# IJST

## INTERNATIONAL

Journal for Sciences and Technology

Vol. (6) No. (4) December 2011

http://www.ijst-jo.com/volumes.html

ISSN: 1816-2509

# IJST International Journal for Sciences & Technology

**I**nternational **J**ournal for **S**ciences and **T**echnology

# المجلة الدولية للعلوم والتكنولوجيا

**Volume 6. No. 4 / December 2011 / ISSN: 1816-2509**

***A Refereed Scientific Journal Since 2006***

مجلة علمية محكمة منذ عام 2006

***Issued By:***

***The International Centre for Advancement of Sciences and Technology***

IJST contact Information:
P.O. Box 2793 Amman 11953 Jordan
Tel. +96265602285
E-mails: info@ijst-jo.com / info.icast@yahoo.com
URL: www.ijst-jo.com

# EDITORIAL BOARD - 2011

# FORWARD

It is my pleasure to welcome you back and present you a new issue of our Journal, Volume 6, No. 4 (2011), the fourth issue of this year, with diversity of researches and elite experts of the Editorial Board and Advisory Group. The members of Editorial Board, the ICAST and TSTC teamwork and I hope you will find this collection of research articles useful and informative.

We are so honored to have all the new editorial board members being joined us in 2011, to strengthen our efforts, raising the prestigious level of the journal, and share in pushing all steps toward shining scientific future in Arab World.

The journal is one of the scientific contributions offered by ***the International Centre for Advancement of Sciences and Technology*** in cooperation with ***Treasure Est. for Scientific Training and Consultations*** to the science and technology community (Arab region with specific focus on Iraq and International).

Finally, on behalf of *the International centre*, I would like to express my gratitude and appreciation to the efforts of the Editorial Board, Advisory group with their valuable efforts in evaluating papers and the Editorial Board Secretary for managing the scientific, design, technical and administrative aspects of the Journal and for preparing this issue for final printing and publishing.

***Editor-in-Chief***

***IJST***

***Abdul Jabbar Al- Shammari***

## The Referees for this Issue

** The referees and advisory group below are listed according to alphabetical order, with deep appreciation for all.*

**Prof. Abdul- Jabbar N. Al- Shammari**

*Faculty of Pharmacy, Al- Isra University. Jordan*

**Dr. Abdullah Sh. M. Al- Shaibany**

*Dept. of food sciences, Faculty of Agriculture, Al- Kufa University. Iraq*

**Dr. Adnan E. Al- Badran**

*College of Sciences . Basra University. Iraq*

**Dr. Akram O. Al- Mashaykhi**

*Dept. of Information Technology, College of Computer Sciences. Amman Arab University. Jordan*

**Prof. Jamal A. Abbas**

*Faculty of Agriculture, Al- Kufa University. Iraq*

**Prof. Jameela Gh. Auda**

*Al- Kindi College of Medicine. Baghdad University. Iraq*

**Prof. Loai Al- Shamaony**

*Faculty of Pharmacy, Misr University for Sciences and Technology. Egypt*

**Prof. Malik Merza**

*Virology Dept. Svanova Biotech University, Uppsala, Sweden*

**Dr. Mohammed A.M. Al- Hajaj**

*College of Sciences . Basra University. Iraq*

**Prof. Taha Al- Samaraei**

*Crown Research Institutes, Palmerston North. New Zealand*

**Prof. Zaki G. Abdul- Ghani**

*Faculty of Pharmaceutical Sciences, Amman Private University, Jordan*

# TABLE OF CONTENTS

** Articles in this issue are listed below according to field specialties order, starting by English section and followed by Arabic section.*

## *(I) ENGLISH SECTION:*

# *ENGLISH SECTION*

# Destruction of *Rhizoctonia solani* by Local Isolates of *Serratia marcescens* Produced Glycolipid and Lytic Enzymes

**Zaid A. H. Al- Shammari**

*Dept. of Biotechnology / College of Sciences/ Baghdad University- Iraq*

***ABSTRACT***

In the present study, the capability of *Serratia marcescens* local isolates from rhizospher of garden soil was studied for biosurfactant and lytic enzyme production. The results exhibited higher capability of isolate *Serratia marcescens* N8 for biosurfactant and lytic enzyme production among seven isolates. The isolate *Serratia marcescens* N8 showed a better emulsifier producer, therefore it selected for biosurfactant production and antifungal activity in the present study. Biochemical analysis of partially purified bioemulsifier demonstrated that the biosurfactant contains amino acid group with *Rf* values of 0.25, glycolipid with *Rf* values of 0.54, and presence of two kind of sugar with *Rf* values of 0.5 and 0.88. Crude biosurfactant efficiency tested for antifungal activity by determination percentage reduction of radial growth, fresh and dry weight and chitinase and protease activity of *Rhizoctonia solani*. Maximum reduction in radial growth 72.2%, and reduction of biomass obtained at by product concentration of 1500 µg/ ml. Maximum chitinase and protease specific activity reached 67 and 62.8 U/mg protein obtained at low by product concentration of 500 µg/ ml, while reduction about 92% and 95% of the enzyme activity respectively recorded at higher by product concentration of 1500 µg/ ml.

***Key words***: Biosurfactant, glycolipid, antifungal activity, enzyme activity

**الملخص باللغة العربية**

تمت دراسة قدرة سبعة عزلات محلية من بكتريا *Serratia marcescens* المعزولة من التربة على انتاج المستحلبات الحياتية والانزيمات المحللة. اظهرت النتائج قدرة عالية للعزلة N8 *Serratia marcescens* في انتاج المستحلب الحياتي والانزيمات المحللة من بين سبعة عزلات. بينت العزلة N8 *Serratia marcescens* قدرة عالية في انتاج المستحلب الحياتي, لذا اختيرت لانتاج المستحلب الحياتي ودراسة فعاليته التثبيطية.اظهرت نتائج الفحوصات الكيموحيوية بان المستحلب الحياتي المنتج يحتوي على مجموعة حامض اميني مع قيمة للحركة النسبية (*Rf*) بلغت 0.25 والدهون السكرية مع قيمة للحركة النسبية (*Rf*) بلغت 0.54 بلغت ووجود نوعين من السكر مع قيم للحركة النسبية (*Rf*) بلغت 0.5 و 0.88. اختبرت كفاءة المنتج الخام للفعالية التثبيطية, وذلك بتحديد النسبة المئوية لاختزال نمو الهايفات, الوزن الطري والرطب وقياس فعالية انزيم الكايتينيز والبروتيز لفطر *Rhizoctonia solani* . اظهرت النتائج اقصى اختزال في نمو الهايفات بلغ 72.2% واختزال عالي في الكتلة الحيوية عند التركيز 1500 مايكروغرام / مل. كما بينت النتائج بان اعلى فعالية نوعية لانزيم الكايتينيز والبروتيز المنتج من الفطر بلغت 67 و 62.8 وحدة/غم بروتين بالترتيب عند اقل تركيز للمنتوج الخام 500 مايكروغرام / مل, بينما سجل انخفاض 92% و 95% في الفعالية النوعية لانزيم الكايتينيز والبروتيز بالترتيب عند التركيز 1500 مايكروغرام / مل من المنتوج الخام.

## *INTRODUCTION*

*Serratia marcescens* is a Gram-negative, enteric bacterium that is able to inhabit a wide variety of ecological niches and cause disease in plant, vertebrate and invertebrate hosts. *S. marcescens* strains produce a range of secreted products, including proteases, nuclease, lipase, chitinases and haemolysin, many of which are likely to represent virulence factors in human infection. A characteristic feature of many S. marcescens strains, particularly those of environmental origin, is production of the red tripyrrole antibiotic prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin). Prodigiosins are currently of great interest because they have been shown to possess antimicrobial, antiprotozoal, immunosuppressive and anti-oncogenic properties (1).

Some strains also synthesize useful secondary metabolites e.g. antibiotics, red pigments and surfactants (The surfactant was identified as arabinolipid), which have potential applications in the pharmaceutical industry and environmental bioremediation .The expression of several *Serratia* virulence factors as well as the synthesis of secondary metabolites is controlled in a cell population density-dependent manner and may also be regulated coordinately with population migration (swarming and sliding) (2).

The microbial surfactants called as biosurfactants are microbial compounds with a distinct surface activity that exhibit a broad diversity of chemical structures such as glycolipids, lipopeptides and lipoproteins, lipopolysaccharides, phospholipids, fatty acids and polimeric lipids. Therefore, it is reasonable to expect diverse properties and physiological functions of biosurfactants such as increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation (3). A host of interesting features of biosurfactants have led to a wide range of potential applications in the medical field. They are useful as antibacterial, antifungal and antiviral agents, and they also have the potential for use as major immunomodulatory molecules and adhesive agents (3).

*Serratia*, a group of gram negative bacteria produces surface active cyclodepsipeptides known as serrawettin W1, W2 and W3. Besides this *Serratia liquefaciens* produces serrawettin W2. Temperature dependant synthesis of two novel lipids – rubiwettin R1 and RG1 is observed in *Serratia rubidaea*. Serrawettin contains only Ser and Thr as hydrophilic amino acid residues. The amino acids were shown to increase cell hydrophilicity by blocking the hydrophobic sites on the cell surface and to promote flagellum – independent mobility (4).

*Serratia marcescens* secretes a variety of extracellular enzymes including chitinases. *S. marcescens* is one of the most effective bacteria for degradation of chitin. When this bacterium is cultivated in the presence of chitin, a variety of chitinolytic enzymes and chitin-binding proteins can be detected. *S marcescens* produces at least three chitinases (ChiA, ChiB and ChiC), a chitobiase and a putative chitin-binding protein (CBP21) (5). The chitinolytic machinery of *S. marcescens* is of great interest because it is one of the best characterized chitinolytic machineries known to date. A synergistic inhibitory activity of prodigiosin and chitinolytic enzymes was observed against spore germination of *Botrytis cinerea*. Selective activity against cancer cell lines enhanced lethal and inhibitory activity of Cry1C BT toxin along with prodigiosin (5).

The aim of this research focused on the study the capability of *Serratia marcescens* isolates from rhizosphere

zone of garden soils, to biosurfactant and lytic enzymes production. Moreover, study the effect of partial purified biosurfactant on mycelium growth, biomass formation and chitinase and protease activity of *Rhizoctonia solani*.

## *MATERIALS AND METHODS*

Microorganisms and growth conditions:

Four bacterial isolates used in this study were isolated from the rhizosphere zone, of the gardens soil, near department of biotechnology /University of Baghdad. Remained isolates of *Serratia marcescens* isolated from soil contaminated with hydrocarbons and its derivatives, obtained from Biology Department of the Baghdad University.
The isolates was maintained on nutrient agar medium (Difco, India) at 30 $^{o}$C. The Fungus isolate *Rhizoctonia solani* used in this study was isolated from infected tomato, obtained from Department of Biotechnology / College of Science /University of Baghdad. The isolate *Rhizoctonia solani* was grown on Potato dextrose agar (PDA) and incubated at 25 $^{o}$C for 72 hrs.

Screening of isolates for lytic enzyme production:

The isolates of *Serratia marcescens* were separately grown on a medium developed by [6] which contained ( g / L): MgSO4 .7H2O,0.2 ; K2HPO4 , 0.9 ; KCl , 0.2 ; NH4NO3 , 1.0 ; FeSO4.7H2O , 0.002 ; MnSO4. H2O, 0.002 and ZnSO4.7H2O, 0.002 (pH 7). The medium was supplemented with dead fungal mycelium (*Rhizoctonia solani*) as inducers for enzymes production at a concentration of 1.0 % and dispensed in Erlenmeyer flasks (250 ml) each flask contained 50 ml of medium.
The flasks were autoclaved and each flask was inoculated with 1% ml of overnight cultures of *Serratia marcescens* isolates. The cultures were incubated in shaker incubator (Basel Switzerland) (150 rpm.), at 30°C. The flasks from each culture were analyzed after 72 hrs of incubation for lytic enzyme production.

Enzymes assay procedures:

Chitinase activity was assayed following the release of N-acetylglucosamine according to the method of Monreal and Reese (6). One ml of 1 % colloidal chitin in 0.1 M citrate phosphate buffer (pH 6.5) was incubated with 1.0 ml of culture filtrate at 37 °C for 2 hrs. One unit of enzyme activity is defined as the amount of enzyme required to produce 0.5 µM/ml of N acetylglucosamine per hour. Specific activity was expressed as units/mg protein.
For protease activity, a reaction mixture containing 1.0 ml of 1 % soluble casein in 0.05M-citrate phosphate buffer (pH 6.5) and 1.0 ml of culture filtrate was used. The reaction mixture was incubated for 1 hr at 37°C then stopped by adding 10 % trichloroacetic acid (TCA), kept for another 20 min at the same temperature, followed by centrifugation at 4000 rpm for 20 min.
Samples of 75 µL were removed and tyrosine was determined according to (7). One unit of the enzyme activity was defined as the amount of enzyme required for the formation of 1.0 µM of the product / min of the reaction, under the standard assay conditions.

Screening of biosurfactant production isolates:

To determine the biosurfactant production ability, S. *marcescens* isolates were inoculated with mineral salt medium (MSM) according to the methods described by (8). In brief, isolates were inoculated into 50 ml of MSM containing 3 % (v/v) of olive oil and incubated with continuous shaking (150

rpm) for 72 hrs at 30 °C using a shaker incubator. Biosurfactant production ability for isolates was determined by extraction of crud by product and determining the weight of crud by product.

Biosurfactant Extraction and production

Production of biosurfactant was performed using method described by (8). Briefly, isolate were grown in 250 ml Erlenmeyer flasks, each containing 50 ml of MSM amended with 3 % (v/v) of olive oil. The flasks were incubated at 30 °C in a shaker incubator for 72 hrs. To isolate the biosurfactant, bacteria were precipitated by centrifugation at 10 000 rpm at 4ºC for 15 min. Biosurfactant was obtained by adjusting the supernatant pH to 2.0 using 6 N HCl and keeping it at 4 °C overnight. The precipitate thus obtained was pelleted at 8000g for 20min, dried and weighted. For further purification, the crude surfactant was dissolved in distilled water at pH 7.0 and dried at 60 °C. The dry product was extracted with chloroform: methanol (65:15), filtered and the solvent evaporated. The red or pinky dried crude biosurfactant was then used for remaining studies.

Biosurfactant analysis**:**

The biosurfactant were isolated according to the method described by (9). The extracted biosurfactant were dissolved in chloroform-methanol (9:1) in the concentration of 10 mg/ml, and 2 µl of the sample was spotted onto thin-layer chromatography (TLC) plates (silica gel 60 F254; Merck) with dimension of (20cm × 20cm × 0.25mm). After development in chloroform-methanol-acetic acid (65:15:2), The amino acid content of surfactant was determined using the ninhydrin method, and the lipid content determined by the phenol-sulphuric reaction method, and glycolipid were determined using the molish test (9), (10).

Antifungal activity

1- Effect of *S. marcescens* glycolipid on radial growth rate of *Rhizoctonia solani*

The effect of glycolipid of *Serratia marcescens* N8 on radial growth rate of *Rhizoctonia solani* was studied on 1/5$^{th}$-strength potato dextrose agar (pH 7). Sterilized growth medium was cooled down to 55 °C and amended with crude glycolipid to final concentrations of 0, 500, 750, 1000, 1250, and 1500 µg/ ml, then the moisture vortexes forcedly to homogenized; each plate contained 20 ml of growth medium. A plug (8 mm) of *R. solani* mycelium, excised from the margin of 3 day old culture PDA plates, and placed in the centre of the 9.0 cm diameter Petri plate containing one-fifth strength PDA amended with partially purified biosurfactant, and incubated at 25 °C. Radial growth rate was measured with a ruler after 3 days. Control plates containing growth medium without addition of glycolipid. Percentage of radial growth inhibition were recorded according to the formula, as follows: Percentage of growth reduction = [(A-B)/A × 100], where: A= diameter of the control hyphal growth (mm), B = diameter of the treated hyphal growth (mm) (6).

2- Effect of crude glycolipid on biomass formation of *R. solani* and lytic enzyme production

The effects of crude glycolipid on biomass of *R. solani* was studied in clarified 1/5$^{th}$-strength potato dextrose broth (PDB, pH 7.0); final concentrations of glycolipid were 0, 500, 750, 1000, 1250, and 1500 µg/ ml. A mycelia plug (8 mm) was transferred to 9.0 cm-diameter Petri plates containing 20 ml of the growth liquid medium. After incubation at 25 °C for 3 days,

mycelium was collected by centrifugation at 5000g for 20 min and blotted to dry on a Whitman filter paper to determining the fresh weight; after drying at 65 $^{o}$C for 24 hrs, mycelium dry weight was determined; obtained weights were corrected for the weight (fresh and dry) of the agar plugs used to inoculate *R. solani* (11). The remained culture precipitates after centrifugations were used for chitinase and protease estimation.

## *RESULTS AND DISCUSSION*

### Lytic enzymes production

Antagonistic microorganisms (*S marcescens* isolates) are able to produce various cell-wall degrading enzymes (Chitinase, *â*-1, 3 and, *â*-1, 4-glucanases, protease and lipase) which may be involved in the cells lysis of phytopathogenic fungi. Antibiosis activity of *S marcescens* isolates were observed on liquid media containing dead mycelia of *Rhizoctonia solani* as a carbon sources to determine the production of lytic enzymes. Data represented in Table 1 showed that the lysis of dead mycelia of *Rhizoctonia solani* were very efficient by *S marcescens* 8 isolate (80% lysis) after 72hrs of incubation at 25± 2°C . On dead mycelium of *Rhizoctonia solani* chitinase, protease was the most lytic enzymes produced by .*S marcescens* 8 antagonistic strains. These results was aggrement with that obtained by (6) who noticed the Antibiosis activity of *Pseudomonas fluorescens* NRC1 and *Pseudomonas fluorescens* NRC3 were observed on liquid media containing dead mycelia of *Phytophthora capsici* or *Rhizoctonia solani* as a carbon sources to determine the production of lytic enzymes.

The results showed that the lysis of dead mycelia of *Phytophthora capsici* or *Rhizoctonia solani* were very efficient by *Pseudomonas fluorescens* NRC1 and *Pseudomonas fluorescens* NRC3 (100% lysis) after 72hrs of incubation at 25± 2°C. On dead mycelium of *Rhizoctonia solani* chitinase, protease was the most lytic enzymes produced by *Pseudomonas fluorescens* NRC1 and *Pseudomonas fluorescens*. On the other hand, lipase was produced at the least rate.(Table 1)

There are many reports on the production of lytic enzymes by microorganism's detected glucanases and chitinase in soil inoculated with *Trichoderma harzianum*. The isolates of *T. harzianum*, which were found to differ in their ability to attack *Sclerotium rolfsii, Rhizoctonia solani*, and *Pythium aphanidermatum,* also differed in the levels of mycolytic enzymes produced by them, as reported by (12). (13) reported that *Serratia marcescens* as a biocontrol agent acts only through the production of pathogen cell wall lysing enzymes, chitinase and *â*-1, 3-glucanase not through any antifungal metabolites.

