a quarter of colonized patients develop invasive infections. Septicemia is associated with a ٢٨٪ increase in mortality, because this bacteria has complex virulence factors include

extracellular enzymes and toxins and cellular compounds such as lipopolysaccharide as well as motility. On the other hand, host–related factors favouring infection with *P.aeruginosa* include the depth and extent of burns, old age, long stay in hospital and systemic or topical treatment with broad–spectrum antimicrobial agents. In recent years there has been a decline in severe *Pseudomonas* infections, which has been ascribed to faster surgical removal of all necrotic tissue, followed by immediate grafting, and the introduction of topical silver compounds. Better isolation facilities presumably also play a role, since they result in a delay in colonization with *P.aeruginosa* to a time when wound healing has decreased the risk of invasive infection (Kolmos,*et al.*,١٩٩٣).

Cross–infection due to failure by staff to disinfect their hands is probably the main route of spread of *P.aeruginosa* in burns units. Contaminated bath equipment and mattresses have been identified as sources of infection, but on the whole there is little evidence that patients become infected with *P.aeruginosa* from inanimate sources, despite the fact that the organism is frequently found in the environment of a burn unit (Sidorenko,*etal.*,١٩٩٩;Igumbor,*etal.*,٢٠٠٠; Bertrand,*et al.*,٢٠٠٠; Fakhridden, ٢٠٠١; Hauser and Sriram, ٢٠٠٥).

Return to the site of infection,*P.aeruginosa* in UTIs less than burns and otitis media infections.These results are relatively acceptable,since they comparable with the results being reported by (Verhaz,*et al.*,٢٠٠٣) and different with (Nihad,*et al.*,٢٠٠٢).Bonaventura and his coworkers (١٩٩٨) who pointed that the presence of an indwelling catheter was associated with a decreased incidence of *E.coli* infections and an increased incidence of *P.aeruginosa*. VanEldere (١٩٩٩) stated that catheters introduce an artificial substratum into the body and cause persistent infections.These

recurrent infections are mainly due to the accumulation of mixed biofilms on the artificial surface of the catheter or other implant.A biofilm on an indwelling urinary catheter consists of adherent microorganism, their extracellular products and host components deposited on the catheter. Biofilm on urinary catheters results in persistent infections that are resistant to antimicrobial therapy. However, the highest percentage of resistance was seen to gentamicin. It was observed that strains which showed resistance to gentamicin also showed significant increase in biofilm production.

(Hauser and Sriram,٢٠٠٥) pointed out that *P.aeruginosa* was the most common bacteria isolated from mild–to–severe form of otitis externa and chronic suppurative otitis media. Moreover, involvement of the ear can present as a mild, superficial, and often self-limited infection (e.g. swimmer's ear) or as malignant otitis externa which presents with a history of non resolving otitis externa, especially in patients who have diabetes or AIDs. However, results can be variable from area to area from patient to patient ,since hygienic conditions, epidemiological conditions, patient status, the severity of infection and irrational use of antibiotics, may contribute in the types and frequencies at causative agents of infection (Verhaz,*et al.*,٢٠٠٣).

٤-Distribution of *P.aeruginosa* Infection between Outpatients and Inpatients :

In attempt to determine the frequency of pseudomonal infection in and out of the hospital environment, the data obtained in this study were analyzed. This analysis may indicate the rate of pseudomonal distribution

within their environments. The results came as it was expected. Figure (٣-١) showed the rate of infection within hospitalized patients accounted for ١٧(٥٦.٧٪) versus ١٣(٤٣.٣٪) in non- hospitalized individuals(out patients).The results were in agreement with almost all results being reported worldwide (Chenoweth and Lynch,١٩٩٧;Blahova, *et al.*,٢٠٠٠;Brooum, *et al.*,٢٠٠٠;Oliver, *et al.*, ٢٠٠٠; Rahal, *et al.*,٢٠٠٢; Normark B.H and Normark K.S,٢٠٠٢; Hauser and Sriram,٢٠٠٥).

From this, it can be conclude that hospital environment perhaps constitutes a habitat for microorganisms and a focus for subsequent infections or what they called (hospital-acquired infections). Furthermore the attention should be drawn to this phenomenon, since the bacterial isolates resident in hospital are more aggressive in relative with the wild types isolate, since there isolates are selectively survive the hospital policies regarding disinfection, sterilization and chemotherapy. For this, *P.aeruginosa* was described as a common pathogen in hospitals, especially in the intensive care units(ICUs) and it has always been difficult to eradicate (Sanders,١٩٨٧; Rahal, *et al.*,٢٠٠٢).

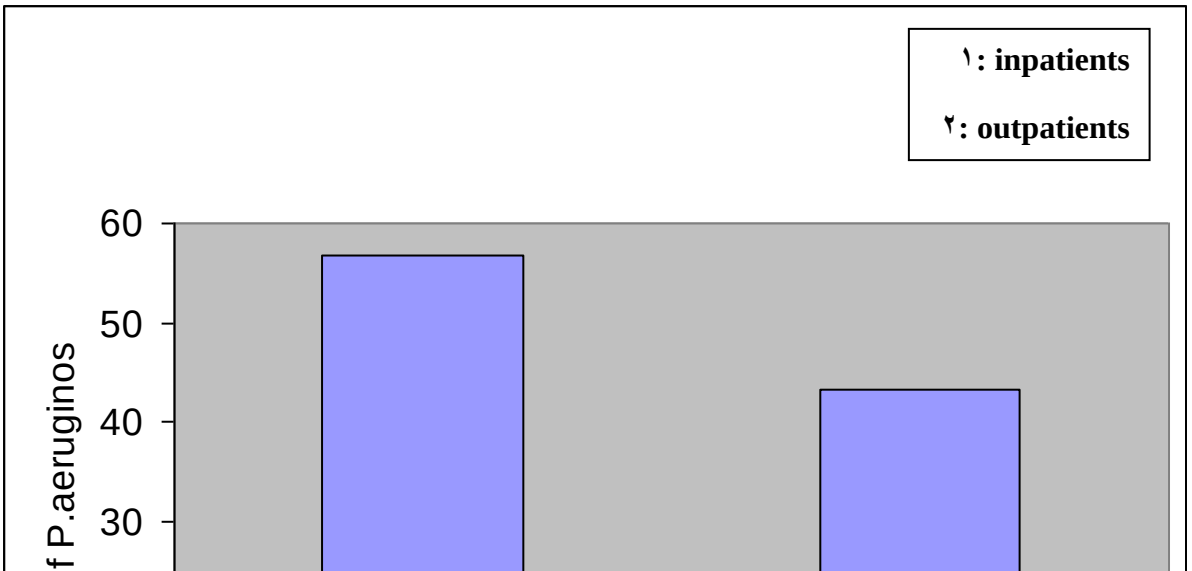


Figure (٣-١) The Ratio of *P.aeruginosa* in Patients.

٥- Antimicrobial Resistance in *P.aeruginosa* :