**Table (1): Lytic enzymes produced by *Serratia marcescens* isolate grown on dead mycelium of *Rhizoctonia solani* after 72 hrs of incubation at 25±2 °C.**

| Isolates | Residual dead mycelium mg/50 ml medium | Percent of dead mycelium lyses (%) | Chitinase (unit/mg protein) | Protease (unit/mg protein) |
|---|---|---|---|---|
| Control | 500 | 0 | 0 | 0 |
| S1 | 380 | 24 | 7.0 | 8 |
| S2 | 300 | 40 | 17 | 19 |
| S5 | 250 | 50 | 20.4 | 21.2 |
| S6 | 350 | 30 | 6.0 | 3.9 |
| S7 | 150 | 70 | 30 | 25.3 |
| S8 | 100 | 80 | 45 | 47.2 |
| S10 | 350 | 30 | 7.8 | 5.9 |

## Screening of isolates for biosurfactant production

Among seven isolates tested for biosurfactant production, four isolates exhibited better biosurfactant production. The results in Table (2) indicated that the isolate *Serratia marcescens* N8 was the best biosurfactant producing among the other isolates based on the capability of isolates to degrade olive oil as the sole source of carbon and energy, and higher biosurfactnt production with emulsification of olive oil to small droplets.

**Table (2): Screening of *Serratia marcescens* isolate for biosurfactant production**

| Isolates | Biosurfactant concentration (g/l) |
|---|---|
| *S. marcescens* N1 | 2.5 |
| *S. marcescens* N2 | 4.5 |
| *S. marcescens* N5 | 5.5 |
| *S. marcescens* N6 | 1.6 |
| *S. marcescens* N7 | 5.0 |
| *S. marcescens* N8 | 6.0 |
| *S. marcescens* N10 | 3 |

The isolate *S. marcescens* N8 was selected in the present study accordance to their higher growth rates and biosurfactant and lytic enzyme production for remained experiments.

## Biosurfactant characterization

The structural analysis of biosurfactants produced by *S. marcescens* N8, were performed by lipid, protein and carbohydrate contents in thin layer chromatography (TLC) analysis (Figure 1). Ninhydrin test was used to detect the presence of amino acids contents of biosurfactants. However when the spots on TLC plates spread with this reagent, the biosurfactant gave positive results, an evident that biosurfactant contained to amino acid group, the presence of amino acid were observed as red-violet spot with $R_f$ = 0.25.

This detection gives an indicator for the presence of amino group in the produced bioemulsifier and it may support the presence of the protein in bioemulsifier. (14) showed that the cells extract surfactant serrawettin W1 of *Serratia* sp. was analyzed by TLC, the treated product with ninhydrin reagent revealed the presence of amino group with $R_f$ = 0.2. (15) mentioned that methanol-soluble products of serrawettin W2 hydrolysis were ninhydrin positive.

Molisch's test (α – Naphthol) is a sensitive chemical test for the presence of glycolipids, based on the dehydration of the carbohydrate by sulfuric acid to produce an aldehyde, which condenses with two molecules of phenol resulting in a red- or purple-colored compound (16). From the results of Molisch's test, it was shown that the biosurfactant contains carbohydrate residues. The phenol – sulphuric acid reaction analyzed for lipid content of biosurfactants. The biosurfactant gave the positive results with phenol – sulphuric acid reaction. This means that the biosurfactant obtained in this study have lipid content. To determine the presence of glycolipids in bioemulsifier of *S. marcescens* N8, the thin layer was treated by orcinol – ferric chloride – sulfuric acid reagent after the diffusion of bioemulsifier, the result appeared as blue-violate spots on TLC plate indicating the presence of glycolipids with $R_f$ = 0.54 (17). This result supports the presence of carbohydrate and lipid in the components of *Serratia* bioemulsifier, which agreed with the study of (18) who reported that *Serratia marcescens* bioemulsifier consists of glycolipid part such as rhamnolipid.(Fig 1)

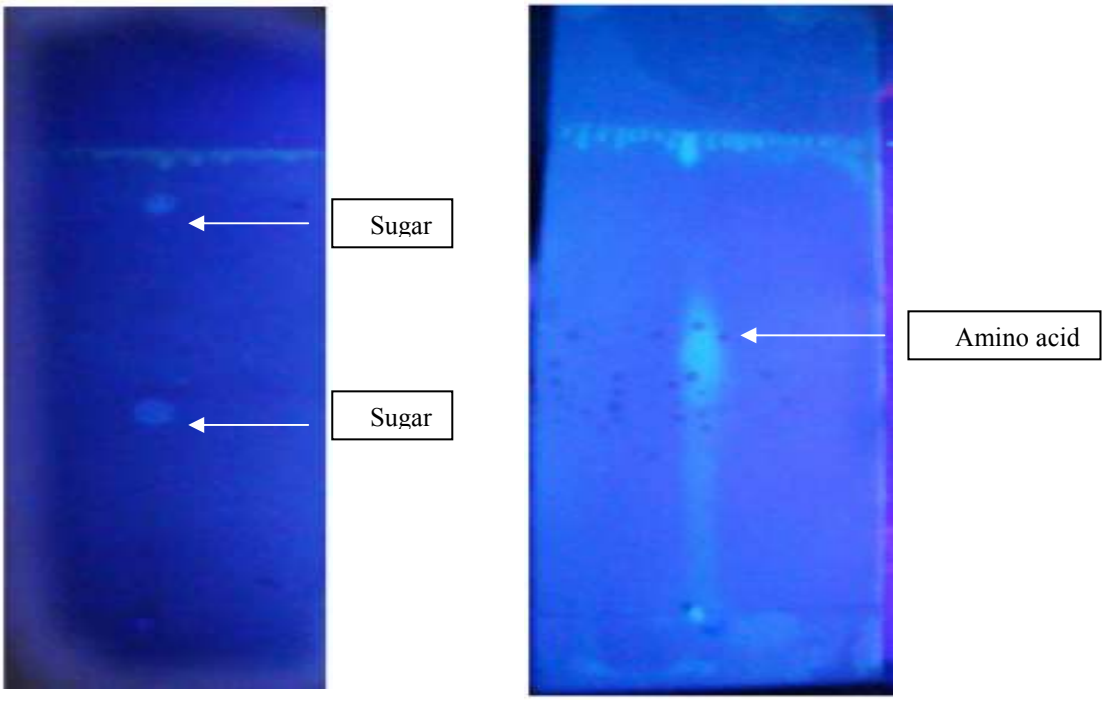



**Figure ( 1 ): TLC analysis for detection of sugar by phenol – sulfuric acid reagent and detection of glycolipids by orcinol-ferric chloride-sulfuric acid using silica gel plate (20 ×20) cm with solvent system chloroform : methanol: acetic acid ( 65 : 15 : 2 , v:v:v ) at room temperature : (A) TLC plate under UV light showed two spots of sugars. (B) TLC under UV light showed blue violate zone of amino acid.**

Thin layer chromatography results suggested that the isolated surface-active product from *S. marcescens* N8 was composed of carbohydrates. The extracted product was separated on TLC plates with a carrier solvent of (chloroform: Methanol: acetic acid, 65:15:2 by volume). When the plats were visualized by reagents, the product of *S. marcescens* N8 was observed to have two spots. Spot 1 with *Rf* = 0.5, and spot two with *Rf* = 0.88. This result may indicate the presence of two kinds of sugars (carbohydrates) that may form basic and functional components of the bioemulsifier. The results observed was in agreement with (19) who reported that the $R_f$ of some standard sugars by using TLC method was: cellobiose $R_f$ =0.30, galactose $R_f$ = 0.43, arabinose $R_f$ = 0.54 and rhamnose $R_f$ = 0.79. From this information we can indicate that the $R_f$ 0.54 for arabinose is very closed for the lower (high molecular weights) spot of *Serratia* bioemulsifier $R_f$ 0.5, while $R_f$ 0.75 of rhaminose is very closed for the higher (low molecular weight) spot of *Serratia* bioemulsifier $R_f$ 0.88.

## Antifungal activity

The antifungal activity of partial purified crude glycolipid and their effects were studied on 1/5$^{th}$ – strength potato dextrose agar (PDA) by determining radial growth rate,

percentage of growth inhibition, of *Rhizoctonia solani*. It found that the partially purified crude biourfactant inhibited radial growth rate of *R. solani*. The antifungal activity increased with increasing concentration of glycolipid (Figure 2). The reduction in radial growth rate ranged from 51.39 to 72.22 % at a by product concentration of 500 to 1500 µg / ml, (Table 3).

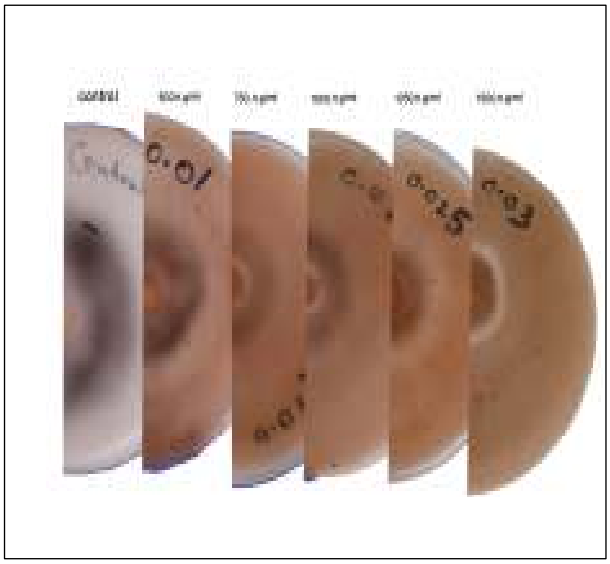


**Figure (2): Effect of different concentrations *S. marcescens* N8 glycolipid on mycelial growth of *Rhizoctonia solani* on PDA after 3 days cultivation at 25 °C**.

**Table (3): Radial growth rate and reduction percentage of radial growth for *Rhizoctonia solani* cultivated on one-fifth strength PDA amended with different concentration of *S. marcescens* N8 glycolipid**

| Concentration of Ciude glycolipid (µg /ml) | Radial growth rate after 3 days (mm) | Reduction of radial growth (%) |
|---|---|---|
| 0 (control) | **72** | **0** |
| 500 | **35** | **51.39** |
| 750 | **33** | **54.17** |
| 1000 | **31** | **56.94** |
| 1250 | **28** | **61.11** |
| 1500 | **20** | **72.22** |

One of the main modes of biosurfactants action, including rhamnolipid and cyclic lipopeptide (CLP) involves the formation of ion channels in the plasma membrane of the target organisms leading to cytolysis (3). CLP produced by soil-inhabiting *Pseudomonas* strain DR54, was shown to induce encystment of *Pythium* zoospores, and also inhibit mycelial growth of *Rhizoctonia solani* and *Pythium ultimum* causing reduced growth and intracellular activity, hyphal swellings, increased branching and rosette formation (11). Thu-Ha *et. al* (11) investigated the effect of purified CLP on mycelial growth of *Phytophthora infestans* on 1/5$^{th}$ strength PDA. The results indicated that mycelial growth of the fungus inhibited at a CLP concentration of 50 µg/ml and higher, caused increased branching of hyphae.

The antifungal activity of *Serratia* bioemulsifier was detected against *Candida albicans, Aspergillus niger and Geotricum*. Inhibition zones were observed around the disks containing bioemulsifier, the inhibition zones of *S. marcescen*s (S10) bioemulsifier against *Aspergillus* and *Geotricum* were (12mm, 10 mm respectively). While the bioemulsifier showed reducing in the growth of *Candida albicans* the inhibition zone of S10 was (9mm) (17).

The fungi possess cell wall composed largely of carbohydrate layers, long chains of polysaccharides, as well as glycoproteins and lipids (20). Bioemulsifier can acts on the lipids in the cell, this interaction alters the membrane fluidity and perhaps produces pores in the membrane through which ions and small molecules are lost.

Surfactin (*Bacillus subtilis* bioemulsifier) was found to be more efficient than iturin A in modifying the *Bacillus subtilis* surface hydrophobic character. This aspect appears essential, in association with the antifungal properties of lipopeptides

involved, in the biological control of plant diseases , also iturin A's mechanism of action is related to the disruption of the plasma membrane by the formation of small vesicles and the aggregation of intramembranous particles in yeast cells. Biosurfactants produced by *Pseudomonas* spp. displayed antifungal activity against yeasts (*Candidia albicans* FMC 17 and *Candida krusei* ATCC 6258), with diameters of zone inhibition ranging between 12 and 17 mm (3).

Results in Figure 3 showed the growth of *R. solani* in liquid media (PDB), growth of *R. solani* was significantly inhibited at glycolipid concentration of 500 µg/ml and higher. Fresh and dry weight of fungus decreased logarithmically with increasing concentrations of by product reaching maximum reduction in biomass at glycolipid concentration of 1500 µg/ml. (11) also studied the effect of CLP on mycelial growth, fresh and dry weight of *Phytophthora infestans* on 1/5$^{th}$ strength PDB. The results showed that the mycelial growth inhibited at a CLP concentration of 10 µg/ml and higher, fresh and dry weight of *Pythium infestans* decreased logarithmically with increasing concentrations of CLP reaching the maximum reduction in biomass at CLP concentration of 50 µg/ml and higher.

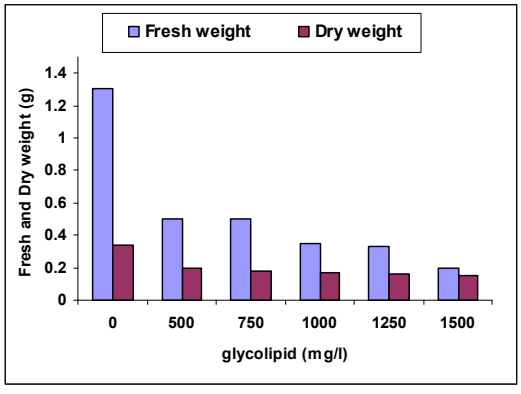



Figure (3): Effect of *S. marcescens* N8 crude by product on biomass (fresh and dry weights) of *Rhizoctonia solani* grown on PDB after 3 days cultivation at 25 °C.

(21) studied antifungal activities of the crude lipopeptide biosurfactant from *Bacillus natto* ASA at different concentrations (0.8-3.2 g/L) using disc diffusion method. Biosurfactant exhibited interesting and antifungal activities. The antifungal activity increased with increasing concentration of biosurfactant and maximum zone of inhibition obtained (48.8 mm) with *Botrytis cinerea* at a biosurfactant concentration of 3.2 g/l. (22) found effective antifungal activity of rhamnolipid and sophorolipid against plant and seed pathogenic fungi. A rhamnolipid mixture obtained from *Pseudomonas aeruginosa* AT10 showed excellent antifungal properties against *Aspergillus niger* at concentration of (16 mg/ml), *Chaetonium globosum, Pnicillum crysogenum, Aureobasidium pullulans* (32 mg/ml), and the phytopathogenic *Botrytis cinerea* and *Rhizoctonia solani* (18 mg/ml).

The antimicrobial effects of biosurfactants can be explained by the structures of biosurfactants resembled to cell membrane. Biosurfactants are amphipathic molecules with hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, or fatty acids. Insertion of fatty acids components of biosurfactants into a cell membrane caused significant ultra structural changes in the cells such as ability of the cell to interiorize plasma membrane. Also, antimicrobial effects of biosurfactants could be that the bleb formation represented an increase in the size of the membrane due to insertion of lipid material. One explanation of the antimicrobial effect of biosurfactants is the adhering property of biosurfactants to cell surfaces caused deterioration in the integrity of cell membrane and also breakdown the nutrition cycle. All these cumulative effects can be

explained the antimicrobial effects of biosurfactants (3).

The produced biosurfactant in the present study was not effective as many of biosurfactants described in the literature, it should be noted that the biosurfactant studied here was not purified enough as the ones described in the literature. The observed results in the present study showed that the maximum reduction (72.22%) of radial growth obtained at crude glycolipid of *S. marcescens* N8 concentration of 1500 µg / ml or (mg/l). Therefore further purification for produced biosurfactant required to increase effectiveness and surface activity of biosurfactant, and decreased the critical micelle concentration.

**Chitinase and protease activity of *R. solani***

The results in Figure 4 showed that the chitinase and protease activity of *R. solani* increased in liquid media of PDB at low concentration glycolipid (500 µg / ml), while at concentration of 1000 µg / ml and higher the enzymes activity was inhibited. Reduction about 92% and 95% of the chitinase and protease activity recorded at higher glycolipid concentration of 1500 µg / ml, compared with low concentration of 500 µg / ml.

A possible structure-function relationship between the hydrophobic fatty acid tail, together with the amphiphilic property of the peptide and glycolipid surfactant, structure may finally play an important role in penetration and binding of biosurfactant within biological membranes. This in turn may support their role as surfactants and as antibiotics, e.g., disrupting membrane functions leading to excess $Ca_2$ influx into target cells, which led to destruction cell wall of microfungus especially at high concentration (23).

In the present study, the glycolipid produced from *S. marcescens* N8 isolate revealed the inhibition towards the *R. solani* chitinase and protease, especially at concentration of 1000 µg/ml and higher. These properties most probably resemble in their action to cell wall degrading enzyme (protease and chitinase) produced by *S. marcescens* N8. In the similar study of (23), the CLP producing *Pseudomonas fluorescens* $V_2$ and $V_3$ demonstrated the largest inhibition towards *R. solani*, also had the most complete array of cell wall degrading enzymes (protease and chitinase).