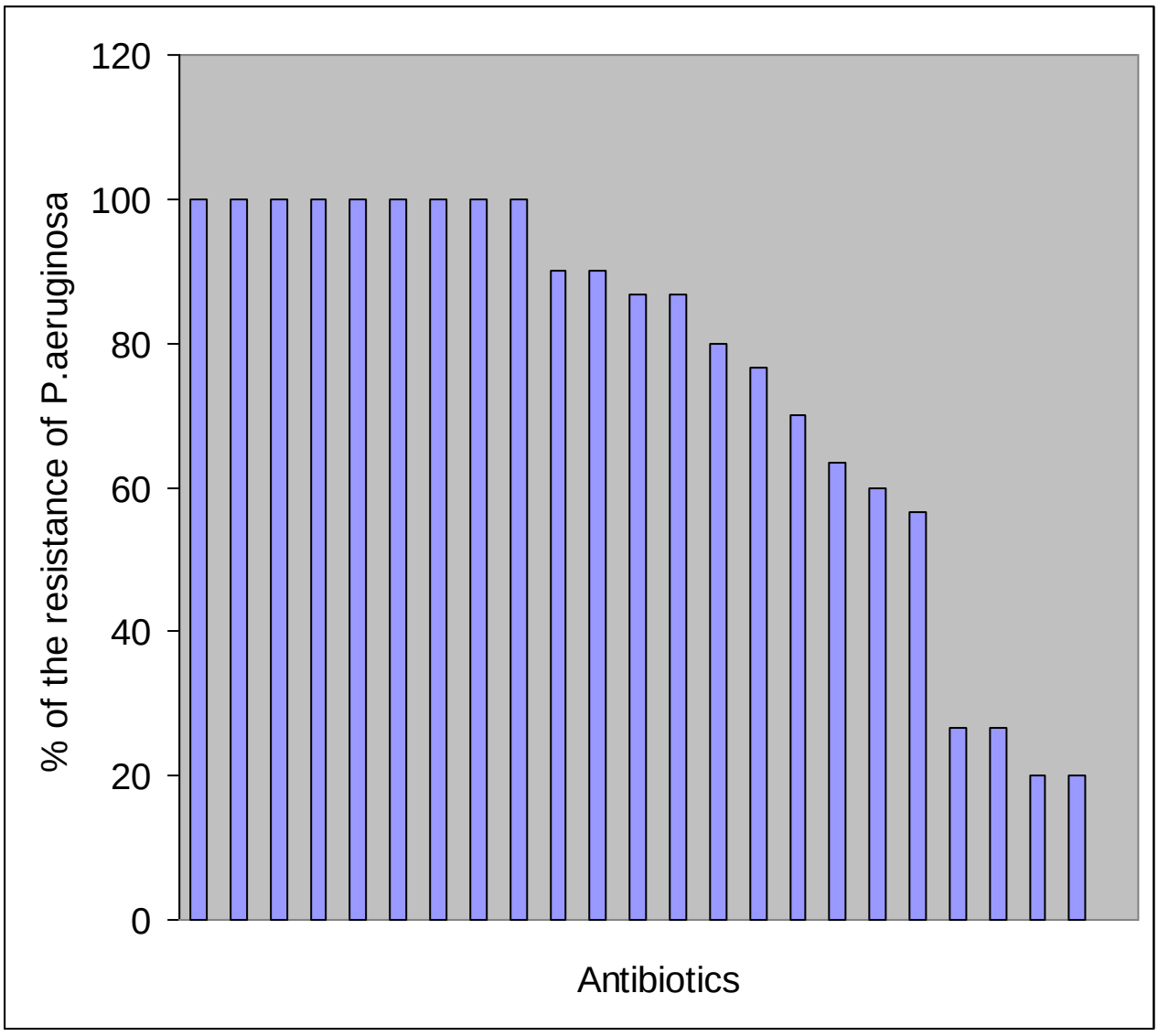
Results shown in figure (٣–٢) reveal a remarkable increase in Pseudomonal resistance to beta-lactam antibiotics represented by penicillin, ampicillin, carbencillin, cefixime, cefotaxime, ceftizoxime, ticarcillin, piperacillin and cefepime, since the level of resistance accounted for the first four antibiotics (١٠٠٪) to for (٩٠٪) , (٨٦.٧٪), (٨٠٪), (٧٦.٧٪)and

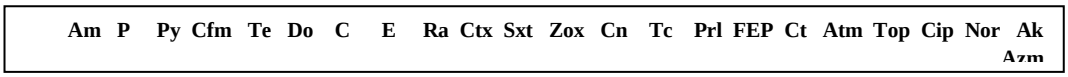
(٧٠٪) for cefotaxime, ceftizoxime, ticarcillin, piperacillin and cefepime respectively. The resistance to the penicillins and cephalosporins has become an important issue in most hospitals in which resistance rate have reached greater levels. These results were inagreement with those results being obtained by other studies of (Jibran,١٩٨٦; Igumbor, *et al.*,٢٠٠٠; Mehta, *et al.*, ٢٠٠١; De–Freitas and Barth, ٢٠٠٢ ; Nihad , *et al.*, ٢٠٠٢; Zhang , *et al.*,٢٠٠٢).

The beta-lactamases have been reported to hydrolyze all antipseudomonal agents.Moreover,*P.aeruginosa* cells particularly in patients with chronic infections can develop a biofilm, in which bacterial cells are enmeshed into a mucoid exopolysaccharide becoming more resistant to beta-lactams as well as decrease the outer membrane permeability that enable bacteria to gain resistance development (Bonfiglio,*etal.*,١٩٩٨;Giamarellou and Antoniadou,٢٠٠١).The problem with *P.aeruginosa* is that, the use of a single antipseudomonal agent, even when *P.aeruginosa* is sensitive, is associated with the development of resistance during therapy(Kaye,*etal.*,٢٠٠٠).Resistance mediated by *P.aeruginosa* can be attributed both to an inducible, chromosomally mediated beta-lactamases that can render broad–spectrum cephalosporins inactive, and to a plasmid–mediated beta- lactamases that can lead to resistance to several penicillins and older cephalosporins (Graig and Ebert ,١٩٩٤; Eltahawy and Khalaf,٢٠٠١).

The results with regard to other antibiotics represented by tetracycline, doxycycline,erythromycin,rifampin,chloramphenicol,co-trimoxazole, gentamicin colistin, aztreonam and tobromycin were variable and the resistance of *P.aeruginosa* against these antibiotics ranged from (١٠٠٪) for the first five antibiotics to (٥٦.٧٪) for the tobromycin. The

resistance toward co-trimoxazole, gentamicin, colistin and aztreonam accounted for (٩٠٪) , (٨٦.٧٪) , (٦٣.٣٪) and (٦٠٪) respectively,while *P.aeruginosa* isolates showed resistance with less percentages to other antibiotics represented by ciprofloxacin, norfloxacin, amikacin and azithromycin accounted for (٢٦.٧٪), (٢٦.٧٪) , (٢٠٪) and (٢٠٪) respectively.





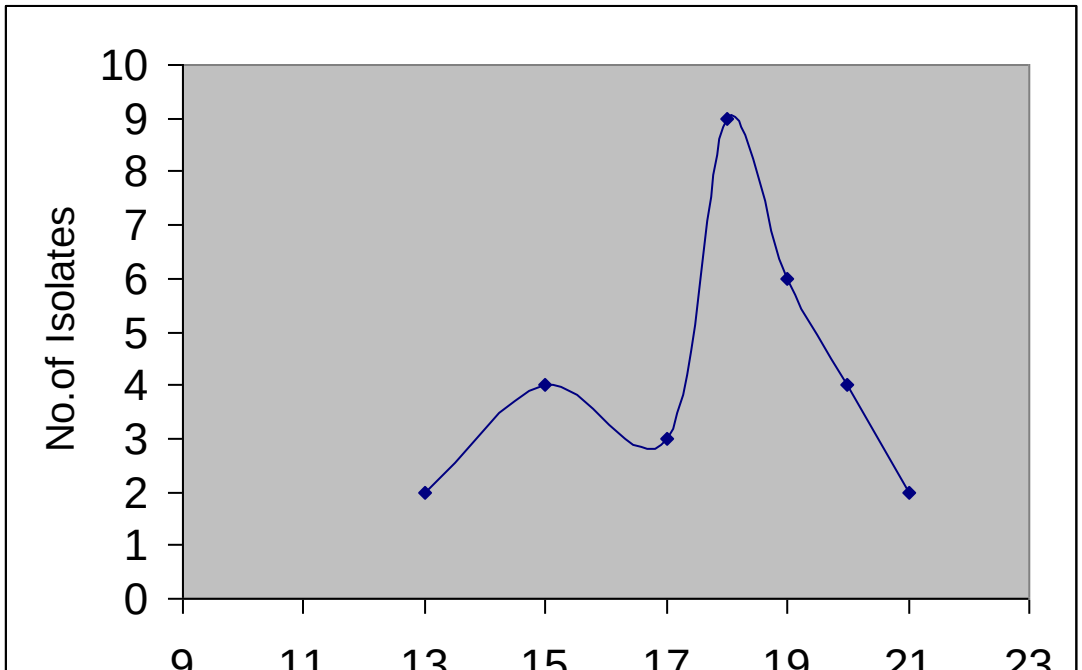
Figure(٣-٢)Rates of Resistance of *P.aeruginosa* for Antibiotics.

P.aeruginosa **resistance can be conferred by the outer membrane which provides an effective intrinsic barrier to accessing the targets are located either in the cell wall or cytoplasmic membrane or within the cytoplasm and modifications in outer membrane permeability via alterations in porin protein channels represent a component of many resistance mechanisms.In addition, inactivating enzymes released from the inner membrane can function more efficiently within the confines of the periplasmic space.The mechanisms by which intracellular concentrations of drugs are limited include decreased permeability through the outer membrane,decreased uptake through the cytoplasmic membrane and active efflux back out across the cytoplasmic membrane (Henwood, *et al.*,٢٠٠١; Po-Ren, *et al.*,٢٠٠٢).**

From these records one can conclude that the azithromycin,amikacin ,norfloxacin and ciprofloxacin are the most effective antibiotics against *P.aeruginosa* and confidently can be suggested for treatment to overcome this bacteria.The results described above were compared with other results

reported by Suzuki and his colleagues(١٩٩٧),Burns and his colleagues (٢٠٠٠) and Sorberg and his coworkers (٢٠٠٢) and were in relative satisfactory accordance, although some other results being reported by other studies (Nihad, *etal*.,٢٠٠٢ ;Boubaker, *et al*.,٢٠٠٣) were in contrast somewhat.

Figure (٣-٣) explains the relationship between the number of isolates and the number of the antibiotics to which are resistant. The results in this study showed that ٢ isolates resistant the less number of antibiotics accounted for ١٣ antibiotics and ٢ isolates resistant the large number of antibiotics accounted for ٢١ antibiotics,while ٤ isolates resistant ١٥ antibiotics and another ٤ isolates resistant ٢٠ antibiotics.The remain isolates resistance antibiotics as follow: ٣ isolates resistant ١٧ antibiotics,٦ isolates resistant ١٩ antibiotics and finally ٩ isolates resistant ١٨ antibiotics.(Gotoh,٢٠٠١ and Singh,*etal*.,٢٠٠٢) mentioned that multiple antibiotics resistance was at first thought to be caused exclusively by the combination of several resistance mechanisms including membrane impermeability, the outer membrane barrier in bacteria and interplay between impermeability and multidrugs efflux pumps.



Figure(٣-٣) Multi-Drug Resistant For *P.aeruginosa* Isolates.

Also (Livermore,٢٠٠٢) pointed out that *P.aeruginosa* carries multiresistance plasmids less often than does *Klebsiella pneumoniae*, develops mutational resistance to cephalosporins less readily than *Enterobacter* species and has less inherent resistance than *Stenotrophomonas maltophilia*. But *P.aeruginosa* consider uniquely problematic by a combination of: the species inherent resistance to many drugs classes, it's ability to acquire resistance, via mutations, to all relevant treatments; it's high and increasing rates of resistance locally; and it's frequent role in serious infections.A few isolates of *P.aeruginosa* are resistant to all reliable antibiotics and this problem seems likely to grow with the emergence of integrons that carry gene cassettes encoding both carbapenemases and amikacin acetyltransferases (Lambert,٢٠٠٢).

٦- Minimum Inhibitory Concentrations (MIC) :

Although a number of factors affect the patient outcome in bacterial infection,the rapid detection of effective antibacterial therapy is known to

reduce severity and mortality as well.The past decade has witnessed a dramatic increase in the prevalence of antibacterial resistance and much of this resistance is related to commonly used antimicrobial agents.The rapid evolution of antibacterial resistance requires that both bacteriologists and physicians have a basic understanding of the mechanisms of resistance to ensure the most appropriate antibacterial choices are made when initiating therapy.Moreover, the proper concentration of antibiotic can be helpful in establishing the level of resistance and can substantially affect the pathogens (Myrna,١٩٩٨; Murray, *et al.*,٢٠٠٣).

In the present study, twelve (١٢) antibiotics were tested for (MICs). These antibiotics are:cefixime,rifampin, doxycycline, penicillin, ampicillin, cefotaxime, gentamicin,co-trimoxazole, ceftriaxone, amikacin, norfloxacin and ciprofloxacin. These antibiotics were chosen as they are traditionally used in the treatment of bacterial infections. In the broth dilution method, various concentrations of an antibiotic were inoculated with a standard suspension of tested bacterial isolate. Following an overnight incubation at ٣٧°c,the(MIC) was determined by observing the lowest concentration of the antibiotic that will inhibit visible growth of the tested bacteria (Baron, *et al.*,١٩٩٤; Murray, *et al.*,١٩٩٩).

Results shown in table (٣-٤) indicated a high degree of pseudomonal resistance to most antibiotics being used in this study. The level of resistance accounted for:(١٠٠٪) regarding the cefixime,rifampin and doxycycline antibiotics to (٩٦.٧٪) and (٩٣.٣٪) for penicillin and ampicillin respectively. Furthermore, the (MICs) ranged from ٤ to <١٢٨ µg/ml for cefixime and ٨ to <١٢٨ µg/ml for the rifampin,doxycycline,penicillin and ampicillin respectively. Reimer and Reller (١٩٨١) obtained resistance for the pseudomonal isolates accounted for (١٠٠٪) against penicillin and

ampicillin with (MICs) ranges reach to (>٥١٢)µg/ml to both antibiotics. The results of the present study which outlined in table (٣-٥) showed that (٦) isolates were sensitive for cefixime with MIC ranged from ٤ to ١٦ µg/ml ,(٧) isolates were sensitive for rifampin with MIC ranged from ١٦ to ٣٢ µg/ml and (٥) isolates were sensitive for doxycycline with MIC ranged from ١٦ to ٣٢µg/ml.

In the present study all the results which related with cefixime,rifampin and doxycycline were recorded as resistance according to the NCCLs guidelines, although these results were in agreement with the results obtained by Alkawash and his coworkers (١٩٩٩) who stated that the MICs for doxycycline and rifampin against *P.aeruginosa* ranged from (٣٢-٦٤)µg/ml were appeared to confer benefit in treatment of human cystic fibrosis (CF) infected by *P.aeruginosa* when they were given in combination with other drugs. These seems to be in agreement with results obtained from the present study as observed in the antibiotics combination experiment.

Table (٣-٤) The Values of Minimum Inhibitory Concentrations (MIC) of some Antibiotics against *P.aeruginosa* Isolates

Antimicrobic agent	Suggested MICs ranges µg/ml *	Percentages for resistant bacteria (n=٣٠)%	MICs values µg/ml **
CFM	٠.٥-١٢٨	٣٠(١٠٠)	٤-<١٢٨
RA	٠.٥-١٢٨	٣٠(١٠٠)	٨-<١٢٨
DO	٠.٥-١٢٨	٣٠(١٠٠)	٨-<١٢٨
P	٠.٥-١٢٨	٢٩(٩٦.٧)	٨-<١٢٨
Am	٠.٥-١٢٨	٢٨(٩٣.٣)	٨-<١٢٨
CTX	٠.٥-٥١٢	٢٦(٨٦.٧)	٨-١٢٨
CN	٠.٠٦-١٢٨	٢٦(٨٦.٧)	٠.٢٥-<١٢٨
SXT	٠.٥-١٢٨	٢٥(٨٣.٣)	٣٢-<١٢٨
CRO	٠.٥-٥١٢	١٦(٥٣.٣)	١-١٢٨
AK	٠.٠٦-١٢٨	٨(٢٦.٧)	٠.٥-٦٤
NOR	٠.١٥-٨	٨(٢٦.٧)	٠.٠٦-<٨
CIP	٠.١٥-٨	٦(٢٠.٠)	٠.٠٦-٤

*** suggested MICs ranges for *P.aeruginosa* (Collee ,*et al.*,١٩٩٦).**

**** experimental MICs values for *P.aeruginosa*, all intermediate resistance isolates recorded resistance (Murray,*et al.*,١٩٩٩).**

Table (٣-٥)Sensitive Isolates of *P.aeruginosa* for Cefixime ,Rifampin and Doxycycline

Antimicrobic agent	Sensitive isolates			Total	MIC ranges µg/ml*	Standard values µg/ml**
	Burns	Otitis	UTI			
Cefixime	١	٣	٢	٦	٤-١٦	≤١ ٢ ≥٤
Rifampin	٠	٣	٤	٧	١٦-٣٢	≤١ ٢ ≥٤
Doxycycline	٠	٣	٢	٥	١٦-٣٢	≤٤ ٨ ≥١٦

***Experimental values .**

****Standard values recommended by (NCCLs,٢٠٠٣).**

The results obtained by this study showed that *P.aeruginosa* isolates revealed high resistance rates (٨٦.٧٪), (٨٦.٧٪) and (٨٣.٣٪) for cefotaxime, gentamicin and Co-trimoxazole respectively. Moreover, the MICs values for these antibiotics ranged from ٨ to ١٢٨µg/ml, ٠.٢٥ to <١٢٨ µg/ml and ٣٢ to <١٢٨ µg/ml respectively, while (Poirel,*etal.*,٢٠٠١) pointed out that the MIC value of cefotaxime ranged from ٤ to ٥١٢ µg/ml with a percentage of (٥٠٪) regarding *P.aeruginosa* strains being isolated by them. These results with gentamicin were agreeable with that results observed by (Po-Ren,*et al.*,٢٠٠٢) who pointed out that the MIC values for this antibiotics against *P.aeruginosa* ranged from ٠.٥ to ١٢٨ µg/ml with a percentage of (٧٥٪).