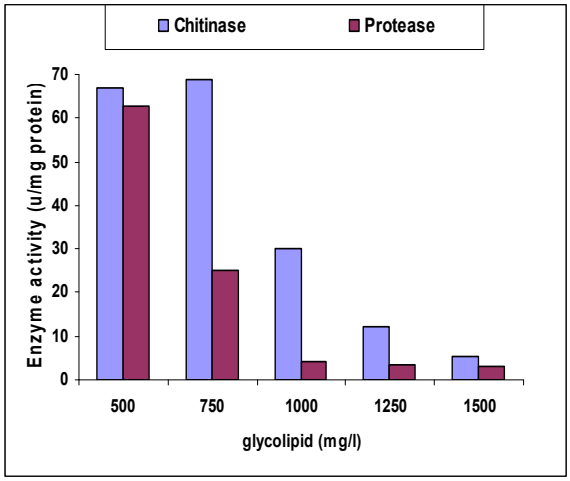



**Figure (4): Lytic enzyme activity of *R. solani* at different concentration of *S. marcescens* N8 glycolipid in PDB medium after 3 days of cultivation at 25 °C.**

The general impact of surfactants in promotion of protein secretion is likely to involve interactions with the lipid components of cell membranes in a manner which facilitates secretion. It should be noted that most of the observations related to the positive effects of surfactants on secretion of extracellular enzymes relate to eukaryotic organisms which release enzymes from intracellular organelles through exocytosis. This observation suggests in our study that surfactants may promote this exocytosis by interaction with cell and organelle lipid membrane components, and enzyme activity increased at low concentration of glycolipid, until reaching inhibitory

concentration at 1000 µg/ml, above this concentration the enzyme activity adversely affected (24). (24) also mentioned that the low concentrations of the nonionic surfactant, polyoxyethylene laurylether; C30 H62 O10, increased the activity of cholesterol oxidases from *Streptomyces hygroscopicus* (SCO) and *Brevibacterium sterolicum* (BCO) in aqueous media containing propanol as a substrate solubilizer while at higher surfactant concentrations the opposite effect occurs.

***ACKNOWLEDGEMENT***

I would like to acknowledge Miss Entissar Faroun Ahmed, Department of Biology /College of Science / University of Baghdad, for valuable help in TLC analysis.

***REFERENCES***

1. Coulthurst SJ., Williamson NR., Harris AK., Spring DR., and Salmond GPC. (2006) Metabolic and regulatory engineering of *Serratia marcescens*: mimicking phage-mediated horizontal acquisition of antibiotic biosynthesis and quorum sensing capacities. Microbiol. 152: 1899–1911.
2. Horng YT. , Deng SC. , Daykin M . , Soo PC. , Wei JR. , Luh K T., Ho SW. , Swift S. , Lai HC., and Williams P. (2002) The LuxR family protein SpnR functions as a negative regulator of N-acylhomoserine lactose –dependent quorum sensing in *Serratia marcescens* . Mol. Microbiol. 45: 1655-1671.
3. Yalçin E. and Ergene A . (2009) Screening the antimicrobial activity of biosurfactants produced by microorganisms isolated from refinery waste waters. J. App. Biol. Sci. 3 (2): 148 – 153.
4. Matsumiya Y. , Wakita D. , Kimura A. , Sanpa S. and Kubo M. (2007) Isolation and characterization of a lipid-degrading bacterium and its application to lipid-containing waste water treatment. J. Biosci. and Bioeng. 103 (4) : 325-330
5. Diby PK., Saju Y., Jisha A., Kumar YS., and Anandaraj M. (2005) Mycolytic enzymes produced by *Pseudomonas fluorescens* and *Trichoderma* spp. Against *Phytophthora capsici*, the foot rot pathogen of black pepper (Piper nigrum L.).Ind. Phytopathol. 58: 10.
6. Moataza MS (2006) Destruction of *Rhizoctonia solani* and *Phytophthora capsici* Causing Tomato Root-rot by *Pseudomonas fluorescences*

Lytic Enzymes. J. Agr. and Biol. Sci. 2(6): 274-281.
7. Lowry O., Rosebrough N, Farr A, and Randall R(1951) Protein measurement with the Folin phenolreagent. J. Biol. Chem. 139: 265.
8. Abouseoud M., Maachi R., Amrane A., Boudergua S., and Nabi A. (2008) Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. Desalin. 223: 143-151.
9. Guerra-Santos L., Kappel O., and Fiechter A. (1984). *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl. Environ. Microbiol. 18(2): 301-305.
10. Vioque E. (1984) Spray reagent for thin-layer chromatography (TLC) and paper chromatography (PC). In: CRC Hand book of chromatography: Lipids. CRC. Press. Inc. 2: 309-317.
11. Thu-Ha TT. (2007) Role of CLP massetolid A: in biological control of *Phytophthora infestans* and in colonization of Tomato plants by *Pseudomonas fluorescens*. PhD thesis. Wageningen University. The Netherlands. 23-35.
12. Elad Y., Chet I., and Henis Y. (1982) Degradation of plant pathogenic fungi by Trichoderma harzianum. Can. J. Microbiol. 28: 719.
13. El- Trabily K., Sykes M., Kurtbohe I., Hardy G., Barbosa A. and Dekker R (2000) Synergistic effect of cellulose producing *Micromonospora carbonacea* and an antibiotic producing *Streptomyces violascens* on the suppression of *Phytophthora cinnamomi* root rot of Banksias grand's. Can. J. Bot. 74: 618.
14. Roberts DP. , McKenna LF. , Lakshman DK. , Meyer SLF. , Kong H. , De Souza JT. , Lydon J. , Baker CJ. , Buyer JS., and Chung S. (2007) Suppression of damping-off of cucumber caused by *Pythium ultimum* with live cells and extracts of *Serratia marcescens* N4-5. Soil Biology & Biochemistry. 39: 2275-2288.
15. Matsuyama T. , Kaneda K. , Nakagawa Y. , Isa K. , Hara-Hotta H., and Yano I. (1992) A novel extracellular cyclic lipopeptide which promotes flagellum – dependent and independent spreading growth of *Serratia marcescens* . J. Bacteriol. 174 (6): 1769-1776.
16. Vioque E . (1984) Spray reagent for thin-layer chromatography (TLC) and paper chromatography (PC). In: CRC Hand Book of Chromatography: Lipids. 2: 309 – 317. CRC. Press. Inc.
17. Al-Mulla EF. (2010) Production and Characterization of Bioemulsifier from Locally Isolated *Serratia marcescens* S10. M.Sc. Theses. College of Science / University of Baghdad. 86-106
18. Bidlan R., Deepthi N., and Manonmani H . (2007) Optimized production of biosurfactant by *Serratia marcescens* DT-1P. Res. J. of Microb. 2 (10) : 705 – 716 .
19. Dudkina MM. , Kotel 'nikova NE. , Gankina ES. , Malakhova II., and Petropavlovskii GA. (1988) Use of thin-layer chromatography for determining sugars in microcrystalline cellulose. J. Chem. of Nat. Comp. 24 (6): 742-743.
20. Brooks GF., Butel JS., and Morse SA. ( 1998 ) Enteric Gram-Negative Rods ( Enterobacteriaceae ) , In : Jawetz , Melnick & Adelberg's

Medical Microbiology, ( 21ed ) Appleton & Lange , Stamford :218-230.

21. Cao X., Liao Z., Wang C., Yang W., and Lu M. (2009) Evaluation of lipopeptide biosurfactant from *Bacillus* natto TK-1 as a potential source of anti-adhesive, antimicrobial and antitumor activities. Brazilian. J. Microbiol. 40: 373-379.
22. Muthusamy K., Gopalakrishnan S., Ravi T., and Sivachidambaram P. (2008) Biosurfactant properties: commercial production and application. Current. Sci. 94(25): 736-747.
23. Nielsen T., Sorensen D., Tobasen C., Anderson J., Christophersen C., Givkov M., and Sorensen J. (2002) Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar Beet rhizospher. Appl. Environ. Microbiol. 68(7): 3416-3423.
24. Singh A., Van Hamme JD., and Ward O. (2006) Surfactants in microbiology and biotechnology: Part 2. Application aspects. Department of Biology. University of Waterloo: Ontario, Canada. 100-116

# Effects of Hemodialysis Treatment on Serum Trace Elements in Patients with Chronic Renal Failure

**Sami A. Mohammed**

*Dept. of Basic Sciences/ College of Dentistry/ Al- Anbar University- Iraq*

*Email: Khalid_awad10@yahoo.com*

***ABSTRACT***

This study was include the effect of chronic renal failure (CRF) disease on undergoing hemodialysis (HD) treatment patients of both sexes and healthy control, Six trace elements which includes (Zn,Fe,Mg,Se,Al ,Ca) were measured in serum of individuals by using Atomic Absorption Spectrophotometer (AAS) methods (flame , flameless ) . Statistically we found that there was a significant decrease in serum level of (Fe, Mg, Se, Zn, Ca) ($p<0.001$) as compared with control group, and there was significant elevation in level of (Al) ($p<0.001$).

***Key words***: Trace element TE, Atomic Absorption Spectrophotometer AAS, Hemodialysis HD

**الملخص باللغة العربية**

يتضمن البحث دراسة تأثير الفشل الكلوي المزمن لمجموعة من المرضى الخاضعين للمعالجة بالديلزة الدموية من كلا الجنسين ومقارنته بمجموعة السيطرة من الأصحاء على ست من العناصر النزرة والتي تتضمن (الخارصين،الحديد،المغنسيوم،السيلينيوم،الألمنيوم،الكالسيوم) حيث تم تقديرها في مصل الدم بواسطة تقنية مطيافية الامتصاص الذري( اللهبي وغير اللهبي) وقد وجد إحصائيا انخفاض معنوي في مستوى كل من (الخارصين،الحديد،المغنيسيوم، السيلينيوم،والكالسيوم)، كما وجد أن هناك ارتفاعا معنويا في مستوى الألمنيوم لدى مجموعة المرضى عند مقارنتها مع مجموعة السيطرة وبمستوى احتمالية ($p<0.001$).

## *INTRODUCTION*

Chronic renal failure (CRF) results from the progressive and chronic deterioration of nephrones, which happens over years. Patients with renal disease have to undergo kidney replacement therapy such as hemodialysis (HD) (1).
The aim of (HD) treatment is to remove metabolic waste products such as urea, and to remove excess fluids from patient's body to restore circulatory volume. (HD) treatment has major effects on the serum composition and fluid balance (2), (3).
Dialysis works on the principles of the diffusion of solutes and ultrafilteration of fluids across a semi-permeable membrane (4).
Trace elements (TE) are those elements that found in human and animals tissues in milligram/Kg amount or less.
The simplest definition of the essential trace elements is that elements required for life .An element is considered essential where a deficient intake produces an impairment of function and restoration with physiological amount of only that element prevents or alleviates the impairment (5).
The basis for this amplification of (TE) action is to interact with enzymes and hormones that regulate the metabolism of larger amounts of biochemical substrates (6)
Essential elements appear to function in such enzymes in one of at least three different ways:
1-The essential elements may already have inherent activity in catalyzing chemical reactions which are greatly enhanced by the enzyme protein, this is especially true of iron and copper metals.
2-The essential metal ions may form a complex with both substrates and enzyme active sites, thus bringing them together in an active form.
3-An essential metal ion may function as a potent electrons withdrawing agent at some point in the catalytic cycle (7).

In patients with uremia, trace element disturbances might occur because of (a) reduced renal function ;(b) proteinurea ,leading to loss of protein bound elements ;(c)alterations in gastrointestinal absorption because of alterations in vitamin-D metabolism and (d)the dialysis procedure per session (8).

## *MATERIALS AND METHODS*

During a period from November (2008) to March (2009), blood samples were collected from ninety persons (61males, 29females) with age range 19-70. Sixty of them were with (CRF) undergoing (HD) treatment at the artificial kidney Department in Baghdad Hospital. All patients had been subjected to (HD) three times per week during a three to four hours session. Thirty healthy persons- 20 males and 10 females- were included in this study as control group.
5cc of blood were collected from each patient in HD-unit and healthy persons. The blood samples were allowed to stand for 15 min then centerifugated at 3000 rpm for 10 min. serum was frozen at -4c till used for estimation trace elements
A standard calibration curve is drowning with two axes: Y-axis represents the Absorbance of the AAS, and X-axis represents the Concentration of the working solution.
For the measurement of (TE) sera in all group, the sample is diluted .Its absorption is measured by AAS and the resultant values are plotted on the standard calibration curve for its concentration to be measured.

## *RESULTS*

Six trace elements Zn, Fe, Mg, Se, Al, and Ca were measured in serum by using AAS method flam-flameless.
The value of Mean and SD for each one of the trace elements serum concentration of patients group and control group are illustrated in table (1)

**Table (1) The Mean value and SD of Trace Elements in patient group and control group in present study**

| | Patients Group | | Control Group | | |
|---|---|---|---|---|---|
| Variables | Mean | ±SD | Mean | ±SD | P-value |
| Ca | 6.5 | ± 0.23 | 9 | ±0.52 | P<0.001 |
| Mg | 0.5 | ±0.14 | 2 | ±0.14 | P<0.001 |
| Zn | 75 | ±8.02 | 105 | ±8.01 | P<0.05 |
| Fe | 19.3 | ±0.85 | 81.7 | ±12.1 | P<0.001 |
| Se | 60 | ±3 | 90 | ±5 | P<0.001 |
| Al | 10.9 | ±0.54 | 6.6 | ±0.11 | P<0.001 |

Our data showed that there was a significant elevation in Aluminum level. Also our studies showed that there was a significant decrease in level of Zn, Fe, Mg, Se, Ca in patients group compared with control group P<0.001 as shown in table (1)

## *DISCUSSION*

Patients with (CRF) undergoing treatment by (HD) are at risk of developing severing trace elements difference. Changes occur in the level of trace elements according to the interdependence of these elements with each other.
The Aluminum, Iron, Zinc, Magnesium, Selenium and Calcium serum contents were determined in (CRF) of dialysis patients, as evidenced by compared with normal renal function (9).
At end-stage, renal dialysis changes occur in the level of trace elements.
In our study there was significant elevation of Al serum level of patients as compared with control group P<0.001 and this found when dialysis dementia was found closely related to arise in Al concentration (10) and this agrees with our result .
Zinc is an essential trace element for life, in our study we show that there was a significant decrease in the serum level of Zn in patients compared with control group P<0.05 and this because uremic case for these patients which are not improved by dialysis treatment (11), (12). This result observed not only in Zn serum level but also Mg serum level , which was low in (HD) patients and this agrees with our study that found the same thing for patients group when compared with control group P<0.001. The result of decrease in Zn serum level was due to loss of Zn through dialysis (13) and other studies reported that Zn bound to alpha-2-micro globulin in plasma which cannot penetrate through (14), (15).
The serum level of iron in our study showed significant decrease for patients group compared with control group and this because at end stage renal disease typically results in anemia, which primarily due to deficient renal production (12), also the iron is hard to be absorbed.
For Selenium level decrease in patients group compared with control group, this occurs because nutritional intakes in patient and the protein bound trace element may be lost more readily in presence of protein urea (16)

***REFERENCES***


1. Greebeer MS and Glick M. (2003) Burkett oral medicine diagnosis and treatment. BC Decker Inc, 10th Philadelphia.417.
2. Shasha SM, Ben Arych H, Angel A, and Gutman D (1983) Salivary content in Hemodialysis patients .Oral Med.38: 60-67.
3. Paraske VA, Agryanius B, and Kopelias L. (2000) Changes in erythrocyte calcium and potassium in patient during HD and CAPD. Int.J.Artif.Organs. 23:750-753.
4. Kishimoto TK, Vismwanathan K, and Gangualy T (2008) Effect of shortly daily versus conventional Hemodialysis on left ventricular hypertrophy and inflammatory markers: aprorospective, controlled study. Int.J.Artif.Organs. 41:341-349.
5. Lehninger A, and Hopkins J (1990) Principles of biochemistry : vitamins and Trace elements in the function of enzymes. Mosby .New York: 270-271.
6. Djordjevic C (1995) Antitumor activity of the aluminum compounds . Met.Ions.Biol.syst. 31:595-616.
7. Huijgen HJ.,Van IHE, and Sanders R ( 1997 ) Precision of the magnesium determination in mononuclear blood cell and erythrocytes Clini.Biochem. 30:203-208.
8. Kentoghiorghes GJ, and Weinberg ED (1995) Iron defense mechanisms of disease and chelating therapy approaches .Blood Rev. 9:33-45.
9. Krizek M, Senft V, and Motan J (2000) Influence of Hemodialysis on selenium blood level . Karlovy University. 101(3):241-248.
10. Hasanoglu E, Altan N, Sindel S, Ongun C, Bail M, and Altintas E(1994) The relationship between erythrocyte superoxide dismutase activity and plasma level of some trace elements (Al,Cu,Zn) of dialysis patients. Gen Pharmac. 25:107-110.
11. Gaw A, Cowan RA, Oreilly DS, Stewart MJ, and Shepherd J(1999) Biochemistry In : Clinical Biochemistry. 2nd edit. .Philadelphia - Churchill Livingstone: 12-72.
12. Bogye G, Tompos G, and Alftan G (2000) Zinc depletion in Hemodialysis patient treated with polysufone membranes .Nephron. 84:119-123.
13. Sunder P, Sanchez-Portugal M, Igles S, De pula A, and Bustamante J(1999) Oxidative stress in chronic renal failure .Ren Fail 21:155-167.
14. Usberti M, Lima G, Arisi M, Bufano G, Vanzo DAL, and Gazzotti MR(1997) Effect of ceruloplasmin on the survival of red blood cells in Hemodialysis patients, Nephron. 10:261-265.
15. Ceballos PI, Witko CV, Merad BM, Nguyen AT, Thevenin M, and Jaudon MC(1996) Glutathione antioxidant system as a marker of oxidative stress in chronic renal failure, Free Radical Bio. Med. 21:845-853.
16. Zachara BA, Gromadzinska J, Wasowicz W, and Zbrog Z(2006) Red blood cell and plasma glutathione peroxides activities and Se concentration in patients with chronic kidney disease: a review .Acta Bioche. pole.53: 63-77.

# The Role of Leadership in IT Project Management Success

**Khalid M. Abu Al- Ghanam (1) Mohammed J. Adaileh (2)**

*Dept. of Computer and Information Technology/College of Al- Kamel/ King Abdul Aziz University- KSA (1) Dept. of Information Systems and management / Al- Qassim University- KSA (2)*

*Emails: khaledganam72@yahoo.com (1) madaileh@aabfs.org (2)*

***ABSTRACT***

Project management improvements have tremendous implications for economic performance.firms are facing challenges to improve success rate of their IT projects,this paper focusing on leadership as one of the most important factors for project success, by presented leadership as the art of project management ,and project mismanegement,factors for success and failure, then the important differences between leadership and management, finally present proposed model for project success.

**الملخص باللغة العربية**

أحدثت التحسينات التي جرت على إدارة المشاريع آثارا هائلة على الأداء الاقتصادي للمنظمات المعاصـــرة، وتواجـــه المنظمات وفقا لذلك تحديات كبيرة لتحسين معدلات نجاح مشاريعها الخاصة بتقنية المعلومات.

تسعى هذه الدراسة إلى التركيز على مفهوم القيادة باعتبارها واحدة من أهم عوامل نجاح المشروع، حيث تظهر الدراسة أن القيادة إنما هي فن وجوهر لإدارة المشاريع بنجاح، كما تتضمن الدراسة تحديدا لأهم مواطن الضعف ومسببات سوء الإدارة للمشروع، وتعرض لعوامل نجاح وفشل المشاريع، وتوضيح الاختلافات بين القيادة والإدارة، وتقترح الدراســـة نموذجا لنجاح المشاريع إداريا وقياديا.