The resistance of Pseudomonas isolates tested in this study against ceftriaxone, amikacin, norfloxacin and ciprofloxacin accounted for (٥٣.٣٪),(٢٦.٧٪),(٢٦.٧٪) and (٢٠٪) respectively. Furthermore, the MIC values ranged from ١-١٢٨ µg/ml for ceftriaxone , ٠.٥-٦٤ µg/ml for amikacin, ٠.٠٦-<٨ µg/ml for norfloxacin and ٠.٠٦-٤ µg/ml for ciprofloxacin. Relatively, these antibiotics seems to be the most effective antibiotics against *P.aeruginosa* and consequently can be suggested for overcoming this organism. This suggestion may require further in vitro and in vivo investigations in order to develop an anti-pseudomonal compound being confidently recommended and safety used for treatment of pseudomonal infections in human. However, the results were in accordance with those results detected by worldwide studies(Livermore and Chen,١٩٩٩;Galloway ,*et al.*,١٩٩٩). All those studies concluded that ciprofloxacin and may all the quinolone members are the only effective oral

antipseudomonal agents available at the present time and their use may help patients to recover.

To confirm the results,the study used (٢) two different techniques for testing the susceptibility of the MDRPA against the tested antibiotics in attempt to detect the proper method and giving a real picture for susceptibility of this organism for these antibiotics.Table (٣-٦) shows a comparison results between disk diffusion and (MIC) techniques.The results revealed no significant difference (p>٠.٠٥) between these two methods.The MIC seem to be more reliable and more acceptable compared with the other method.This result may confirm the results obtained by (Murray,*etal.*,١٩٩٩) who proposed that the MIC method is a well-standardized and reliable reference method that is useful for research purposes.The results may conclude that MIC method provides accurate and precise results.This can be attributed to the objectivity of the presence or absence the growth which is visually detected, therefore this method is desirable in selected cases.

The results with disk diffusion method may be affected by some factors such as the culture media (type,nature and concentration), growth density, temperature and pH of the medium in addition to the technical difficulties. Furthermore, the results with disk diffusion method may vary according to the qualification and experience of the demonstrator who measured the results (the inhibition zone)(Garrod,*etal.*,١٩٧٨).Even though, the disk diffusion method remains applicable, simple, inexpensive, does not require any special equipments and it provides category results that are easily understood by clinicians (Murray,*et al.*,٢٠٠٣).

Table (٣-٦)Comparison of Susceptibility Testing Results between Techniques for (٣٠) Isolates of *P.aeruginosa*

Antimicrobic agent	Degree of susceptibility	Susceptibility testing methods	
		Disk diffusion method n(%)	MIC method n(%)
P	S	٠	١(٣.٣)
	I	٠	٠
	R	٣٠(١٠٠)	٢٩(٩٦.٧)
Am	S	٠	٢(٦.٧)
	I	٠	٠
	R	٣٠(١٠٠)	٢٨(٩٣.٣)
CFm	S	٠	٠
	I	٠	٠
	R	٣٠(١٠٠)	٣٠(١٠٠)
RA	S	٠	٠
	I	٠	٠
	R	٣٠(١٠٠)	٣٠(١٠٠)
Do	S	٠	٠
	I	٠	٣(١٠)
	R	٣٠(١٠٠)	٢٧(٩٠)
SXT	S	٣(١٠)	٥(١٦.٧)
	I	٠	١(٣.٣)
	R	٢٧(٩٠)	٢٤(٨٠)
CN	S	٤(١٣.٣)	٤(١٣.٣)
	I	٢(٦.٧)	٥(١٦.٧)
	R	٢٤(٨٠)	٢١(٧٠)
CTX	S	٣(١٠)	٤(١٣.٣)
	I	٨(٢٦.٧)	٩(٣٠)
	R	١٩(٦٣.٣)	١٧(٥٦.٧)

NOR	S	٢٢(٧٣.٣)	٢٢(٧٣.٣)
	I	٠	١(٣.٣)
	R	٨(٢٦.٧)	٧(٢٣.٣)
CIP	S	٢٢(٧٣.٣)	٢٤(٨٠)
	I	٤(١٣.٣)	٣(١٠)
	R	٤(١٣.٣)	٣(١٠)
AK	S	٢٤(٨٠)	٢٢(٧٣.٣)
	I	٦(٢٠)	٧(٢٣.٣)
	R	٠	١(٣.٣)
CRO	S	-(N)	١٤(٤٦.٧)
	I	-	٩(٣٠)
	R	-	٧(٢٣.٣)

(N): Non-dun.

٧- Antimicrobial Combination :

The idea behind the use of drugs combinations to treat bacterial infections is to reduce the chance of resistant mutants arising during the treatment. Theoretically, if the rate of mutation to one drug is, for example,$١٠^{-٨}$ and to the second is $١٠^{-٧}$, the probability of mutation to resistance to both drugs occurring in a single cell is $١٠^{-١٥}$ (Wilson and Miles,١٩٧٥). A bacterial population of $١٠^{١٥}$ cell/ml is beyond the range normally expected in vivo and the danger of resistant mutants emerging should be largely or totally removed (Jibran,١٩٨٦).

Another reason for the use of drugs in combination status is that the combination may be more effective than either drugs when used alone. The interaction between any two drugs in combination may be either synergistic, partially synergistic (i.e.additive), indifferent or antagonistic (Collee,*et al.*,١٩٩٦). Combinations that can achieve synergistic or additive effects are of great value when toxicity of one or both members of the combination is considered because with the enhanced effect one can use low concentrations to produce the desired therapeutic action. Several important antimicrobials have significant dose-related toxicities which

seriously limit their use (e.g. chloramphenicol and aminoglycosides) (Cunha,٢٠٠٠).

The use of combinations might couple a broad-spectrum of antimicrobial activity. In some polymicrobial (multiple) infections, it may be necessary to treat with agents active against each of the major pathogens (Obritsch,*et al.*,٢٠٠٥). Antimicrobial combinations have been of particular interest clinically for the treatment of infections caused by organisms that were resistant to acceptable concentrations of single antimicrobials. Although those antimicrobials have been used to treat a wide varieties of infections, there are very few controlled trials of their efficacy and only a small number of instances in which there appears to be clear agreement between the invitro laboratory data and clinical applications. These include the use of piperacillin, mezlocillin, ticarcillin or other pecillins plus tobromycin or gentamicin for serious pseudomonas infections (Cunha,٢٠٠٠).

Accordingly the antibiotics combination for six selected isolates of *P.aeruginosa* was investigated and the results were shown in tables (٣-٧ to ٣-١٣). The results indicated for synergistic effects between beta-lactam antibiotics represented by penicillin, ampicillin, cefotaxime and ceftriaxone. These antibiotics are the safest and mostly widely used class of antibiotics ever developed were known to bind with specific receptors in the cell wall which called penicillin binding proteins (PBPs), at least some of which are enzymes involved in transpeptidation reactions, from three to six (or more) PBPs per cell can be present. After antibiotic molecules have attached to the receptors, peptidoglycan synthesis is inhibited as final transpeptidation is blocked. A final bactericidal event is the removal or inactivation of an inhibitor of autolytic enzymes in the cell wall. This

activates the autolytic enzymes and results in cell lysis (Obritsch, *et al.*,٢٠٠٥), but Cunha, (٢٠٠٠) stated that the double beta-lactam combinations were popular years ago for the treatment of fever in neutropenic patients have not shown any advantage over the association of antimicrobials with different targets and most probably should not be used in the treatment of severe infections caused by *P.aeruginosa*.

Table(٣-٧) Antibiotics Combination Effect on Burn Isolate No.١٢ of *P.aeruginosa*

Antibiotics	P	Am	CTX	CRO	AK	CN	CIP	NOR	SXT	RA
P	+ +	+	-	-	-	-	-	-	+	+
Am		+ +	+	-	-	+	+	+	+	+
CTX			+ +	-	-	-	(+)	(+)	+	(+)
CRO				(+)	-	-	(+)	+	-	-
AK					+	-	+	+	-	+
CN						++	+	+	++	+
CIP							+	+	+	+
NOR								(+)	(+)	(+)
SXT									++	++
RA										++

Table (٣-٨) Antibiotics Combination Effect on Burn Isolate No.٣٠ of *P.aeruginosa*

Antibiotics	P	Am	CTX	CRO	AK	CN	CIP	NOR	SXT	RA
P	+ +	+	-	-	-	-	-	-	+	+
Am		+ +	+	-	-	+	+	+	+	+
CTX			+	-	-	-	-	+ +	+ +	-
CRO				+	-	-	-	(+)	-	-
AK					+	-	+ +	+	-	+
CN						+ +	(+)	+ +	+ +	-
CIP							+	+	+	-
NOR								+ +	+ +	+ +
SXT									+ +	+
RA										+ +