## *INTRODUCTION*

The tendency to overlook "art" of project management is one reason why so many projects fail, as well as, the science project managers can increase the success rate of their projects, and well be better able to complete projects(on time, cost, scope, and quality) successfully. This can be achieved by the awareness of the project management process groups, which can lead to project integration management between the nine knowledge areas of projects to improve project success rate . This can be done by focusing on leadership skills

In this paper, the researchers try to illustrate the role of leadership in project management success, through introducing the leadership as the art of project management in integrating and managing the nine knowledge areas, then represent the project mismanagement ,reasons for project success and failure, explain the evolving requirements of IT leadership skills, and illustrate the main differences between management and leadership, and finally proposed a model for project management success.

## Leadership: the art of project management

Perhaps because project management is closely associated with information technology (IT), many researchers had associated the term project management with technical skills. Corporations typically ensure that their employees have the technical skills, they need to work on, whatever tasks they are assigned, but, as Jin Johnson, chairman of the Standish group international, Inc. has said: when projects fail, its rarely technical projects, like businesses, often fail because they are not properly managed (1).

So , the significant driver of project management success is the effective and intelligent leadership, communicated through an inspiring vision of what the project is meant to achieve and how it can make a significant positive impact. According to this fact, the main role of the leadership is establishing the attitude by developing a vision of the future, aligning people and providing logic for how the vision can be achieved , and directing the role of the executive managers of the project management to activate successfully (beginning with initiating process, planning process , monitoring and controlling process, closing process )through enabling the integration of the nine knowledge areas, as shown in figure (1).

Many organizations show little interest toward project management. According to the project management net work, only 17,6% of organizations used project management processes through their organizations in 2002, compared with 22.5% in 2001 and 9,3%.in 2000. The employees appointed to manage project may be the persons who suggested it, volunteers, an individual perceived as having the time to manage the project or the person with the most in-depth technical knowledge needed for project (2).

Managing a project may require technical knowledge, but, as well as managing a business, it also requires business knowledge. The project manager must be well organized and self-confident, and must have the right attitude. Technical knowledge is important, but so are business acumen, an understanding of the corporate culture and an ability to lead people to do what is expected of them. In other words the leadership skills is the art of understanding and executing the science of project management, so knowledge is important, but so is the ability to execute it.

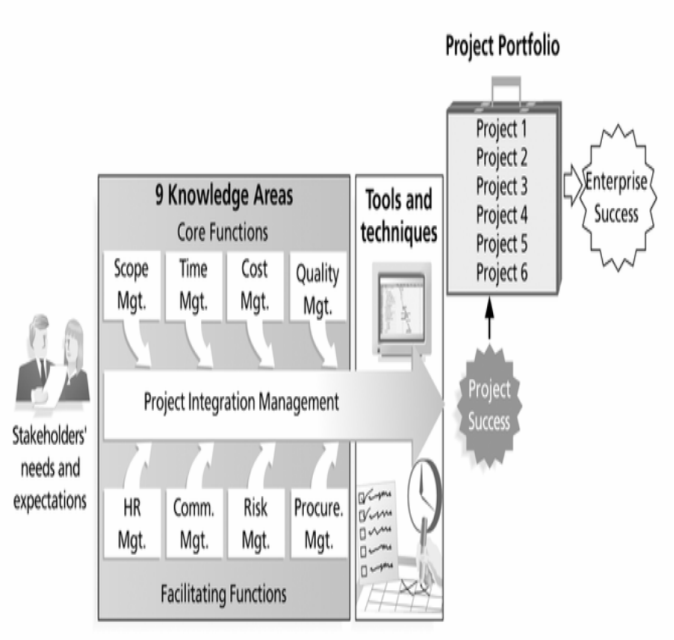



**Figure (1) the project management and the nine knowledge areas**

No one would claim that every project that fails is the result of poor management, a poorly funded or ill-conceived project, will fail regardless of the skills of the project manager or project team. Projects that lack by-in from top management are doomed, as are projects that lack ties with company objectives or that have no clear return on investment.

Sometimes a shift in business priorities requires that certain projects be abandoned. But project mismanagement plays significant role in many projects failures. The high failure rate for projects has been well documented, although signs of improvement are encouraging. In1995, the Standish group reported that 31%of all information technology (IT) projects were canceled before completion, that only16%of projects were completed successfully, and that 88%of all projects were over budget, over schedule or both. Standish also reported an average cost overrun of 189% and an average time overrun of 222% of original estimates.

In its most recent report, in 2001, Standish found that time overruns have dropped to an average 36%, cost overruns have dropped to an average of 45%, and the percentage of IT projects that are completed successfully has changed to 28%.(3 ).

**Reasons for project failure**

A review of 1,000 projects by the UK office of government commerce (OGC) found that technology was one of the likely reasons for a project to fail. Program fails for management reasons, not technical reasons. The OGC found the main reasons for failure to be (3):

-lack of leadership

- lack of knowledge at the top of the organization about what the technologists are trying to explain and lack of knowledge among technology its about what business users want.

- Poor risk management- not in terms of whether program code is accurate but rather in terms of understanding of complexity of business process and human change.

- Inability to break down programs into bit- size chunks. Programs or projects talking 12-18 months are too long, because things change. There is also problem that people might not tell the project that things have changed.

**Success factors**

so what is needed to make change programs successful ?

-In complex programs the management must come from the very top, because such programs threaten the entire organization. So the folowing involved points must have the attention of the chief executive:

1. Clear leadership from the top is especially important if some of the stakeholders must be kept engaged throughout, especially the customers or business users.

2. The need for hybrid managers who can build communication bridges between technologists and top management was articulated many years ago, and is still best advice. Hybrid managers are people who can cope with the business, aren't afraid of the technology to the extent that they have been there, don that and have the personality to talk to a wide range of people be credibility to talk to a wide range of people be credible to users and technologists, and get their cooperation's. finally clarity vision of what it to be achieved is the out . such vision clarifies what programs is not going to do, which is also an important issue. In addition, vision leads the team to start splitting the work into chunks, because stages towards the vision are identified.

**The evolve of the requirements for leadership of IT projects**

As the role of the chief information officer (CIO) has evolved, to involve the strategic nature of the (CIO) position, and align business and IT strategy as well as connect the IT strategy and business strategy. The requirements for the skills, education, and experience of the CIO also have evolved. A brief comparison of the CIO of 1985 as compared to today is shown below (4) table (1)

**Table (1): comparison of the CIO in 1985 and today**

| 1985 | today |
|---|---|
| *hierarchical kingpin | *visionary leader |
| *dictator | *relationship manager |
| *technology guru | *marketer |
| *mainframe bigot | *open systems-oriented |

Luftman and Brier (4), in their work at the IBM advanced business institute, continue to survey business and IT executives on their views of a CIO qualifications, (4) .
Their findings are presented in figure (2). it is important to note that tow qualifications are viewed by 80 percent or more of the executives questioned as key to the CIO job from both the IT and business function (e.g., finance , marketing, R&D) perspective. The first is knowledge of applying IT to business, and the second is leadership. CEO views leadership as the primary qualification for ACIO.

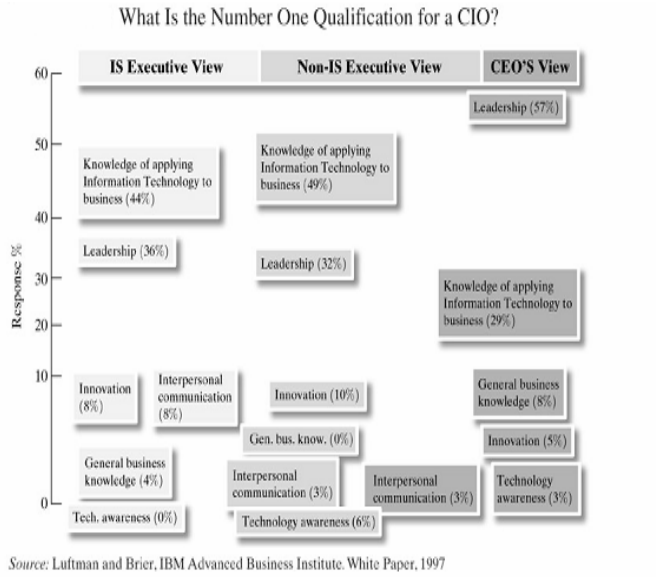
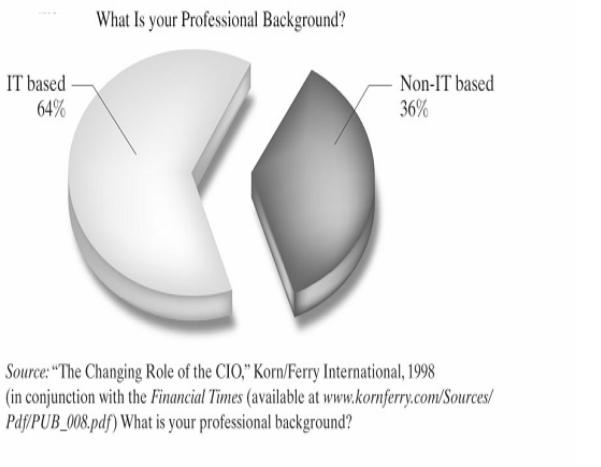


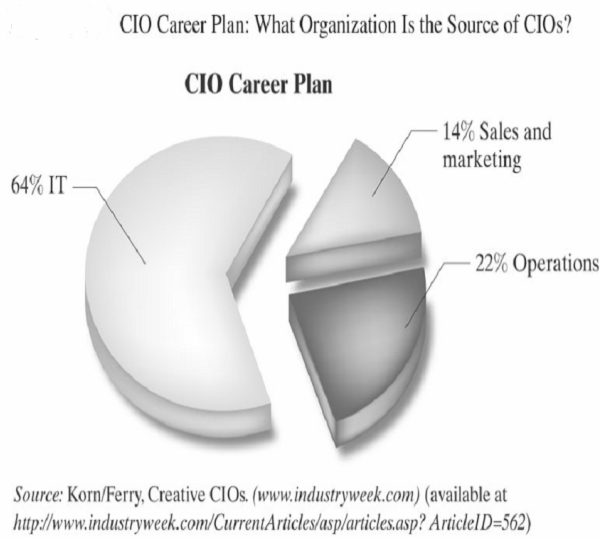

**Figure (2): survey business and IT executives on their views of a CIO qualifications**

The CIO as the leader of IT projects in the firm must be well-rounded individual, technically astute, people-oriented, with excellent business skill. Current statistics indicate that the majority of CIOs and IT executives continue to come primarily from IT back ground. Approximately two-thirds of CIOs have IT backgrounds from a professional education and experience perspective as shown in figure (3) and figure (4). The korn/ferry results have been expanded to indicate other business function back grounds CIO may have if thy are not career IT professionals. (4).




*Source:* "The Changing Role of the CIO," Korn/Ferry International, 1998 (in conjunction with the *Financial Times* (available at *www.kornferry.com/Sources/Pdf/PUB_008.pdf*) What is your professional background?

**Figure (3): CIOs' IT backgrounds from a professional education perspective**




*Source:* Korn/Ferry, Creative CIOs. *(www.industryweek.com)* (available at *http://www.industryweek.com/CurrentArticles/asp/articles.asp? ArticleID=562*)

**Figure (4): CIOs' IT backgrounds from an experience perspective**

## Leadership versus management

Management and leadership are not the same, because leadership is : (4)

1. concerns primarily with establishing direction , developing a vision of the future-often the distant of future-and strategies for producing the changes need to achieve the vision

2. aligning people : communicating direction in words and deeds to all those whose cooperation may be needed so, as to influence the creation of teams and coalitions that understand the vision and strategies and the accept their validity.
3. Motivating and inspiring energizing people to overcome major political, bureaucratic, and resource barriers to change by satisfying basic, but often unfulfilled, human needs.

Where as management is about: (4)

1. Planning and budgeting: establishing detailed steps and time tables for achieving needed results, then allocating resources necessary to make it happen.
2.Organizing and staffing: establishing some structure for accomplishing plan requirements, staffing that structure with individuals, delegating responsibility and authority for carrying out the plan, providing policies and procedures to help guide people, and creating methods or systems to monitor or implementation.
3. Controlling and problem solving ; monitoring results, identifying deviations from plan, then planning and organizing and organizing to solve their problems.

In other words, leadership produces change, often to a dramatic degree, and has the potential to produce extremely useful change (new products that customers want, new approaches to labor relations that help make affirm more competitive). While management produces a degree of predictability and order and has the potential to consistently produce the short- term results expected by various stake-holders (for customer, always being on time: for stakeholders, being on budget).

**The Project Management Model**

Regardless of technological development, it is still true – and will always be true – that "humans lie at the heart of any organization and its systems," as Kendall and Rollins note (1). It takes a combination of business systems, providing strategy, structure and control, and human systems, providing clarity, competence and commitment, to create business success. Figure (5)

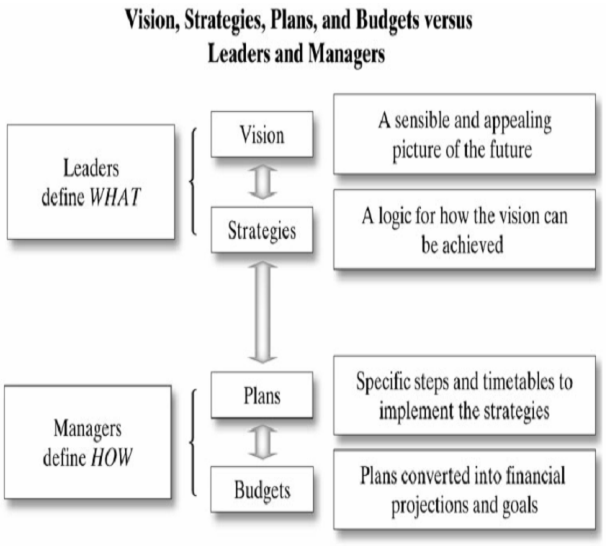



**Figure (5): vision, strategies, plans and budgets versus leaders and managers**

As such, it is important to choose the right people to manage projects. As much care should be given to the appointment of a project manager for a mission critical project as is given during the hiring process for a key position within the company. And yet, most organizations have no process for choosing project managers. They also have little idea what skills and personality traits are needed by project managers to help them succeed.

The characteristics of a successful project manager are consistent, regardless of industry sector, corporate culture or other factors. The model (see figure 6) divides project management skills into three major Categories – "technical," "personal," and "leadership."

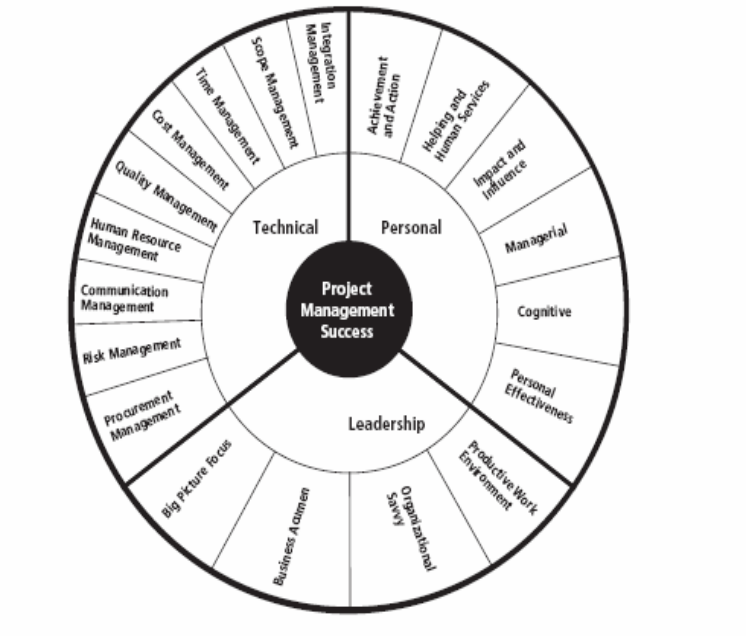



**Figure (6): the model for project success**

These three Categories combine the art and science of project management. The technical skills focus on the science of project management. The other two thirds of the model – "personal," and "leadership" – focus on the art, adding" management" to project management. Even those organizations that follow best practices for project management and have highly developed PMOs often fail because they ignore the art of project management. Think of project management as an iceberg. Above the water are the technical skills that are needed. They are easy to measure and demonstrate. The art of project management is more difficult to recognize and measure. You have to find out how people work with other people to complete projects and build a model around their skills.

To accomplish this, we've broken down the three skills Categories into Clusters that further describe the specific behaviors required for successful project management (see figure 7).

The Clusters are divided into Units, which are then broken into Elements.


**Figure (7): the specific behaviors required for successful project management**

As you assess potential project management leaders, you will not find anyone who perfectly meets all of the criteria outlined in the model. Such an individual may not exist. However, the model can help you identify likely candidates that embody many of the skills needed for project management competency, after which you can provide the training necessary to make them effective leaders. The model can also help you identify development gaps in your current project managers.

*Technical Skills*

We've divided technical competency into the nine widely accepted skills identified by the Project Management Institute that make upthe Project Management Body of Knowledge :

> Integration Management
> Scope Management
> Time Management
> Cost Management
> Quality Management
> Human Resource Management
> Communications Management
> Risk Management
> Procurement Management

The project manager must understand: how to manage procurement and human resources, so that the resources needed to implement a project are available; risks, ranging from technical to political challenges that can ground a project; cost, time and quality, so that the project can be completed on time and on budget, while maintaining or exceeding the necessary quality; communications, so that progress is reported accurately and knowledge is shared with all stakeholders; scope management and integration management, so that the project is understood in its proper context and is aligned with business goals.

The nine knowledge areas are used to carry out 39 processes that make up the PMBOK®. Each process uses information from the previous process, and, with the help of various tools and techniques, enhances it before beginning the next process. These processes are divided into five phases: initiating, planning, executing, controlling and closing. Review these phases, and you will recognize that they require not only technical skills, but business skills, embodying both art and science. Planning, for example, requires technical expertise to understand and implement the processes involved, but it also requires an understanding
of business strategies. Tying the project to the overall business strategy and understanding its impact on the company's bottom line, for example, should be part of the planning phase.

Kendall and Rollins, authors of *Advanced Project Portfolio Management and the PMO*, recommend adding Senior Management Oversight, PMO Management and Portfolio.(1) Management to the nine project management qualities. These skills, and others, are assumed in the proposed model.

*Leadership Skills*

Just as the technical skills outlined in project management model overlap with business skills, the business skills outlined by the model require a degree of technical competency. To be an effective communicator, for example, the project leader must understand technical language and jargon, but must also have the business skills to translate such language to business strategies and objectives for non-technical management.

Business and leadership skills are needed by project managers to link their projects to the relationships, resources and infrastructure of the organization. These skills, as identified in the competency model, include:

> A big picture focus
> Business acumen
> Organizational savvy
> Productive work environment

These Clusters are defined by their corresponding Elements as follows: A "big picture" focus requires leading through vision, strategic positioning and a systematic perspective. "Leading through vision" and "strategic positioning" are the ultimate business attributes. They measure the success of a project manager, just as they measure the success of a chief executive officer. Project managers can't live in a silo. It is not enough for the project manager to focus on a specific project. An effective leader must also be able to align the project with the needs of the enterprise. A "systematic perspective," the "science" part of this Cluster, integrates strategic planning with business processes.

"Business acumen" divides into the Elements of industry awareness and business operations knowledge. Industry awareness is self-descriptive and relates to an individual's knowledge of the company's position relative to its competitors. By comparing technology, marketing efforts, financial strength and management strength, the project leader should have a grasp of his organization's competitive advantages – and disadvantages.

Business operations knowledge complements industry awareness, and is as internally focused as industry awareness is externally focused. It requires intimate knowledge of the company's culture, its organization, and its business processes and practices. In addition to understanding the business, the project manager needs to know how to change it.

Organizational savvy requires an understanding of the company's politics and how to use them to advantage to advance the project. It also requires an ability to build coalitions and networks, which can create interdepartmental project support. While company resources can fund only a limited number of projects, it is important for project managers to remember that their co-workers are not their competitors. Project managers must be able to sell ideas, not only to their project team, but also throughout the organization. To accomplish this, they must understand how to motivate stakeholders.

To create a productive work environment, the project manager must be able to rapidly develop an effective project team and establish a collaborative culture within the team. Speed is critical. Completing projects on time is the number one factor in determining project success, because it improves time-to-market, which can create a competitive advantage and increase market share.

*Personal Characteristics*

Personal characteristics include:
> Achievement and action
> Helping and human services
> Impact and influence
> Managerial
> Cognitive
> Personal effectiveness

An achievement-oriented person is typically someone who is always ready to take action, rather than procrastinating until just before a project's deadline. Such people seek the information they need to take action, rather than waiting for the information to come to them. They show initiative, but maintain a concern for order, quality and accuracy.

Helping and human services characteristics include a customer-service orientation and strong interpersonal understanding. The individual shows compassion, and would feel comfortable mentoring or coaching others. His or her people skills extend beyond

the project team to the customer. Project deadlines are met not only to satisfy managers, but also to satisfy customers.

The ideal project manager is a role model for others, demonstrating a positive influence on other employees and making an impact on their productivity and performance. Organizational awareness and the ability to build relationships are also part of this Cluster.The project leader must know who to go to for project resources and how to obtain those resources, which might otherwise be used for other projects.

Managerial skills range from an ability to be assertive and use positional power effectively, to cooperation and teamwork. Team leadership, directness and an ability to develop others are other characteristics identified for this Cluster. Cognitive skills combine analytical and conceptual thinking, requiring a balance of right-brain and left-brain skills. It is, again, art and science in balance.

Finally, personal effectiveness includes self-control, self-confidence, flexibility and organizational commitment. The effective project manager is loyal to the organization, the project team and the project goals. Effective project managers lead by doing.

## *CONCLUSION*

The results of the proposed model will only improve when organizations begin taking care in defining the competencies of their project leaders. As leadership skills are the art of understanding and executing the science of project management.

So the model can help organizations to identify likely candidates that embody many of the skills needed for project management competency, after which we can provide the necessary training to make them effective leaders. the model can also help us to identify development gaps in our current project managers. Finally, all mentioned above is to improve project management success rate.

## *REFERENCES*

1. Christenson D., Derek H., and Walker T. (2004) Understanding the role of vision project management. Proj. Manag. J. : 39-51.

2. sumner M., Bouk D., and Giamortino A. (2006) exploring the linkage between the characteristics of IT project leaders and project success. www.ISMjournal.com. :43-49.

3. Gould M., and Freeman R.(2004) The art of project management. Boston University. www.propoint.com

4. Jerry N., and Luftmanand O.(2006) Managing the information technology resources. Leader. in the inform. Age. J.: 95-113.

# Incidence of Whooping Cough in Diyala Province- Iraq

**Dawood S. Al-Azzawi, Kareem A. Obaid, and Najdat S.Mahmoud**

*Pediatrics Dept. / Medical College/ Diyala University- Iraq*

*Email: dawoodalazawi@yahoo.com*

***ABSTRACT***

Whooping cough is still a cause for persistent cough in school age children, however, many doctors expose their patients to a various laboratory investigations spending more time & cost, further distressing them, on the other hand, implicating whooping cough as a cause for any persistent cough may hide more serious condition. So we are really facing a problem that we don't know the true incidence of pertussis in our community. The objective of the study is to estimate the incidence of whooping cough in school age children who were presenting with persistent cough.

Over 2 years from the 1st of October 2008- 1st of October 2010, patients 5- 16 years old who presented with persistent cough for more than 2 weeks to a private clinic in Baquba city Iraq had been studied, whooping cough was diagnosed clinically according to the case definition of European Union/ World Health Organization (EU/ WHO) for pertussis.

A total of 1659 patients studied, 106 ( 6.4 %) of them were met the criteria of WHO & European union, distributed equally between male & female, but early school age group children were more affected than older age group. Some of those patients were fully vaccinated, while the remainders were only partially vaccinated.

Although changes in the vaccination program have reduced pertussis morbidity in childhood, there is still a significant infection rate in children's and adolescents.

***Key Words:*** pertussis, whooping cough, vaccine

**الملخص باللغة العربية**

السعال الديكي لايزال سببا لاستمرار حالات السعال لدى الاطفال في سن المدرسة بحيث يتعرض العديد من الحالات للفحوص المختبرية المختلفة وانفاق المزيد من الوقت والمال ومزيد من الضغط النفسي لهم ومن ناحية اخرى يعزى للسعال الديكي لكثير من حالات السعال المستمر لاكثر من اسبيوعين وقد تخفي حالة اخرى اكثر خطورة ,لذلك نحن نواجه مشكلة تتمثل بعدم معرفة حقيقة انتشار حالات السعال الديكي في مجتمعنا ولذلك جاء الهدف من هذة الدراسة لغرض تقدير حالات السعال الديكي لدى الاطفال في سن الدراسة الذين يعانون من حالات سعال مستمرة لاكثر من اسبوعين وعلى مدى اكثر من سنتين من الاول من اكتوبر 2008 ولغايةالاول من اكتوبر 2010 وللاطفال مابين 5-16 سنة ويعانون من سعال لاكثر من اسبوعين من المراجعين لعيادة خاصة ب طب الاطفال في مدينة بعقوبة وهي مركز محافظة ديالى وحسب تعريف حالة السعال الديكي من منظمة الصحة العالمية والاتحاد الاوروبي لمرض السعال الديكي ,وقد جمعت 1659 حالة مرضية لدراستها ومنها 106 حالات (6.4%) انطبقت عليهم هذة المعايير وكانت موزعة بالتساوي بين الذكور والاناث والبعض كان قد تم تطعيمهم كاملا والبعض الاخر تلقوا تطعيم جزئي (حسب نظام التطعيم الدولي المعتمد في العراق)وقد تبين بانه بالرغمك من التغييرات والتطوير في برامج تطعيم المرض قد نجحت بتخفيض معدلات المرض في مراحل مبكرة الا انه لايزال معدل اصابة ملحوظ في بعض الاطفال والمراهقين.

## *INTRODUCTION*

Pertussis—whooping cough—is a highly infectious respiratory tract infection caused by Bordetella pertussis bacteria , also known as whooping cough, is a highly contagious bacterial disease caused by Bordetella pertussis. Symptoms are initially mild, and then develop into severe coughing fits, which produce the namesake high-pitched "whoop" sound in infected babies and children when they inhale air after coughing.(1) The coughing stage lasts for approximately six weeks before subsiding. In some countries, this disease is called the 100 days' cough or cough of 100 days (2). The first symptoms of Pertussis, which is spread by sneezing or coughing, are similar to those of a common cold. Prevention via vaccination is of primary importance as treatment is of little clinical benefit to the person infected (3). Antibiotics, however, do decrease the duration of infectiousness and are thus recommended (3). The disease currently affects 48.5 million people yearly, resulting in nearly 295,000 deaths (4).

The classic symptoms of pertusis are a paroxysmal cough, inspiratory whoop, and vomiting after coughing (5). The cough from pertussis has been documented to cause subconjunctival hemorrhages, rib fractures, urinary incontinence, hernias, post-cough fainting, and vertebral artery dissection (5). Then, after 1–2 weeks, severe outbursts of coughing develop that end with a characteristic “whoop.” Whooping cough is most common in infants and young children although adolescents and adults can develop a mild, often undiagnosed version of the disease. By contrast, more than half of children who develop pertussis before they are a year old need hospitalization. Complications of pertussis include pneumonia, a brain condition called encephalopathy and, in rare cases, death. Treatment with antibiotics can shorten the illness and can stop it spreading to other people. Whooping cough can be prevented by vaccination, Pertussis vaccines are effective (6), routinely recommended by the World Health Organization (7) and the Center for Disease Control and Prevention (8), and saved over half a million lives in 2002 (8). The multi-component a cellular Pertussis vaccines for example is between 71-85% effective with greater effectiveness for more severe disease(6).

The duration of protection is between five to ten years. This covers childhood, which is the time of greatest exposure and greatest risk of death from Pertussis (5), (9).

An injection of inactive B. pertussis that “primes” the immune system so that it can deal quickly and effectively with any subsequent infections. Globally, more than 80% of children are now vaccinated against pertussis in the first few months of life.

## *AIM OF THE STUDY*

The study is a trial to estimate the incidence of whooping cough in school age children who were presenting with persistent cough, with showing of the characteristics of those patients.

Widespread pertussis vaccination since the 1950s has greatly reduced the incidence (the number of new cases in a population) of whooping cough. However, the disease is still present in all countries and its incidence is increasing again in many Western countries. One possible reason for vaccination failing to eliminate whooping cough might be B. pertussis transmission through mild infections in adolescents and adults. Because the protection provided by vaccination wanes over time, people who were vaccinated as infants can develop mild pertussis as adolescents or adults and unknowingly infect unvaccinated babies. Currently, pertussis vaccination involves several injections in the first few months of life and a preschool booster but some countries have recently introduced booster doses of pertussis vaccine for adolescents and adults to try to improve pertussis control, even though the transmission dynamics of pertussis in adults and adolescents are poorly understood. In this study, the researchers estimate the incidence and basic reproduction number (the

expected number of new cases of a disease caused by an infected individual) to discover more about the transmission dynamics of pertussis.

## *PATIENTS AND METHODS*

Over 2 years from the 1st of October 2008- 1st of October 2010, patients 5-16 years old who presented with persistent cough for more than 2 weeks to a private clinic in Baquba city Iraq had been studied, whooping cough was diagnosed clinically according to the case definition of European Union/ World Health Organization (EU/ WHO) for pertussis.

A confirmed case was defined as a patient with at least one specific sign of pertussis (whooping, paroxysmal cough, vomiting, increased coughing at night), whose B. pertussis infection was confirmed by either PCR or ELISA.

A clinical case was defined as a patient with at least one specific sign of pertussis (whooping, paroxysmal cough, vomiting and increased coughing at night) whose disease was diagnosed clinically by a GP, without laboratory confirmation of B. pertussis infection.

Because of absence of the laboratory facilities which were depended on at other studies to diagnose pertusis ( culture of throat or nasopharyngeal swab &/ or serological tests), we depend on clinical diagnosis according to the case definition of European Union/ World Health Organization ( EU/ WHO) for pertusis (cough lasting at least two weeks with one or more of the followings: paroxysms of cough , inspiratory whoop, or post-tussive vomiting without other apparent cause), exclusion of other differential diagnosis's was done by history, clinical examination, & by sending all patients for WBC count & differential, ESR, & chest x- ray.

So, every child presented with persistent cough with paroxysmal attacks, whoop, or post-tussive vomiting & had normal ESR & chest X-ray with no previous similar attack was included as a case of pertusis, most of those children had WBC count more than 15,000/cmm with lymphocyte predominance.

Regarding immunization, the medical records had been depended on, but because of the small sample size, we concentrate on describing the immunization status of the patients of whooping cough & avoiding their comparison with others in order not to under- or over-estimate of the role of vaccination in preventing whooping cough.

Other data had been collected were characteristics of the patients (age, sex, & residence).

## *RESULTS*

During the two year of the study, 1659 patient with persistent cough visited the private clinic searching for management, out of them, 106 (6.4 %) child met the criteria of the case definition of European Union/ WHO for pertusis, while the others were distributed among asthma, pneumonia, & foreign body inhalation.

The cases of whooping cough were equally affect both sexes, while it has been found that 5- 10 year age group children were more affected than 11- 16 year age group children. The following tables (1,2,3,4) will show those characters of the patients.

The frequency of clinical symptoms (such as vomiting, increasing coughing at night, paroxysmal cough, fever) observed in patients with a diagnosis of pertussis was similar to that observed in those who were not diagnosed as having pertussis.

The investigations (WBC and differential count, Hb% and ESR) were done for all patients included in the study.

Regarding the residence, only 15 (14.2 %) patient were from Baquba (centre of the governorate), while the others were from the peripheries (22 from Bany-saad, 17 from Khalis, 24 from Baladrouz and the remaining 28 were from Mukdadya)

In spite of the availability of vaccination for pertusis at all health centers of the province all over the year, only 40 (37.7 %) of the patients of pertussis had been fully vaccinated.

**Table (1) characteristics of patients enrolled in the study**

| Age Group | Fully Vaccinated n (%) | Partially vaccinated n (%) | Unvaccinated n (%) | Cough more than 2 weeks n (%) |
|---|---|---|---|---|
| 5-10y | 21 (19.8%) | 20 (18.9%) | 18 (17%) | 936(56%) |
| 11-16y | 19 ((17.9) | 18 (17%) | 10 (9.4%) | 723(44%) |
| Total | 40 (37.7%) | 38 (35.9) | 28 (26.4%) | 1659 |

**Table (2) Characteristics of enrolled pts. From whom suitable samples were obtained for group A patients (11-16y old) total no. (723 patients)**

| Characteristics | Pat's without dx. Of pertussis | Pts. With pertussis dx | Total No. obtained 723 n (%) |
|---|---|---|---|
| **Paroxysmal cough** | 122 | 88 | 210 (29%) |
| **whoop** | 85 | 51 | 136 (18.8%) |
| **Vomiting** | 82 | 68 | 150 (20.7%) |
| **Cyanosis** | 36 | 8 | 44 ((6%) |
| **Increasing coughing at night** | 224 | 68 | 292 (40.3%) |
| **Asthma** | 84 | 6 | 90 (12.4%) |
| **fever >38$^0$c** | 40 | 12 | 52 (7%) |
| **Contact with a pertussis case** | 34 | 12 | 46 (6.3%) |
| **Contact with symptomatic patient** | 34 | 12 | 46(6.3%) |
| **History of whooping cough in infancy** | 13 | 3 | 16 (2.2%) |
| **Median number of days of cough** | 26 | 18 | 44 (6%) |
| **Total** | 617 | 106 | 723 |

**Table (3) Characteristics of enrolled patients. From whom suitable samples were obtained for group B patients (5-10y old) total no. (936 )**

| Characteristics | Pat's without dx. Of pertussis n(%) | Pts. With pertussis dx n (%) | Total No. obtained n (%) |
|---|---|---|---|
| **Paroxysmal cough** | 142 | 98 | 240 (25.6%) |
| **whoop** | 93 | 56 | 149 (15.9%) |
| **Vomiting** | 84 | 72 | 156 (16.6%) |
| **Cyanosis** | 33 | 6 | 39 (4%) |
| **Increasing coughing at night** | 334 | 102 | 436 (46.5%) |
| **Asthma** | 96 | 8 | 114 (12.2%) |
| **fever >38$^0$c** | 40 | 12 | 52 (5.5%) |
| **Contact with a pertussis case** | 13 | 28 | 41(4.4%) |
| **Contact with symptomatic patient** | 40 | 18 | 58(6.2%) |
| **History of whooping cough in infancy** | 11 | 2 | 13 (1.4) |
| **Median number of days of cough** | 20 | 18 | 38 (4%) |
| **Total** | 830 | 106 | 936 |

**Table (4): Distribution of patients with whooping cough by age & sex**

| Age/Sex | Male n (%) | Female n (%) | Total n (%) |
|---|---|---|---|
| **5- 10 year** | 32 (50 %) | 32 (50 %) | 64 (60.4 %) |
| **11- 16 year** | 21 (50 %) | 21 (50 %) | 42 (39.6 %) |
| **Total No.(%)** | 53 (50 %) | 53 (50 %) | 106 (100 %) |

## *DISCUSSION*

Countries' case definitions, vaccination strategy and coverage, and surveillance systems differ, making incidence comparisons difficult (12-14). In our study, (23%) of enrolled patients from whom suitable samples were obtained were diagnosed by their GP as having pertussis. The incidence of pertussis in our study population could have been underestimated due to a number of unexpected problems that occurred during sample collection.

A study on whooping cough in school age children with persistent cough in school age children with persistent cough, published in BMJ 2006 JULY22;333 (7560) 174-177 reveals that : study of 172 cases 37% 64 patients had evidence of recent Bordetella Pertussis infection, 55 patients (85.9%)of these children had been fully vaccinated (15)

As substantial proportion of immunized school age children presenting to primary care with persistent cough had evidence of infection with Bordetella Pertussis, thus doctors should be alert to a potential diagnosis of whooping cough in any child who presents with a persistent cough, however parents are more likely to worry about their child cough and want further investigation if they don't have a clear diagnosis

The epidemiology of pertussis in adolescents and adults is not well defined because of the broad spectrum of clinical manifestations.

In our study, no clinical differences were observed between patients with and without a pertussis diagnosis. It has previously been reported that most (80%) adolescents and adults with pertussis had a cough that lasted more than 21 days and that many were still coughing at 90 days (16). this shown that pertussis is an endemic disease among adolescents and adults.

This finding is important because secondary attack rates of pertussis in non-immunised household contacts have been estimated to be high percentage (90%).

In our study, patients were followed to record the number of days that they continued coughing after their visit.

The gold standard treatment in French pertussis guidelines is macrolides,

## *CONCLUSION*

1. For school children presenting with cough lasting 2weeks and more whooping cough should be considered even if the child is immunized fully or not, making the diagnosis of whooping cough to prevent the unnecessary investigations and treatment.
2. Although changes in the vaccination program have reduced pertussis morbidity in childhood, there is still a significant infection rate in children's and adolescents.