++ : static effect only + : partial cidal effect

(+) : incomplete cidal effect - : fully cidal effect

Table (٣-٩) Antibiotics Combination Effect on Otitis Media Isolate No. ٢٥ of *P.aeruginosa*

Antibiotics	P	Am	CTX	CRO	AK	CN	CIP	NOR	SXT	RA
P	+ +	+	+	-	-	-	+ +	+ +	+ +	(+)
Am		+ +	+	+	+	+	+ +	+ +	+ +	(+)

CTX			+	-	-	-	+	+ +	+ +	-
CRO				+	-	-	+	+ +	+ +	-
AK					+	-	+ +	+ +	+	(+)
CN						+ +	+ +	+ +	+ +	(+)
CIP							(+)	+ +	+ +	+
NOR								(+)	+ +	-
SXT									+ +	+
RA										+ +

Table (٣-١٠) Antibiotics Combination Effect on Otitis Media Isolate No.٥٥ of *P.aeruginosa*

Antibiotics	**P**	**Am**	**CTX**	**CRO**	**AK**	**CN**	**CIP**	**NOR**	**SXT**	**RA**
P	+ +	+	-	-	-	-	+ +	+ +	+	+
Am		+ +	+	+	+	+	+ +	+ +	+ +	+
CTX			+ +	+	-	-	+ +	+ +	+ +	(+)
CRO				+ +	-	-	+ +	+ +	+ +	(+)
AK					(+)	-	+ +	+ +	+	(+)
CN						+ +	+ +	+ +	+ +	+
CIP							+ +	+ +	+	+
NOR								+ +	+ +	+ +
SXT									+ +	+
RA										+ +

++ : static effect only + : partial cidal effect

(+) : incomplete cidal effect - :fully cidal effect

Table (٣-١١) Antibiotics Combination Effect on UTI Isolate No.٢٤ of *P.aeruginosa*

Antibiotics	P	Am	CTX	CRO	AK	CN	CIP	NOR	SXT	RA
P	+ +	+	-	-	-	-	-	-	+	+
Am		+ +	-	-	-	-	+ +	+ +	+	-
CTX			+	-	-	-	+	+	+	-
CRO				(+)	-	-	+	+	-	-
AK					(+)	-	+ +	+ +	+ +	-
CN						+ +	+	+	-	-
CIP							+ +	+	+	+
NOR								+ +	+	+
SXT									+ +	+
RA										+ +

Table (٣-١٢) Antibiotics Combination Effect on UTI Isolate No.٢٥ of *P.aeruginosa*

Antibiotics	P	Am	CTX	CRO	AK	CN	CIP	NOR	SXT	RA
P	+ +	+	-	-	-	-	-	-	+	+
Am		+ +	-	-	-	-	+ +	+ +	+	-
CTX			(+)	-	-	-	+	+ +	-	+
CRO				(+)	-	-	+	+	-	-
AK					(+)	-	+	+	-	-
CN						+	+	+ +	+	+
CIP							+ +	+ +	+ +	+
NOR								+ +	+ +	+ +
SXT									+ +	+
RA										+ +

++ :static effect only + :partial cidal effect

(+) :incomplete cidal effect - :fully cidal effect

Table (٣-١٣) Fully Cidal Synergism cases in Antibiotic Combination on the six Selected Isolates of *P.aeruginosa*

Antibiotics	P	Am	CTX	CRO	AK	CN	CIP	NOR	SXT	RA
P			-	-	-	-	-	-		
Am			-	-	-	-				-
CTX				-	-	-	-		-	-
CRO					-	-	-		-	-
AK						-			-	-
CN									-	-
CIP										-
NOR										-
SXT										
RA										

- : fully cidal effect .

Beta-lactam antibiotics which were used in this study revealed another synergism case with amikacin and gentamicin from one side and with ciprofloxacin and Norfloxacin from other side. This may due to the ability of beta-lactam antibiotics enhance the absorption of aminoglycosides by increase the permeability and by hydrophilic uptake pathway which responsible for the transport of beta-lactams ,and during the outer membrane by channels filled with water so called transmembrane diffusion (Brooks,*et al.*,٢٠٠١) and help the quinolone antibiotics by inhibition cell wall synthesis, enable the quinolones molecules from

entering to the bacterial cell and reaching to the target site (DNA gyrase) leading to kill the bacteria (Steven, *et al.*,١٩٩١).

The potency of trimethoprim and the sulfonamides against *P.aeruginosa* is limited, since the MICs of this compound toward *P.aeruginosa* is typically in the resistance is not well understood yet ,but this may be to the poor affinity for the target enzymes and low outer membrane permeability (Kohler,*et al.*,١٩٩٦) and although this bacteria resistant Co-trimoxazole when used alone, but when combined with beta-lactam antibiotics from one side and with aminoglycosides from other side it revealed synergistic effect. (Aboud,٢٠٠١)observed that the combination of bacteriostatic antibiotic with bactericidal antibiotic led to synergistic effect that may due to the penetration mechanism of the antibiotic in to the bacterial cell being do not be affected by mutations occur in the organism ,that may because of it's structure properties.

Rifampin is particularly active against gram-negative bacteria and Mycobacteria. It acts by inhibiting bacterial DNA dependent RNA polymerase. Point mutations in the chromosomal genes confer resistance to rifampin. The frequency of point mutations occurrence precludes the use of rifampin as a single agent for the treatment of bacterial infections resulted by *P.aeruginosa* (Murray,*et al.*,٢٠٠٣). Cunha,(٢٠٠٠) stated that ,invitro synergism has been observed if rifampin was added to the combination of penicillins and/or aminoglycosides and results have improved in some experimental animal models using these combinations, so that rifampin represented synergism case when combined with every of (ampicillin, cefotaxime, ceftriaxone ,amikacin ,gentamicin ,ciprofloxacin and norfloxacin) each one on side in this study.

٨- The Combined Effect of the Ceftriaxone Plus Gentamicin on *P.aeruginosa* isolates :

Despite the advent of newer antipseudomonal compounds such as carbapenems and the fluoroquinolones, aminoglycosides have an important role in the therapy of serious *P.aeruginosa* infections. They continue to be used because of (١) their excellent and fast concentration-dependent bactericidal activity, a determining factor in the prognosis of severe *P.aeruginosa* infections in neutropenic patients or in ICU life-threatening nosocomial infections, particularly during the first ٢٤ hours of treatment ; (٢) their limited tendency to develop resistance during therapy; (٣) the synergistic effect when combined with antipseudomonal penicillins and antipseudomonal cephalosporins invitro and invivo; (٤) the protective effect toward resistance development in the beta-lactams whenever they are given simultaneously in vivo; (٥) their lack of inoculum effect; (٦) their prolonged and concentration-dependent post antibiotic effect (>٢ hours) ; (٧) their antimicrobial activity at levels below their MICs; (٨) the possibility to be given once daily despite a half-life that demands twice-daily or three daily administration (Giamarellou and Antoniadou,٢٠٠١).

Regarding to ceftriaxone, it is used in high volume worldwide and is not associated with significant resistance problems (Cunha,٢٠٠٠),as well as the antibacterial spectrum of activity for it is similar to that of ceftizoxime and cefotaxime, but ceftriaxone has the longest half-life of all the third-generation cephalosporins (about ٩ hours), which allows for once-daily dossage (Ellsworth, *et al.*,٢٠٠٣). For all these aspects ,gentamicin and ceftriaxone were used as combination against *P.aeruginosa* isolates in the present study.

The invitro susceptibility of (٦) six selected isolates of *P.aeruginosa* to gentamicin in combination plus ceftriaxone was determined by means of a checker board technique which recommended by (Garrod,١٩٧٨) as shown in table (٢-٦). The results of the present study showed that the combination of gentamicin and ceftriaxone resulted in synergistic activity against ٥(٨٣.٣٪) of the six tested isolates. The growth of these isolates was inhibited by combining gentamicin and ceftriaxone in concentrations of one-fourth of the amount of each antibiotic required to inhibit the growth of these isolates when tested alone (table,٣-١٤),while partial synergy (i.e. additive effect) was observed between gentamicin and ceftriaxone against one isolate (No.O٥٥), inhibition of growth was observed when one-fourth the effective concentration of ceftriaxone was combined with one-half the effective concentration of gentamicin.

Cunha (٢٠٠٠) stated that the presence of all cell wall-active agents and in many species such as *P.aeruginosa* with aminoglycosides such as streptomycin ,gentamicin and amikacin resulting in bactericidal synergism. Owlia and his colleagues (٢٠٠١) showed that with the exception of beta-lactams, alginate did in fact impede the penetration of antibiotics such as aminoglycosides, therefore the use of beta-lactams antibiotics in combination with aminoglycosides enhancement the activity of these antibiotics.

It has been suggested that patients with severe MDRPA infections should be treated with combination therapy, consisting of an antipseudomonal beta-lactam with an aminoglycoside or fluoroquinolone rather than aminoglycosides and fluoroquinolone combinations, to provide adequate therapy and improve patient outcomes (Obritsch,*etal.*,٢٠٠٥). As the prevalence of MDRPA increases and treatment options become limited

, various antimicrobial combinations have been proposed as an alternative in clinical practice despite resistance to one or both agents in the combination.

Table (٣-١٤) The MIC Effect of Gentamicin and Ceftriaxone Singly and in Combination

Synergy test	Isolate	CN µg/ml	CN + CRO µg/ml	CRO µg/ml
Synergy	U٢٥	١	٠.٢٥-٠.٥	٢
	U٢٤	٢	٠.٥-١	٤
	B١٢	٢	٠.٥-١	٤
	B٣٠	٢	٠.٥-١	٤
	O٢٥	٢	٠.٥-١	٤
Partial synergy	O٥٥	٤	١-٢	٤

٩- Synergistic Effect of EDTA with Some Antibiotics on *P.aeruginosa* isolates :

Egorov ,(١٩٨٥) stated that using antibiotics in combination with other preparations may help in prevention of development of antibiotics-resistant microorganisms such as some biologically active compounds referred to the ethylene diamine tetra acetate. He supposed that such compound may decrease the resistance of bacteria to be antibiotics, perhaps by prevention of drug resistance factor (DRF) to transferred through bacteria by conjugation and/or transformation. The results of the present study showed that *P.aeruginosa* isolates were able to produce the metallo-beta-lactamases (MBLs) and this phenomenon has been clearly and extensively demonstrated (Nicas and Hancock,١٩٨٠; Lee, *et al.*,٢٠٠٢ Walsh, *et al.*,٢٠٠٢ ;Butt, *et al.*,٢٠٠٣ ;Akpolat,*et al.*,٢٠٠٣; Hemaltha ,*et al.*,٢٠٠٥).

The results of the present study showed that positive result for MBLs included a total in of ٩(٣٠٪) isolates from the all (٣٠) thirty isolates when followed by the agar dilution method. These were ٧(٢٣.٣٪) isolates of burns and ٢(٦.٧٪) isolates of UTIs. The growth of these isolates was inhibited by ampicillin in concentration ٣٢µg/ml with presence of EDTA while these isolates had not been inhibited by ampicillin when used the (٤) four concentrations (٦٤,٣٢,١٦,٨)µg/ml in absence the EDTA. When cefixime was used instead of ampicillin with the concentrations (١٦,٨,٤,٢)µg/ml showed positive result for MBLs included a total in of ١٢(٤٠٪)isolates from the all (٣٠) isolates. These were ١٠(٣٣.٣٪) of burns isolates and ٢(٦.٧٪) of UTI isolates. The growth of these isolates were inhibited by cefixime in concentration ٨µg/ml with the presence of EDTA but were not inhibited if EDTA remove.

From these MBLs positive isolates, burn isolate (No.١٢) was selected to experiment using the same antibiotics (ampicillin and cefixime) with the same concentrations by well-agar diffusion method. This isolate exhibited a significant zone size enhancement with a mixture of EDTA plus ampicillin in concentration ١٦µg/ml and with a mixture of EDTA plus cefixime in concentration ٤µg/ml, results outlined in table (٣-١٥), figures (٣-٤),(٣-٥). MBLs enzymes belong to group of beta-lactamases divalent cations of zinc as co-factor for enzyme activity. They have potent activity not only against carbapenems group but also against other beta-lactamases (Nicas and Hancock,١٩٨٠; Poirel,*etal.*,٢٠٠١). MBLs are not inhibited by the commercially available inhibitors such as sulbactam and tazobactam (Walsh, *et al.*,٢٠٠٢), but the production of MBL was detected by EDTA, because EDTA can interact with *P.aeruginosa* LPS ,since the EDTA can remove ions from it's LPS sites, whereas the antibiotic molecules being

cationic ,would compete with these ions for these sites (Nicas and Hancock,١٩٨٠).

There are reports on MBLs production in *P.aeruginosa* from various countries like Brazil ,Korea ,Singapore and France. MBL was first reported as a zinc dependent enzyme in *Bacillus cereus* in mid ١٩٦٠s (Hemaltha, *et al.*,٢٠٠٥) and since has been described in different gram-negative bacteria. All these enzymes were produced by chromosomal genes. However ,in ١٩٩١, the first plasmid–mediated MBLs from *P.aeruginosa* have been reported from Japan, while another type of acquired beta-lactamase was first reported from Italy in ١٩٩٩ (Butt, *et al.*,٢٠٠٣).

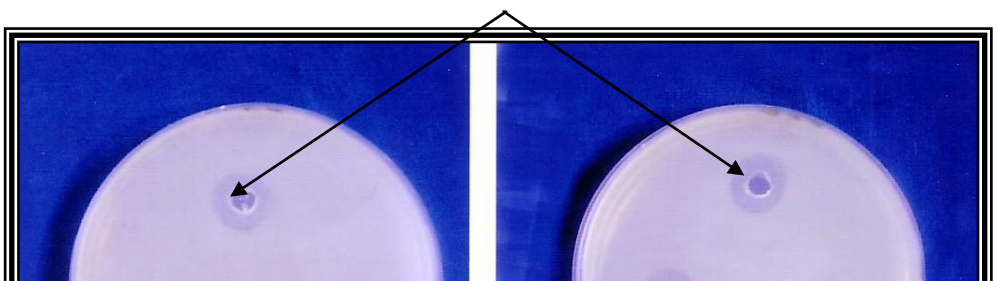
Table (٣-١٥)Susceptibility of Burn Isolate No.١٢ to Ampicillin and Cefixime with and without EDTA

Antimicrobial agent	Concentration µg/ml	Diameter of the inhibition zone(mm)	
		Without EDTA	With EDTA
Am	٨	٠	١٣
	١٦	٠	١٤
	٣٢	٦	٢٠
	٦٤	٧	٢٣
CFM	٢	٠	١٦

١٩	٠	٤	
٢٦	٨	٨	
٢٧	٨	١٦	

MBL production is a significant problem in hospital isolates of *P.aeruginosa*. The mobility of beta-lactamase genes associated with integrons and being disseminated throughout bacterial populations is of great concern to microbiologists and physicians alike (Walsh, *et al.*, ٢٠٠٢). So the rapid detection of MBL-positive gram-negative bacilli is necessary to aid infection control and prevent their dissemination.