## *REFERENCES*

1. Carbonetti NH. (2007). "Immunomodulation in the pathogenesis of Bordetella pertussis infection and disease". Curr Opin Pharmacol 7 (3): 272–278.

2. Heininger U (2010) "Update on pertussis in children.". Expert review of anti-infective therapy 8 (2): 163-73.

3. Bettiol S, Thompson MJ, Roberts NW, Perera R, Heneghan CJ, and Harnden A (2010) Bettiol, Silvana. ed. "Symptomatic treatment of the cough in whooping cough". Cochrane Database Syst Rev (1): CD003257. doi:10.1002/14651858.CD003257.

4. Cornia PB, Hersh AL, Lipsky BA, Newman TB, and Gonzales R (2010) "Does this coughing adolescent or adult patient have pertussis?". JAMA 304 (8): 890–896.

5. Zhang L; Prietsch SO, Axelsson I, and Halperin SA (2011). "Acellular vaccines for preventing whooping cough in children.". Cochrane database of systematic reviews (Online) (1): CD001478. PMID 21249646.

6. World Health Organization (WHO) "Annex 6 whole cell pertussis. "http://www.who.int/biologicals/publications/trs/areas/vaccines/whole_cell_pertussis/Annex%206%20whole%20cell%20pertussis.pdf. Retrieved 5 June 2011
7. Centre for Disease Control and Prevention. "Pertussis: Summary of VaccineRecommendations" http://www.cdc.gov/vaccines/vpd-vac/pertussis/recs-summary.htm. Retrieved 5 June 2011.
8. Versteegh FGA, Schellekens JFP, Fleer A, and Roord JJ. (2005) "Pertussis: a concise historical review including diagnosis, incidence, clinical manifestations and the role of treatment and vaccination in management.". Rev Med Microbiol 16 (3): 79–89.
9.http://www.revmedmicrobiol.com/pt/re/revmedmicrob/abstract.00013542-200508000-00001.htm
10. Barret AS., Ryan A., Breslin A., Cullen L., Murray A., and Gogan J. (2010) Pertusis outbreak in northwest Ireland. Euro surveill. 15 (35):121-126
11. Couzigou C., & Flanault A. (2003) Is pertusis being considered as a cause of persistent cough among adult. Eur. J. epidemiol.18: 1013- 1015.
12. Lasserre A, Tison C, Turbelin C, Arena C, Guiso N, and Blanchon T. (2010) Pertussis prevention and diagnosis practices for adolescents and adults among a national sample of French general practitioners. Prev Med. 51(1): 90-91.
13. Tondella ML, Carlone GM, Messonnier N, Quinn CP, Meade BD, and Burns DL.(2009) International Bordetella pertussis assay standardization and harmonization meeting report. Centers for Disease Control and Prevention, Atlanta, Georgia, United States.Vaccine. 27(6):803-814.
14. Von König CH, Halperin S, Riffelmann M, and Guiso N.(2002) Pertussis of adults and infants. Lancet Infect Dis. 2(12):744-750.
15. Tozzi AE, Pandolfi E, Celentano LP, Massari M, Salmaso S, and Ciofi degli Atti ML (2007) Comparison of pertussis surveillance systems in Europe. Vacc. 25(2):291-297.
16. Cherry JD.(2005) The epidemiology of pertussis: a comparison of the epidemiology of the disease pertussis with the epidemiology of Bordetella pertussis infection. Pediat.115(5):1422-1427.

# Detection of metallo-β-lactamases and inhibitor resistant Temoniera enzyme in β-lactam resistant *Klebsiella* spp. and *Enterobacter cloacea* isolated from wound infections: A Genetic study

**Mushtak T.S. Al- Ouqaili**

*Dean of College of Pharmacy / Al- Anbar University- - Iraq*

*E-mail: dr.mushtak_72@yahoo.com*

***ABSTRACT***

The emergence of acquired metallo-beta-lactamases (MBLs) is of clinical concern since they confer resistance to all available beta-lactams except aztreonam. This study had been undertaken for detecting the ability of *Klebsiella* species and *Enterobacter cloacea* to produce inhibitor-resistant Temoniera beta-lactamases (IRT) and metallo-β-lactamase. Further, for determining the genetic elements encoding for such enzymes whether chromosomally or conjugated plasmid mediated. Furthermore, for detect the potency of some curing agents on conjugated plasmids DNA. The study was performed on 75 wound patients admitted to Department of Surgery, Ramadi Teaching Hospital during the period from February to July, 2010. Rapid iodometric β-lactamase production test in addition to phenotypic and confirmatory tests for production of IRT, and metallo-β-lactamase were performed. Plasmid DNA extraction, conjugation and curing experiments were performed. Our result showed that Fourty isolates were bacteriologically identified as *Klebsiellae* spp. including 26 (34.7%) *Klebsiellae pneumoniae* and 14 (18.7%) *Klebsiellae oxytoca*, 10 *(*13.3%) *Enterobacter cloacea*, and etc. In the screening test for IRT production, 6 (24%) isolates of *K. pneumoniae* were resistant to inhibition by clavulanic acid. In the screening test for metallo-beta-lactamase production, the study revealed that 1(10%), 1(4%) isolates of *Klebsiella oxytoca* and *K. pneumoniae* respectively were produced this enzyme. In the genetic part of study, Five (83.3%) of inhibitor-resistant TEM β-lactamase and 2 (100%) of metallo-β-lactamase producer isolates of *Klebsiella* spp., were plasmid mediated while the remaining were chromosomally mediated. The result of curing experiments showed successful curing for all the study isolates by both methods. The study suggested that the production of β-lactamases of type IRT, and metallo appeared to be the major mechanisms of resistance of *Klebsiellae* spp. and *Enterobacter cloacea* to β-lactams. Also, conjugative plasmids were found to be the main genetic elements encoding for such enzymes produced by these bacteria. Finally, the study suggested that sodium dodecyl sulphate was of higher potency than elevated growth temperature in the curing of plasmid DNA.

***Key words:*** metallo-β-lactamases, IRT, *Klebsiella*, Plasmids, Conjugation, Curing

**الملخص باللغة العربية**

ان الهدف من الدراسة هو لتحديد قابلية بكتريا الكلبسيلا والانيروباكتر كلوشيا على انتاج انزيمات البيتالاكتاميز من نوعي تيمونيرا والميتالوبيتالاكتاميز وتحديد العناصر الوراثية المشفرة لإنتاجها سواء كانت كروموسومات او بلازميدات مقترنة بالاضافة الى تأثير العوامل المحيدة في تحييد البلازميدات المقترنة. اجريت الدراسة على 75 من مرضى الجروح الداخلين الى قسم الجراحة في مستشفى الرمادي التعليمي. تم اجراء طريقة اليود السريعة بالاضافة الى الاختبارات المظهرية والمؤكدة لتحديد انتج الانزيمات اعلاه. انجزت تجارب استخلاص الحمض النووي البلازميدي والاقتران والتحييد. تم عزل اربعون عزلة من بكتريا الكلبسيلا مقسمة الى (34.7%) 26 بكتريا الكلبسيلا الرئوية و (18.7%) 14 للكلبسيلا اوكسيتوكا تليها بكتريا الانتيروباكتر كلوشيا 10 (13.3%). اظهر اختبار المسح لأنزيمات التيمونيرا بيتالاكتاميز بان (24%) 6 من بكتريا الكلبسيلا الرئوية بان كانت مقاومة للتثبيط باستخدام حمض الكلافيولانك فيما اظهر اختبار المسح انتاج انزيمات الميتالو من قبل عزلة واحدة من بكتريا الكلبسيلا الرئوية واخرى من الكلبسيلا اوكسيتوكا. اظهر اختبار التحليل البلازميدي بان خمس عزلات كلبسيلا (83.3%) من مجموع ستة منتجة لأنزيمات تيمونيرا وكافة عزلات الكلبسيلا المنتجة لأنزيمات الميتالو كانت مشفرة بواسطة بلازميدات. كما اظهرت تجارب التحييد نجاح طريقتي التحييد المستخدمة في الدراسة. تستنتج الدراسة بان انتاج الانزيمات من كلا النوعين كان الية رئيسية في مقاومة كلا من بكتريا الكلبسيلا والانتيروباكتر كلوشيا لمضادات البيتالاكتام. كذلك فان البلازميدات المنتقلة بالاقتران الجنسي كانت العنصر الوراثي الرئيسي المشفر لإنتاج انزيمات الدراسة. أخيرا تستنتج الدراسة بان مادة سلفات دوديسيل الصوديوم اظهرت كفاءة اعلى من طريقة درجة حرارة النمو المتزايدة في تحييد الحمض النووي البلازميدي.

## *INTRODUCTION*

The emergence of acquired metallo-beta-lactamases (MBLs) is of clinical concern since they confer resistance to all available beta-lactams except aztreonam, and they are not inhibited by class A β-lactamase inhibitors(1). MBLs have been categorized in different groups of which, Imipenemases (IMPs) and Verona integron-encoded metallo-β-lactamases (VIMs) are the most commonly identified types and they are predominant in Asia and Europe respectively. VIM enzymes have been grouped in three main clusters designed as VIM-1, VIM-2 and VIM-7 (2). Although they are more prevalent among non-fermenting Gram-negative bacteria, they are increasingly recognized among members of the Enterobacteriacea family (2).

Carbapenemases are a diverse group of enzymes. They are currently uncommon but are a source of considerable concern because they are active not only against oxyimino-cephalosporins and cephamycins but also against carbapenems (3). Plasmid-mediated IMP-type carbapenemases, 17 varieties of which currently known, became established in Japan in the 1990s in both enteric gram-negative organisms and in *Pseudomonas* and *Acinetobacter* species. IMP enzymes spread slowly to other countries in the Far East, were reported from Europe in 1997, and have been found in Canada and Brazil.

A second growing family of carbapenemases, the VIM family, was reported from Italy in 1999 and now includes 10 members, which have a wide geographic distribution in Europe, South America, and the Far East and have been found in the United States (4).The class A enzymes, notably the plasmid-mediated KPC enzymes, are effective carbapenemases as well. Finally, some OXA-type β-lactamases have carbapenemase activity, augmented in clinical isolates by additional resistance mechanisms, such as impermeability or efflux (3), (5)

Resistance to carbapenems is an emerging problem among gram-negative hospital pathogens. A transferable plasmid encoding the VIM-4 metallo-β-lactamase was detected in isolates of *Klebsiella pneumoniae* and *Enterobacter cloacae* obtained from a single patient under carbapenem therapy. Thus, enterobacteria appear to increasingly contribute to the spread of VIM-type enzymes (6).

It is well realized that plasmids are responsible for the spread of most of the new β-lactamases, but the genes encoding these enzymes may also be located on the bacterial chromosome. The genes encoding some β-lactamases are carried by transposons(7). Genes for many of the new β-lactamases are found in integrons, which often include genes conferring resistance to other antibiotics. For this reason, the new β-lactamases are usually produced by organisms that are resistant to multiple antimicrobial agents. Carbapenemases of the IMP and VIM families are also found within integrons, but the origin of their genes is not yet known. Therefore, the concern for the detection and occurrence of IRT and carbapenemases is due to main reasons. Firstly, the ubiquitous prevalence in nosocomial infections intensive care unit (ICU) and its association with therapeutic failure especially in the life-threatening infections. Secondarily, many strains producing these enzymes demonstrate an inoculum effect in that the MICs of antimicrobials rise as the inoculum increases. Therefore, this study has been under taken for screening the production of inhibitor-resistant Temoniera beta- lactamases (IRT) and carbapenems hydrolyzing enzymes (metallo-β- lactamase). Further, to detect the genetic elements coding for these enzymes whether chromosomally or conjugative plasmid mediated. Furthermore, to confirm the plasmid profile phenomenon by curing experiments.

## *PATIENTS AND METHODS*

Swabs or aspirates were taken from different anatomical sites of 75 patients wounds like bone, joints, connective tissues. The swabs were taken during the period from February to July, 2010 from patients admitted to Ramadi General Hospital. The swabs and aspirate were inoculated immediately on routinely used culture media nutrient agar, blood agar, MacConkey agar and chocolate agar plates and incubated overnight at 37ºC. The suspected *Klebsiellae* and *Enterobacter cloacae* colonies were identified bacteriologically according to criteria laid down by Baron and associated (8), (9). Bacterial standardization was adjusted by using McFarland 0.5 turbidity standard (barium sulfate) (10)

### Beta-Lactamase production test:

The capability of bacterial isolates of *Klebsiella* species and *Enterobacter cloacea* for production of beta lactamases was detected by cell suspension rapid iodometric method mentioned by Miles and Amyes (11). Negative control strain (*Escherichia coli* ATCC 25922) was used in this experiment.

### Determination of minimal inhibitory concentration (MIC):

The double fold dilutions of antimicrobial in five ml volumes of broth were prepared. A starting range of about eight fold higher than the normal MIC for the species has been tested and extended to at least one dilution below that of the control organism. A drug free control tube was included, thereafter one set of tubes was inoculated with a drop of well grown broth culture of the test organism diluted one in hundred (about $10^5$ organism) and the other with the control organism similarly diluted and then incubated overnight. After incubation, the last tube which shows no growth will represent the minimal inhibitory concentration (12).

### Screening for IRT and metallo-β-lactamase production:-

Standard disk diffusion technique:

The β-lactamase producer isolates were screened for their susceptibility to ceftazidime, ceftriaxone, cefoxitin and metallo-β-lactams represented by imipenem. 5ml of Brian heart infusion broth medium was inoculated with the bacterial isolates, incubated at 37 Cº for 3-4 hr (mid log phase). 10 µl of broth suspension to 10 ml normal saline, 0.1 ml of the suspension was transferred to Muller-Hinton agar plates and spreaded with sterile swab on the agar surface in three different planes by rotating the plate approximately 60º each time to obtain an even distribution of the inoculum (13). With sterile forceps, the selected antibiotic disks (CAZ, CTX, FOX and IMP) were placed on the inoculated plates and incubated at 37 Cº for 18 hours. After incubation, the results were interpreted according to the criteria laid down by Clinical Laboratory Standard Institute, (CLSI) (14). *Escherichia coli* 25922 and Escherichia *coli* MM294 were used as the reference strains.

### Phenotypic Confirmatory Testing Part I:

### For IRT and metallo beta-lactamase production:

A-Double disk synergy test:

Double disk synergy test was used to demonstrate the effect of clavulanic acid in the presence of cefotaxime and ceftazidime. In this test, 5ml of sterile nutrient broth was inoculated with the bacterial isolates, incubated at 37 ºC for 4 hours. 10µL of suspension broth to 10 ml normal saline, 0.1 ml of this dilution was spread by sterile spreader on the entire surface of Muller-Hinton agar in 3 different planes by rotating

the plate approximately 60º each time to obtain an even distribution of the inoculum[15]. The selected antibiotic disks (containing 30 µg of ceftazidime and 30 µg of cefotaxime with and without 10 µg of clavulanic acid) was placed on the sensitivity pate.. The disks are arranged so that the distance between them is approximately twice the radius of the inhibition zone produced by the later generation cephalosporins test on its own. After overnight incubation, the inhibition zones were measured and synergism occurred in some isolates between CAZ and CA and CTX (15). The results were interpreted according to the criteria established by CLSI (14). *E. coli* ATCC 25922 was used as the quality control isolate.

B- IPM-EDTA-disk synergy test:

The procedures were done according to (16) as follow:-186.1 g of disodium EDTA was dissolved in 1000 mL of distilled water to prepare 0.5 M EDTA solution and pH was adjusted to 8.0 by using NaOH. The mixture was then sterilized by autoclaving. EDTA-imipenem disks were prepared by adding EDTA solution to 10-µg-imipenem disks to obtain a concentration of 750 µg. The disks were dried immediately in an incubator and stored at 4°C in an air tight vial without desiccant. Bacterial isolates were adjusted according to McFarland 0.5 turbidity standard and were inoculated to Mueller Hinton agar. A 10-µg-imipenem disk and an imipenem plus 750 µg EDTA were placed on Mueller Hinton agar. Another disk containing only 750 µg EDTA was also placed as a control. After overnight incubation, the established zone diameter difference of ≥ 7 mm between imipenem disk and imipenem plus EDTA was interpreted as EDTA synergy positive. Non-MBL producer isolate of *Klebsiella* species was used as negative control (17).

**Phenotypic Confirmatory Testing Part II:**

Minimal inhibitory concentration tubes containing ceftazidime or cefotaxime with and without clavulanic acid were prepared in nutrient broth. 5 ml of sterile nutrient broth medium was inoculated with the bacterial isolates, incubated at 37ºC for 4 hours. 10 µl from suspension broth was placed in 10 ml normal saline, 10 µl from the suspension broth distribution to the tubes containing ceftazidime, cefotaxime (in concentration of 0.5 to 256 µg/ml) were tested combination with 4 µg of CA per ml. After incubation for overnight at 37 ºC, the lowest concentration was measured visually and compared with positive and negative control.

**Plasmid Profile:**

DNA isolation was performed using alkaline-lysis technique of Birnboim and Doley (18) mentioned with some modifications by (9) as follows: Bacterial isolate was grown in blood agar plate for 18 hr. at 37ºC. Then, the growth was collected with sterile swab, then, inoculated in 50 ml brain heart infusion broth supplemented with 100 µg/ml ampicillin and incubated over night at 37ºC. Afterthat, the growth was distributed in tubes (10 ml), and then centrifuged 10000 round per minute (r.p.m) for 15 minutes at room temperature. The pellet was resuspended in 1.5 ml of TEG-solution, mixed by gentle inversion and stood on crushed ice for 30 min. 2.5 ml of freshly prepared 0.2N NaOH containing 1% SDS was added and then mixed by gentle inversion and stood on crushed ice for 5 min. 1.5 ml of chilled 5M potassium acetate was added, mixed inversion, stood on crushed ice for 1 hr. at 14000 r.p.m for 15 min at room temperature; the supernatants were centrifuged and transferred to new micro-centrifuge tubes. Phenol-chloroform solution was added to DNA extract, centrifuged by micro-centrifuge for 10 min.

The water was layer drawn and transferred to new micro-centrifuge tube and precipitated with ice-cold absolute ethanol, incubated over night at 37 ºC. The solution was centrifuged for 10 min. by micro-centrifuge, then supernatants were discarded, and the extracted DNA was dried and dissolved in 10 µl TE buffer. The resulting extracts were electrophoresed in 1% agarose gels at 50 volt for 1.5 hr. The samples were stained with ethidium bromide and visualized by Ultraviolet transilluminator.