٠.١ml mixture



an

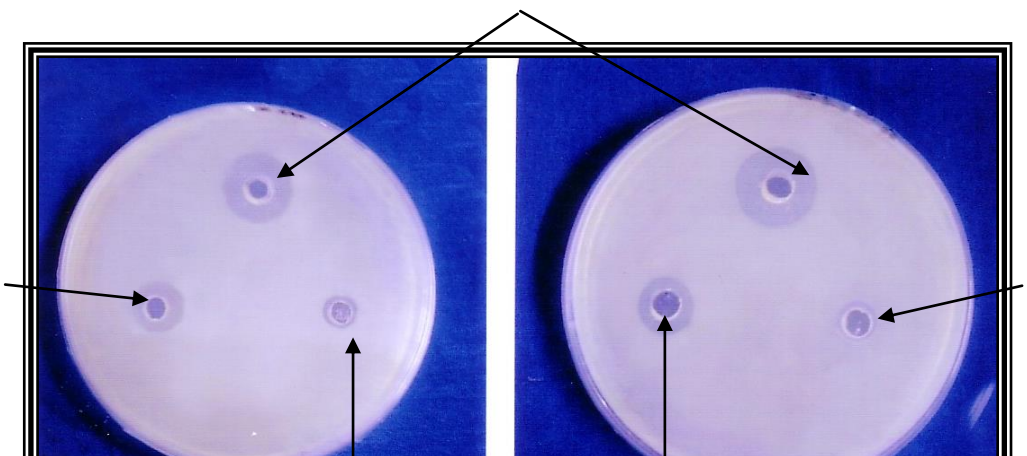
٠.١ml
٠.١ml

antibiotic EDTA

٠.١ml antibiotic ٠.١ ml EDTA

١٦ µg /ml ٨ µg /ml

٠.١ ml mixture



٠.١ ml ٠.١ml

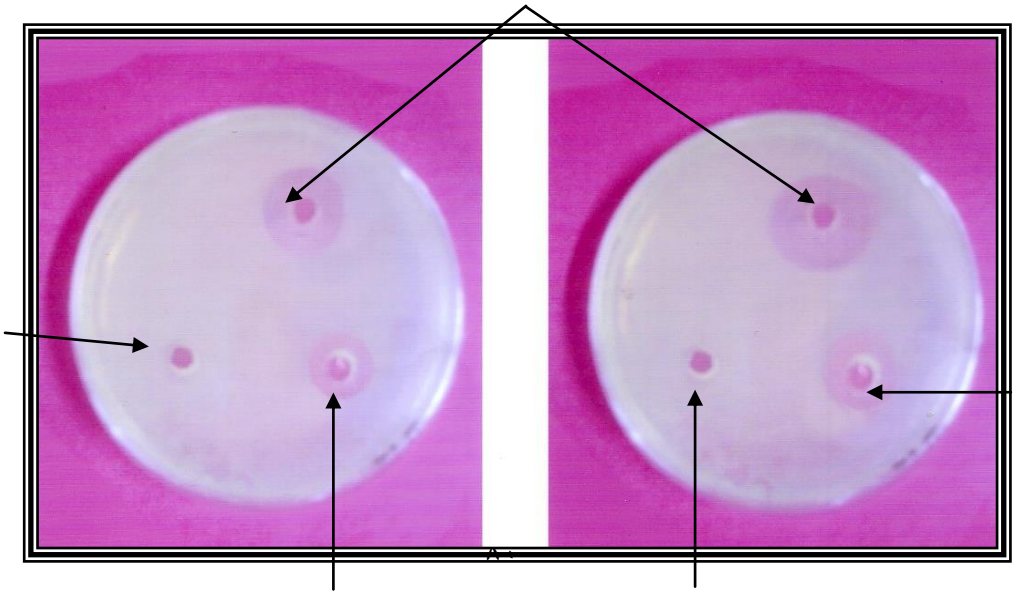
EDTA
antibiotic

٠.١ ml antibiotic ٠.١ ml EDTA

٣٢ µg /ml ٦٤ µg /ml

Figure (٣-٤) synergistic effect of EDTA plus ampicillin with different concentrations on *P.aeruginosa* .

٠.١ ml mixture



٠.١ml

antibiotic

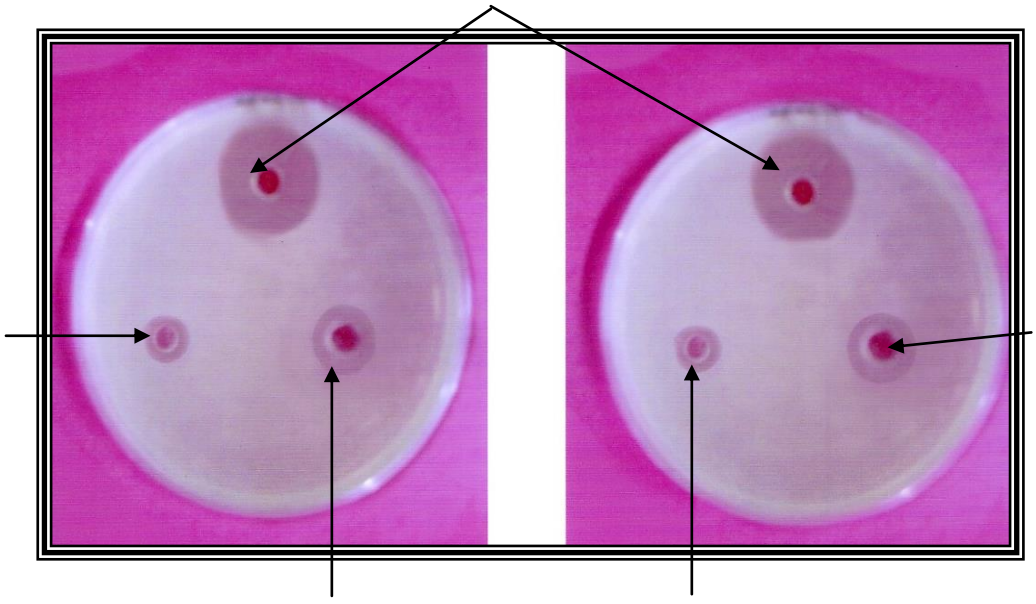
٠.١ml

EDTA

٠.١ ml antibiotic ٠.١ ml EDTA

٤ µg/ml ٢ µg /ml

٠.١ ml mixture



٠.١ml ٠.١ ml

antibiotic

EDTA

٠.١ ml antibiotic

٠.١ ml antibiotic ٠.١ ml EDTA

١٦ µg /ml ٨ µg /ml

Figure (٣-٥) synergistic effect of EDTA plus cefixime with different concentrations on *P.aeruginosa* .

١٠- Synergistic Effect of B-complex with Some antibiotics on *P.aeruginosa* isolates :

The effect of vitamins with some antibiotics was also tested in this study. The experiment was carried out on ٥ five isolates of *P.aeruginosa* which resistance or intermediate resistance to some antibiotics used in this study as follow: (amikacin, cefotaxime, gentamicin ,ciprofloxacin and ceftriaxone) by using macro-broth dilution (MIC) method. The results of this experiment showed that reduced MIC values from resistance or intermediate resistance to sensitive values, outlined in table (٣-١٦). The first burn isolate No.١٢ has intermediate resistance for amikacin and ceftriaxone with MIC ٣٢µg/ml, in presence of B-complex converted to sensitive with MIC ٨µg/ml for both antibiotics .Secondly, otitis media isolate No.٣٣ has resistance for gentamicin with MIC ١٦µg/ml converted to sensitive with MIC ٤µg/ml and resistance to cefotaxime with MIC ٦٤µg/ml convert to ٤µg/ml in presence the B-complex .Regarding, UTI isolate No.٢٤ has resistance to gentamicin with MIC ١٦ µg/ml and to ciprofloxacin with MIC ٢µg/ml convert to sensitive with ٤µg /ml and ٠.١٢µg/ml for

gentamicin and ciprofloxacin respectively. UTI isolate No.٢٥ has resistance to ciprofloxacin with MIC ٢µg/ml and ceftriaxone with MIC ٣٢ µg/ml convert to sensitive to ciprofloxacin with MIC ٠.٢٥ µg/ml and ceftriaxone with MIC ٨µg/ml .Finally, otitis isolate No.٥٥ has resistance for ceftriaxone with MIC ٦٤µg/ml and intermediate resistance for cefotaxime with MIC ١٦ µg /ml. This isolate did not affect when these antibiotics mixed with B-complex.

Table (٣-١٦)Susceptibility of *P.aeruginosa* Isolates to B-complex With Some Antibiotics

Type & number of the isolate	Antimicrobial agent	MICs values µg/ml without B-complex	MICs values µg/ml with B-complex
B١٢	AK	٣٢(I)*	٨(S)**
	CRO	٣٢(I)	٨(S)
O٣٣	CN	١٦(R)***	٤(S)
	CTX	٦٤(R)	٤(S)
U٢٤	CN	١٦(R)	٤(S)
	CIP	٢(R)	٠.١٢(S)
U٢٥	CIP	٢(R)	٠.٢٥(S)
	CRO	٣٢(R)	٨(S)
O٥٥	CRO	٦٤(R)	٦٤(R)
	CTX	١٦(I)	١٦(I)

*I : Intermediate

**R :Resistance

***S :Sensitive

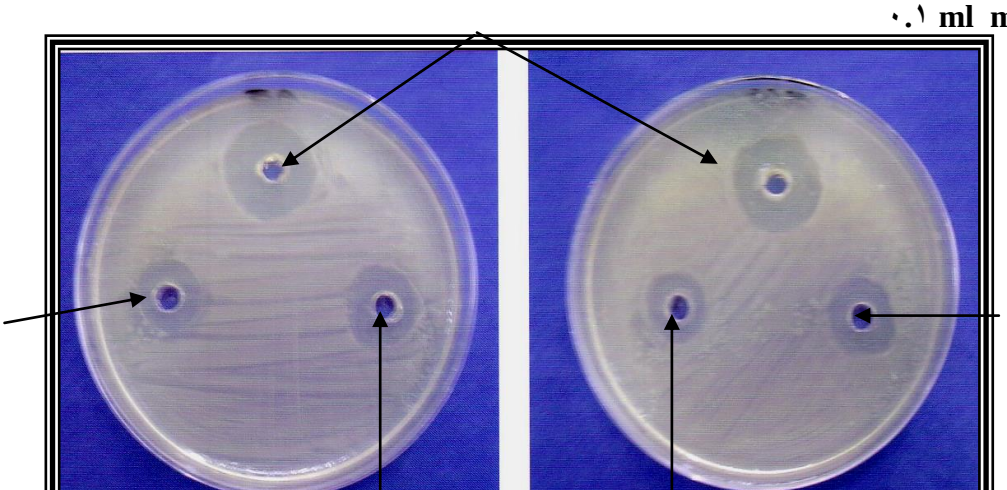
From these positive isolates selected UTI isolate No.٢٥ to experiment the effect of B-complex with ciprofloxacin and ceftriaxone by well-agar diffusion method .As shown in table (٣-١٧),figures (٣-٦),(٣-٧) results exhibited a significant zone size enhancement with a mixture of B-complex plus ciprofloxacin in concentration ٠.١٢ µg/ml and B-complex plus ceftriaxone in concentration ٨µg/ml .The mechanism of this action seems to depend on the outer membrane which contains a set of less abundant proteins which are involved in the transport of specific molecules vitamins and iron siderophore complexes (Brooks ,*et al.*,٢٠٠١) .These proteins show high affinity for substrates and probably function like the classic carrier transport systems of the inner cytoplasmic membrane .This experiment carried out according to study of (Ichimiya, *et al.*,١٩٩٤) who investigated the activity of some vitamins with penicillin G, gentamicin,quinolone, cephalosporin and imipenem. They observed a remarkable enhancement in the antibiotics activity except penicillin G .

Generally the results obtained in detection the effect of EDTA were more effect on *P.aeruginosa* isolates from the results obtained in detection the effect of B-complex.

Table(٣-١٧)Susceptibility of *P.aeruginosa* UTI Isolate No.٢٥ to Ciprofloxacin and Ceftriaxone with and without B-Complex

Antimicrobial agent	Concentration µg/ml	Diameter of the inhibition zone(mm)	
		Without B-complex	With B-complex
CIP	O.١٢	١٢	٢٢
	٠.٢٥	١٢.٥	٢٣
	٠.٥	١٣	٢٥
	١	١٤	٢٧
CRO	٨	١٠	٢١
	١٦	١١	٢١
	٣٢	١٤	٢٣
	٦٤	١٦	٢٥

٠.١ ml mixture



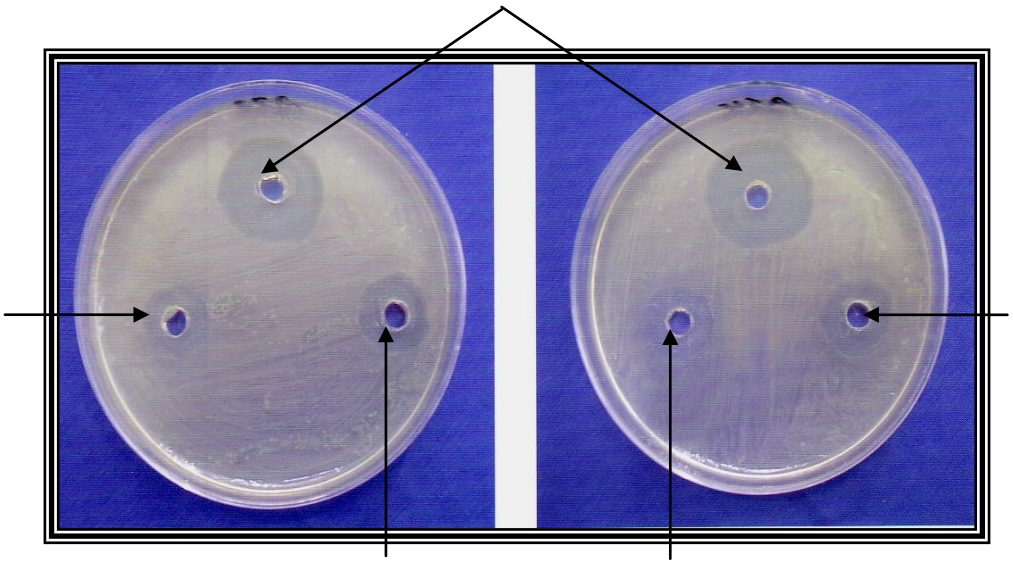
٠.١ ml ٠.١ml

B-complex
antibiotic

٠.١ ml B-complex ٠.١ ml antibiotic

٠.٢٥ µg /ml ٠.١٢ µg /ml

٠.١ ml mixture



٠.١ ml ٠.١ml

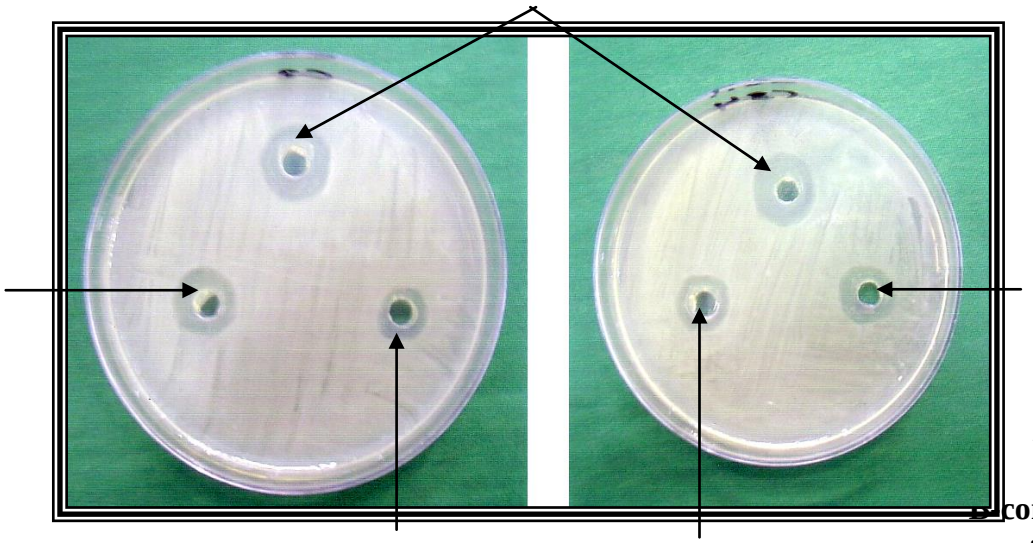
B-complex
antibiotic

٠.١ ml antibiotic ٠.١ ml B-complex

٠.٥ µg /ml ١ µg /ml

Figure (٣-٦) synergistic effect of B-complex plus ciprofloxacin with different concentrations on *P.aeruginosa* .

٠.١ ml mixture



٠.١ml ٠.١ ml

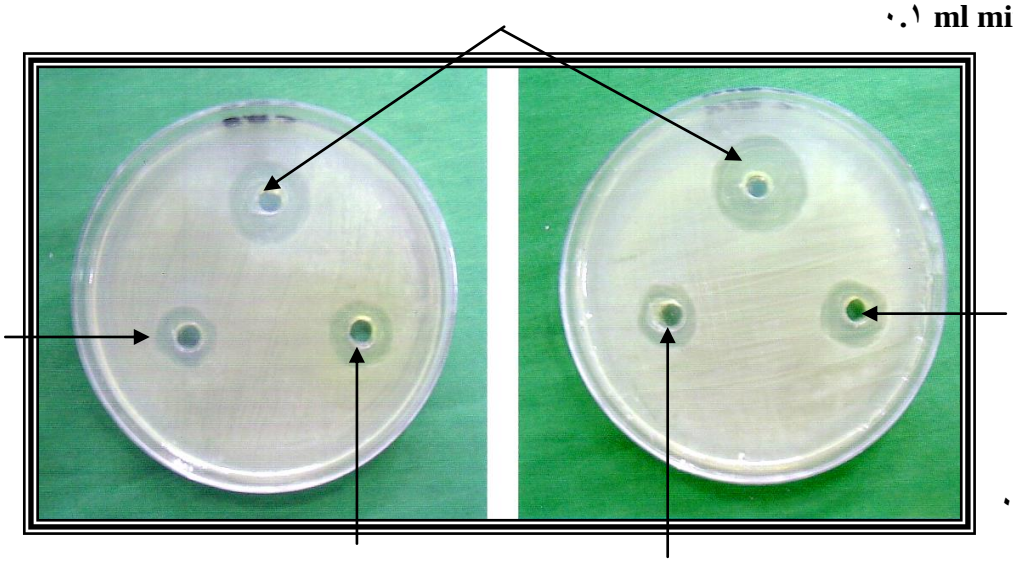
B-complex

antibiotic

٠.١ ml antibiotic ٠.١ ml B-complex

٨ µg /ml ١٦ µg /ml

٠.١ ml mixture



٠.١ ml

٠.١ ml antibiotic

B-complex

٠.١ ml antibiotic ٠.١ ml B-complex

٣٢ µg /ml ٦٤ µg /ml

Figure (٣-٧) synergistic effect of B-complex plus ceftriaxone with different concentration on *P.aeruginosa* .

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