**Conjugation:**

Mating experiments were performed by using conjugation in broth medium method described by (19). The selective media used were brain heart infusion broth, brain heart infusion agar supplemented with 100 µg/ml Rifampicin and ampicillin respectively and brain heart infusion agar supplemented with 100 µg/ml Rifampicin . the procedure was as follow:- *Escherichia coli* MM294 rifr was used as the recipient strain and donors strain was separately inoculated into 5ml brain heart infusion broth, then incubated at 37 ºC for overnight. The mixture was centrifuged at 10000 r.p.m for 10 min, washed for one time by brain heart infusion broth removing the supernatant (O.D 0.5 at 540 nm). 1ml from donor strain and 1ml from recipient strain were placed in sterile tube, incubated 1-2 hr at 37 ºC. Then, the mixture was mixed on the vortex shaker for 1 min only. Afterthat, 0.1 ml of 10-1 to 10-6 dilutions were plated by spreading on appropriate selective media (containing rifampicin and ampicillin 100 µg/ml) and incubated for 24 hr at 37 ºC. 0.1 ml of 10-7, 10-8 dilutions were plated by spreading on appropriate selective media (containing rifampicin only) and incubated for 24 hr at 37ºC. Finally, conjugation Frequency was determined as follows:- Conjugation frequency =No. of transconjugation cell /No. of recipient cell. β-lactamase test was used to confirm the transferring of plasmid from donor to recipient cell.

**Plasmid Curing:**

Curing experiments were performed by using two types of curing agents (sodium dodecyl sulphate according to (20) and elevated growth temperature according to (21).

A-Sodium Dodecyl Sulphate

Cells were grown in 5 ml of brain heart infusion broth to mid log phase (3.5 hr), 0.1 ml of young culture of *Klebsiella* spp. were inoculated in a series of 5 ml fresh brain heart infusion broth universals containing various concentration of SDS from the stock solution 50 mg/ml (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2400, 2800, 3000) µg/ml. The growth density of different universals was measured visually and compared with the control to determine the effect of SDS on bacterial growth. The lowest concentration of SDS that inhibited bacterial growth was considered as the minimal inhibitory concentration (MIC). Samples were taken from universals containing the highest concentration of each curing agent that still allows bacterial growth which is known as (subminimal inhibitory concentration) and serially diluted. 0.1 ml of 10-4, 10-5, 10-6, 10-7, 10-8 dilution was spreaded on nutrient agar plates and incubated over night at 37ºC to score the survived colonies.

B-Elevated growth temperature:

Cells were grown in 5 ml of Brain heart infusion broth for 24 hr. at 37 ºC. 0.1 ml form inoculum in 5ml brain heart infusion broth incubated for 3 hr. at 44 ºC. This step was repeated for 5 to 7 times. 0.1 ml of $10^4$-$10^8$ dilution was

spreaded on nutrient agar plates and incubated overnight at 37 °C.

**Selection of cured cells**:

The cured cells were selected according to the following procedure: Isolated single colonies obtained were picked by sterile tooth picks by procedure (Picking and patching) on plates of nutrient agar, incubated overnight at 37 °C. These were regarded as master plates and from nutrient agar plates containing 100µg/ml ceftazidime and incubated overnight at 37°C. The number of colonies that lost resistance phenomenon to this antibiotic was determined. Curing frequency was determined from the following equation: Curing frequency = Induced – spontaneous / total count.

## *RESULTS AND DISCUSSION*

Out of 75 patients, 73 (97.3%%) of them revealed bacterial infection while the specimens of two (2.7%) were sterile specimens. Fourty isolates were bacteriologically identified as *Klebsiellae* spp. including 26 (34.7%) *Klebsiellae pneumoniae* and 14 (18.7%) *Klebsiellae oxytoca*, 10 (13.3%) *Enterobacter cloacea*, 8 (10.7%) *Pseudomonas aeruginosa*, 8 (10.7%) *Staphylococcus aureus*, 4 (5.3%) anaerobic *Bacteroides fragilis*, 3 (4.0%) *Peptosreptococcu*s spp. The clinical data regarding the distribution of *Klebsiellae* isolates, type of specimens and type of infection are presented in the following table. (1)

**Table (1): The distribution of clinical isolates of *Klebsiellae pneumoniae*, *Klebsiellae oxytoca* and *Enterobacter* spp. according to the type of specimens and type of infection**

| Study isolates no ( %) | Type of specimen | Type of infection |
|---|---|---|
| *Klebsiellae pneumoniae* 26 (65%) | | |
| 10 (38.5%) | Wound swab | Osteomyelitis |
| 2 (7.7%) | Wound swab | Diabetic foot infection |
| 6 (23.0%) | Wound swab | Burn |
| 4 (15.4%) | Knee aspirate | Septic arthritis |
| 2 (7.7%) | Hip aspirate | Septic arthritis |
| 2 (7.7%) | Mid-stream urine | Urinary tract infection |
| *Klebsiellae oxytoca* 14 (35%) | | |
| 3(21.4%) | Wound swab | Osteomyelitis |
| 4(28.6%) | Wound swab | Diabetic foot infection |
| 2(14.3%) | Wound swab | Burn |
| 2(14.3%) | Knee aspirate | Septic arthritis |
| 2(14.3%) | Hip aspirate | Septic arthritis |
| 1(7.1%) | Blood culture | Bacteremia |
| *Enterobacter cloacae* 10 (13.3%) | | |
| 2(20%) | Wound swab | Osteomyelitis |
| 6(60%) | Wound swab | Burn |
| 2(20%) | Knee aspirate | Septic arthritis |

Fourty isolates were bacteriologically identified as *Klebsiella* spp. including 26 (34.7%) *Klebsiella pneumoniae* and 14 (18.7%) *Klebsiella oxytoca*, 10 (13.3%) *Enterobacter cloacea*, 8(10.7%) *Pseudomonas aeruginosa*, 8 (10.7%) *Staphylococcus aureus*, 4(5.3%) anaerobic *Bacteroides fragilis*, 3 (4.0%) *Peptosreptococcus* spp. and two (2.7%) were sterile specimens.

In the screening test for inhibitor-resistant TEM β-lactamase (IRT) production, 6 (24%) isolates of *K. pneumonia* were resistant to inhibition by CA. Also, it was observed that these isolates were resistant to CTX, ceftriaxone (CRO), CAZ and imipenem (IMP), but remained susceptible to cefoxitin and cephalothin.table (2)

**Table (2): The result of screening tests (Broth microdilution and disk diffusion techniques) for potential inhibitor resistant Temoniera β-lactamase producing isolates of *Klebsiella* spp.**

| Study isolates no. | MIC screening test (µg/ml) | | | | Disk diffusion test (mm) | | | | Effect of CA | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CAZ | CTX | CRO | KF | CAZ | CTX | FOX | IMP | MIC | DD |
| SKP 1 | 16 | 256 | 16 | 4 | 6 | 6 | 16 | **8** | -ve | -ve |
| SKP 2 | 64 | 32 | 32 | 8 | 6 | 6 | 20 | **6** | -ve | -ve |
| SKP8 | 256 | 256 | 32 | 4 | 6 | 7 | 19 | 6 | +ve | -ve |
| SKP17 | 16 | 16 | 32 | 2 | 6 | 6 | 18 | 7 | -ve | -ve |
| SKP24 | 64 | 8 | 32 | 4 | 6 | 8 | 18 | 8 | +ve | -ve |
| SKP30 | 8 | 16 | 32 | 2 | 6 | 6 | 16 | 6 | -ve | -ve |

**CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone, FOX: cefoxitin, KF: cephalothin, IMP: imipenem, DD: Disk Diffusion`**

In the screening test for metallo-beta-lactamase production, our result showed that 1(10%) and 1(4%) isolates of *Klebsiella oxytoca*, *K. pneumoniae* respectively were resistant to CAZ, CTX, CRO and carbapenem (represented by imipenem) considering the break point for zone of inhibition and minimal inhibitory concentration for meropenem was < 13 mm and 8 µg/ml respectively (see table 3).

**Table (3): The result of screening tests (Broth dilution and disk diffusion techniques) for potential metallo-β-lactamase producing isolates of *Klebsiella* spp.**

| Study isolates no. | MIC screening test (µg/ml) | | | | Disk diffusion test (mm) | | | | Effect of CA | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CAZ | TX | RO | MEP | CAZ | CTX | FOX | IMP | IC | D |
| SK 10 | 34 | 18 | 128 | 16 | 8 | 8 | 10 | 7 | -ve | -ve |
| SK 12 | 18 | 256 | 64 | 32 | 7 | 8 | 8 | 6 | -ve | -ve |

**CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone, FOX: cefoxitin, MEP: imipenem, DD: Disk Diffusion**

In the genetic part of this study, the result of plasmid profile analysis revealed that out of Six inhibitor-resistant TEM β-lactamase producing isolates of *Klebsiella* spp., Five (83.3%) were plasmid mediated (the genes coding for these enzymes were located on single plasmid as reflected in bands no 3, 4, 6, and 7 and multi-plasmid in the 5$^{th}$ band) (see figure 1). The 5$^{th}$ band harbored two plasmids one large in molecular weight and the other was small in molecular weight. The genetic elements coding for IRT-β-lactamases at 8th band was chromosome. On the other hand, Further 2(100%), of metallo-β-lactamase were also plasmid mediated (the gene was harbored on one plasmid (band no 2) and the other by multi-plasmid (band no 1).

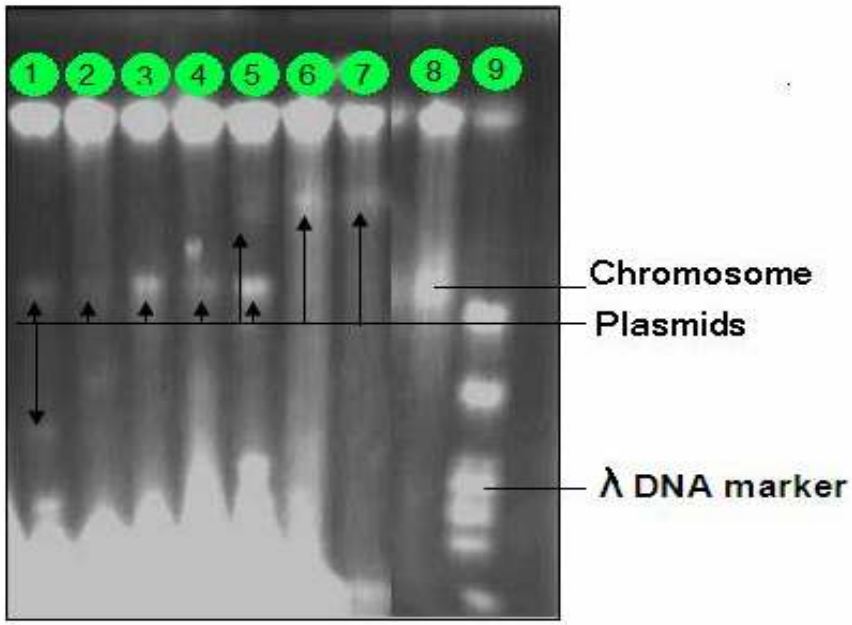



**Figure (1): The bands from one to seven represent plasmids produced by *Klebsiella* spp. (The first two) encoding for metallo-β-lactamases and IRT-β-lactamases (From three to seven) . Each of the 1st and 5th bands harbored two plasmids one large in molecular weight and the other was small in molecular weight while the others harbored single plasmid. The genetic elements coding for IRT-β-lactamases at 8th was chromosome. The first right band represents DNA marker (λ DNA phage). Agarose concentration is 1% (W/V), voltage 50 and time is 1.5 hr.**

Conjugation experiments were performed by using broth mixture technique for 5 (83.3%) and 2 (100%), of IRT enzymes and metallo-β-lactamase respectively (Table 4).

**Table (4) : Conjugation between multi-resistant bacterial donor strains and the recipient *Escherichia coli* MM 294.**

| Donor strains | Mechanism of Resistance | No. of transconjugants Cells/ml | No. of recipient cell | Conjugation frequency |
|---|---|---|---|---|
| SKP1 | IRT-β-lactamase production | $30 \times 10^{-5}$ | $3 \times 10^{-9}$ | $10 \times 10^{-4}$ |
| SKO2 | IRT-β-lactamase production | $16 \times 10^{-7}$ | $5 \times 10^{-9}$ | $3.2 \times 10^{-2}$ |
| SKP8 | IRT-β-lactamase production | $9 \times 10^{3}$ | $3 \times 10^{8}$ | $3 \times 10^{-5}$ |
| SKP10 | Metallo-β-lactamase production | $5 \times 10^{4}$ | $7 \times 10^{8}$ | $7.1 \times 10^{-5}$ |
| SKO17 | IRT-β-lactamase production | $3 \times 10^{3}$ | $9 \times 10^{7}$ | $1.4 \times 10^{-7}$ |
| SKP24 | IRT-β-lactamase production | 4 X 104 | $7 \times 10^{8}$ | $5.7 \times 10^{-5}$ |
| SKO12 | Metallo-β-lactamase production | $16 \times 10^{3}$ | $2 \times 10^{8}$ | $8 \times 10^{-5}$ |

Curing experiment showed successful curing for all the study isolates by using SDS and elevated growth temperature as curing methods (figures 2-5).

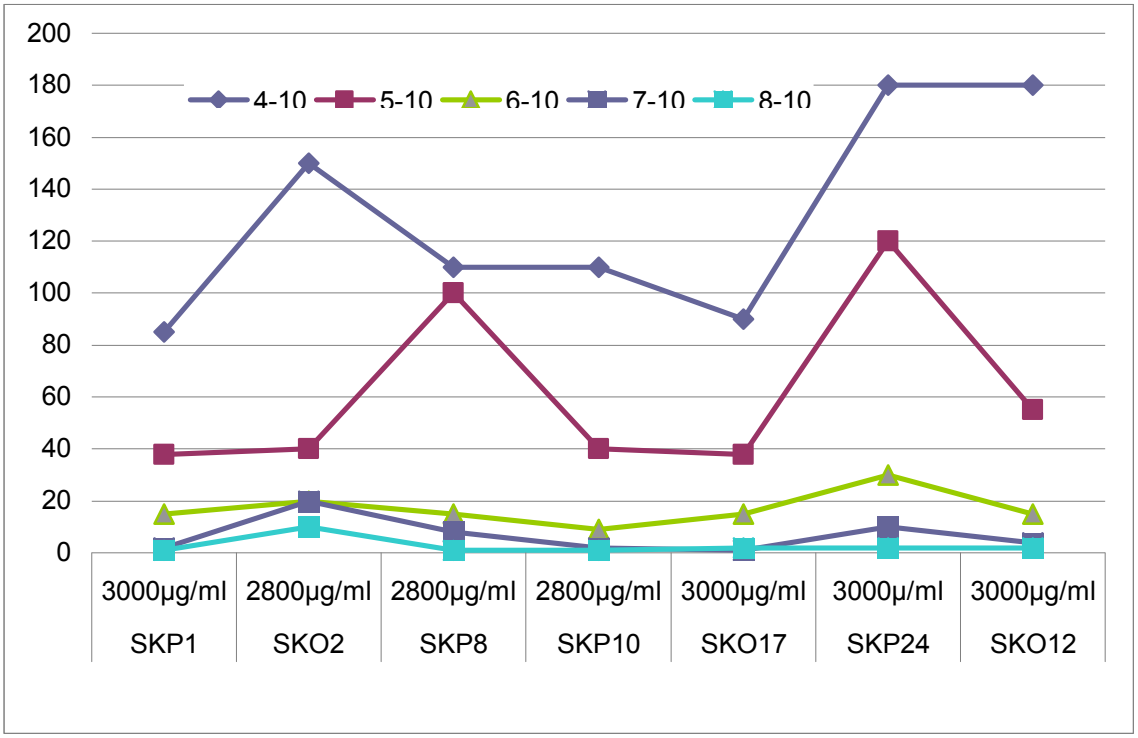



**Figure (2): Effect of Sodium Dodecyl Sulphate as curing agent**

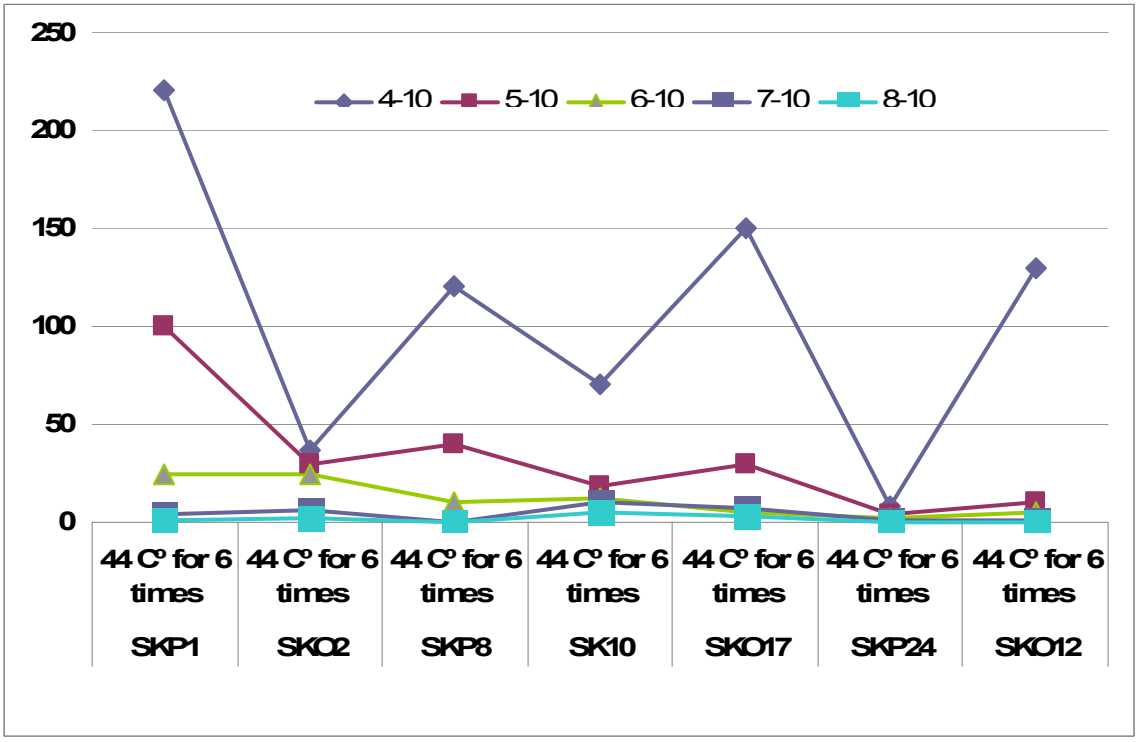



**Figure (3): Effect of elevated growth temperature as curing agent.**

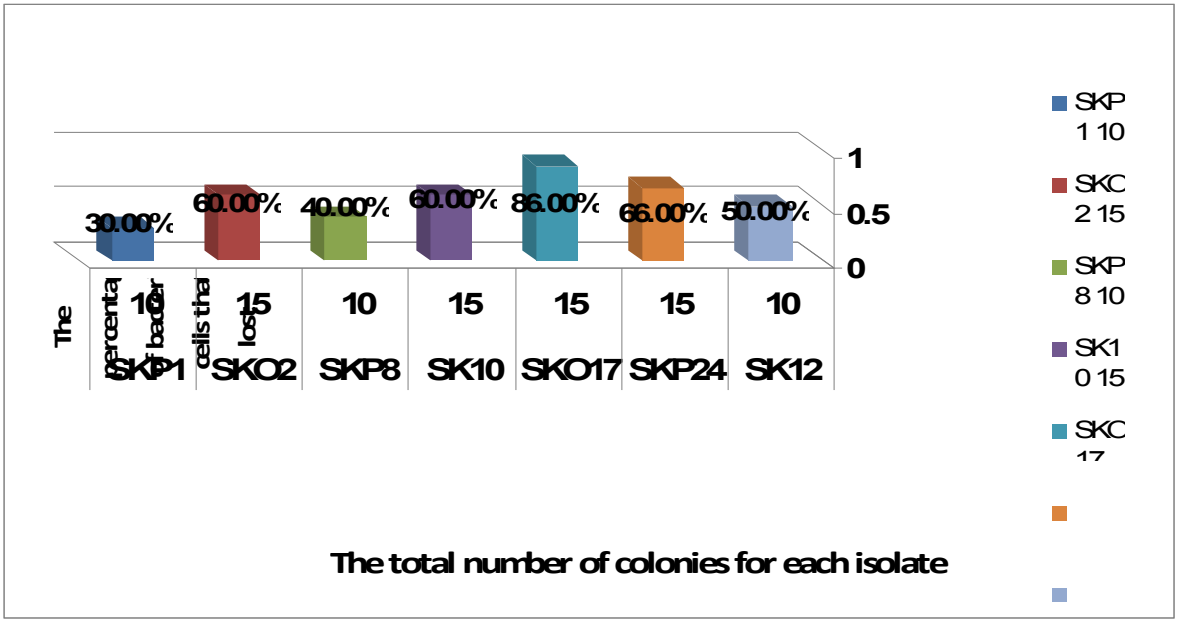



**Figure (4): Number of bacterial cell that lost resistance to antibiotic (ceftazidime) after treatment with sodium deodecyl sulphate.**

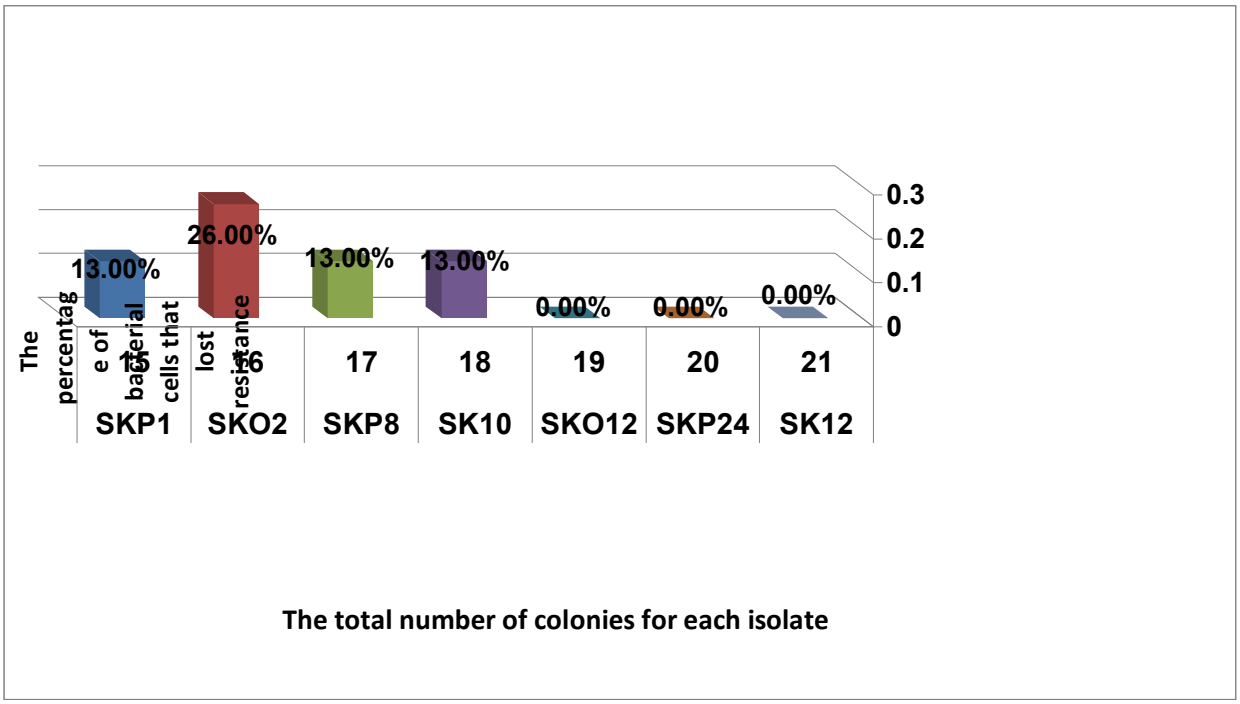



**Figure (5): Number of bacterial cell that lost resistance to antibiotic (ceftazidime) after treatment with elevated growth temperature (44C°) for 6 times.**

## *DISCUSSION*

It is well realized that the spreading of new resistance determinants among nosocomial pathogens represents a worldwide problem. In addition to extended spectrum beta lactamase, the most important class of broad-spectrum-lactamase has been emerging among gram-negative pathogens over the last decade: the metallo-β-lactamases (MBLs), which are most prevalent in gram-negative bacteria[22]**.** It is well known that life threatening infections due to extended spectrum β-lactamases (ESBLs), Ambler class C enzymes (AmpC) and carbapenemases producing isolates of *Klebsiella* spp. are continuing to be one of the major leading causes of morbidity and mortality in the community and hospital setting. This pattern involved the consequences of a complex interaction between the patient's underlying diseases, severity of illness, type of Intensive Care Unit (ICU), duration of stay, and the number, type and duration of invasive devices and procedures (23), (24).

With the widespread use of extended-spectrum cephalosporins throughout the world, strains that produce ESBLs have been detected on every inhabited continent. These enzymes are most commonly found in *K. pneumoniae*, and other gram-negative bacilli. The emergence and spread of ESBL-producing strains have led to questions regarding the optimal therapy for infections caused by ESBL-producing strains (25). The extended spectrum cephalosporins were rapidly adopted and they played an important role in the treatment of potentially life-threatening infections caused by a wide range of bacteria. Because of their broad spectrum of activity, they are of particular value in the initiation of empirical therapy (26).

In the screening test for metallo-beta-lactamase production, our study plan used the cefotaxime, ceftriaxone, ceftazidime and carbapenem (represented by imipenem). Our result showed that 1(10%), 1(4%) isolates of *Klebsiella oxytoca, K. pneumoniae* respectively were resistant to CAZ, CTX, CRO and carbapenem (represented by imipenem) considering that the break point zone of inhibition of imipenem was < 13 Mm (27). (28) reported that the emergence of carbapenemases in *K. pneumoniae* and *E. cloacae* poses relevant clinical problems. It could be argued that carbapenems maintain clinical efficacy on MBL-positive enterobacteria, since these drugs appear to allow a low-level expression of MBL enzymes, while carbapenem MICs remain below the susceptibility breakpoint (29). *Escherichia coli* with a self-transferable, multiresistant plasmid coding for metallo-β-lactamase VIM-1. However, in our isolates, a significant increase of carbapenem MICs was observed at higher inoculum sizes. This finding strongly suggests the possibility of a clinical failure of carbapenem therapy.

It is well documented that Inhibitor resistant TEM-β-lactamases (IRT) were resistant to CTX, CAZ, CRO and IMP but remained susceptible to cefoxitin and cephazolin. Since 1991, Inhibitor-resistant TEM (IRT) β-lactamases have been described to be present in *Escherichia coli* clinical strains (30). Also, the IRT-producing strains are resistant to penicillins and to their combinations with β-lactamase inhibitors.

Our results revealed that, in the screening test for IRT production, 6(24%) isolates of K. pneumoniae were resistant to inhibition by CA. Further, these isolates were resistant to CTX, CAZ, CRO and IMP but remained susceptible to cefoxitin. Furthermore, the MIC for cephalothin against the potential IRT producer isolates of Klebsiellae pneumonia was 3.7 ±2.3 µg/ml considering that the

susceptibility break point of cephalothin was 8 µg/ml. This result is in agreement with the criteria published by (31). In another study, (32) isolated clinical strains of K. pneumoniae which was highly resistant to amoxicillin (MIC > 1.024 mg/liter), however, it was susceptible to cephalothin (MIC of 8 mg/liter). The IRT β-lactamases have been found almost exclusively in strains of *E. coli*; however, our study confirms the diffusion of this mechanism of resistance *to K. pneumoniae*, a species frequently implicated in nosocomial infections.

In the genetic part of this study, the result of plasmid profile analysis revealed that out of 8 ESBL producer isolates of *Klebsiella* spp. 7(87.5%) were plasmid mediated (the genes coding for these enzymes were located on the plasmid). Further 1(100%), 1(33.3%) of metallo-β-lactamase and IRT respectively were also plasmid mediated. However, 1(12.5%), 2(100%), 2(66.7%) of ESBL, AmpC, and IRT enzymes respectively failed to show plasmid bands in agarose gel electrophoresis. It is well recognized that conjugation is considered a major pathway for horizontal gene transfer among bacteria. Conjugation requires cell-to-cell contact and operates by DNA replication resulting in unidirectional transfer of genetic material from a donor to a recipient cell. It is mediated mainly by conjugative plasmids, although conjugative transposons are also capable of triggering the process of conjugation (33).

Two aspects of conjugative plasmids have contributed to their importance as mediators of DNA transfer. First, it has been observed that conjugative plasmids mediate gene transfer in various environments such as soil and rhizosphere, plant surfaces, water or human gut. Second, conjugative plasmids are highly promiscuous: donor and recipient cells may belong to different genera or even to different kingdoms. A conjugative plasmid can infect different bacterial species if they coexist in the same habitat because conjugation requires contact between donor and recipient cells (34). In this study, conjugation experiments were performed by using broth mixture technique for 5(83.3%), 1(100%), 1(33.3%) of ESBL, metallo-β-lactamase and IRT enzymes respectively. Successful conjugation between isolates of *K. pneumoniae* and *Klebsiella oxytoca* with *E. coli* MM 294, as it is observed in this study, clearly indicated that conjugation is one of the important factors in spreading plasmids coding for antibiotic resistance among bacteria and that such transfer can occur not only by the strains of the same species but also between strains of closely related species especially those belong to the family of enterobacteriaceae. Their ability to pass between different bacterial strains or species has been considered to be of lower efficiency than their ability to pass between similar bacteria, due to diverse barriers such as restriction systems (35).

Our study showed that the conjugation has been detected and conjugation frequencies represented previously. These results indicated that Ceftazidime resistance marker is isolated on self-transmissible or mobilized plasmids which are curable by SDS. Our results on curing experiments revealed that *Klebsiella* spp. SKP1, SKP2, SKP8, SKP10, SKO12, SKO17, and SKO24 were very resistant and the MIC was higher than the highest concentration used in this study (3000 µg/ml). Samples from sub-minimal inhibitory concentrations sub MIC (the highest concentration allows bacterial growth) were taken to select cured cells. It is well recognized that Sodium dodecyl sulphate (SDS) is capable of curing certain plasmids. Some plasmid containing cells are presumed to be more sensitive to SDS because of plasmid-specific pili on their cell surface. The concentrations of SDS used in this study were (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2400, 2800, 3000 µg/ml), However, other researcher reported that the most effective concentration of a particular curing agent can vary considerably in the range of 100-to 1000-fold depending on the species

being treated, curing agent efficiency and the mode of action of curing agent. After treatment of bacterial strains with curing agents, survivors were analyzed for the loss of resistance to antibiotics by plating them on agar media containing the proper antibiotic. Then the curing percentage and efficiency of each agent were analyzed. (20) reported that the use of sodium dodecyl sulphate (SDS) proved very effective in curing the plasmid with a relatively high frequency ($6.25 \times 10^4$ of indigenous *Klebsiella pneumonia*.

Elevated incubated temperature (5-7Cº) above the normal or optimal growth temperature was also used in this study as a curing method. Results of this method revealed that Amp C producing isolates of *Klebsiellae* spp. were cured at higher ratio than ESBLs producing isolates.. It is well recognized that the high level of antimicrobial drug resistance in this bacterium is conferred by a plasmid-encoded KPC, which confers resistance to all cephalosporins, monobactams, and carbapenems (36). Infection with carbapenem-resistant *K. pneumonia* is associated with an increased proportion of deaths compared to carbapenem-susceptible *K. pneumoniae (37).* The efficiency of curing generally varies from less than 0.1% to more than 99% depending upon the agent involved, the bacterial strain, and the conditions used considering the curing activity is generally related to the ability of these compounds to intercalate into the DNA molecule.

The study suggested that the production of β-lactamases of type IRT, and metallo appeared to be the major mechanisms of resistance of *Klebsiella* spp. and *Enterobacter cloacea* to β-lactams. Also, conjugative plasmids were found to be the main genetic elements encoding for ESBLs produced by *Klebsiella* spp. and *Enterobacter cloacea*. Finally, the study suggested that sodium dodecyl sulphate was of higher potency than elevated growth temperature in the curing of plasmid DNA.

***REFERENCES***

1. Tato, M., Coque1 T. M., Baquero, F., Cantón, R. (2010). Dispersal of carbapenemase blaVIM-1 gene associated with different Tn402 variants, mercury transposons and conjugative plasmids in Enterobacteriaceae and *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 54:320-327.

2. Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann. (2005). Metallo-beta-lactamases: the quiet before the storm? Clin. Microbiol. Rev. 18:306-325.

3. Nordmann P, Poirel L.(2002) Emerging carbapenemases in gram-negative aerobes. Clin Microbiol Infect. 8:321-331.

4. Toleman MA, Rolston K, Jones RN, Walsh TR. (2004). blaVIM-7, An evolutionarily distinct metallo-β-lactamase gene in a *Pseudomonas aeruginosa* isolate from the United States. Antimicrob Agents Chemother. 48:329-332.

5. Poirel L, Heritier C, Tolun V, Nordmann P. (2004). Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. Antimicrob Agents Chemother.48:15-22

6. Luzzaro, F., Denis Docquier, J., Colinon, C., Endimiani, A., Lombardi, G., Amicosante, G., Rossolini G.M., Toniolo, A. (2004). Emergence in *Klebsiella pneumoniae* and *Enterobacter cloacae* Clinical Isolates of the VIM-4 Metallo-β-Lactamase Encoded by a Conjugative Plasmid. Antimicrob Agents Chemother. 48: 648–650

7. George A. Jacoby, M.D., and Luisa Silvia Munoz-Price, M.D. (2005). The New β-Lactamases. N Engl J Med. 352:380-390.

8. Baron EJ, Peterson LR, Finegold SM. (1994). Method for testing antimicrobial effectiveness. In: "Diagnostic Microbiology, (Bail and Scott's Ed.)". Toronto: C.V. Mosby company., 9th ed:168-193.

9. Sambrook J, Fritisch EF, Maniatis T. (1989). Molecular cloning: Laboratory manual. Cold Spring Harbor Laboratory. New York. 1659.
10. National Committee for Clinical Laboratory Standards (NCCLS). (2004). Performance standards for antimicrobial susceptibility testing. Fourteenth Informational Supplement Document M100-S14. Wayne, PA: NCCLS.26-32
11. Miles RS, Amyes SG. (1996). Laboratory Control of Antimicrobial Therapy. In: "Mackie and McCartney Practical Medical Microbiology, (Collee JG, Fraser AG, Marmion BP, Simmons A. Eds.)". 14th Ed., Churchill Livingstone, New York: 151-178.
12. Al-Ouqaili MT. (1999). Quantitative susceptibility test for antimicrobial agents against *Pseudomonas aeruginosa* obtained from Ramadi Teaching Hospital in Ramadi. M.Sc. Thesis. College of Medicine, University of Al-Anbar. 39.
13. Al-Kubaisy, SH. (2004). Genetic study of the variety of β-lactamases produced by β-lactam-resistant *Klebsiella* spp. M.SC. Thesis. College of Medicine, University of Al-Anbar. 44.
14. Clinical laboratory Standard Institute. (CLSI). (1999). Performance standards for antimicrobial susceptibility testing. Ninth Informational Supplement Document M100-S9. Wayne, PA: NCCLS.
15. Al-Ouqaili MT. (2002) In terms of $MIC_{50}$ and $MIC_{90}$, the role of extended and non-extended spectrum beta-lactamase in the resistance of Enteropathogenic *Escherichia coli* to beta-lactams. Al-Anbar Med J. 4:16-20.
16. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. (2002). Imipenem-EDTA disk method for differentiation of metallo-beta-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J Clin Microbiol, 40:3798-3801.
17. Clinical and Laboratory Standards Institute. (2007). Performance standards for antimicrobial susceptibility testing; seventeenth informational supplement. M100-S17. Clinical and Laboratory Standards Institute, Wayne, PA.
18. Birnboim HG, Doly J. (1979). Rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res., 7: 1513-1523.
19. O'connell M. (1984). Genetic transfer in prokaryotes transformation, transduction and conjugation. By: Puhler A., Timmis, K. Advanced molecular genetics. Springer verlug Berlin. 2-13.
20. Mansi M, Anderson KJ, Inche CA, Knowles LK, Platt DJ. (2000). Isolation and curing of the *Klebsiella pneumoniae* large indigenous plasmid using sodium dodecyl sulphate. Res. Microb. 151: 201-8.
21. Al-Saeed M S. (1997). Aerobic bacteria infection of the respiratory tract in Babel Governorate. Genetic study on *Klebsiellae* ssp. Ph.D. Thesis. College of Medicine, University of Baghdad. 58.
22. Bush, K. (2001). New β-lactamases in gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin. Infect. Dis. 32:1085-1089.
23. Pena C, Pujol M, Ardanuy C, Ricart A, Pallares R, Linares JAJ, Gudiol F. (1998). Epidemiology an successful control of a age outbreak due to *Klebsiella pneumoniae* producing extended-spectrum β-lactamases. Antimicrob Agents Chemother .42:53-8.
24. Babini GS, Livermore DM. (2000). Antimicrobial resistance amongst *Klebsiella* ssp. collected from intensive care units in southern and western Europe in 1997-1998. J Antimicrob Chemother. 45:183-89.
25. Kim YK, H, Lee HJ, Park SE, Choi EH, Kim J, kim Jh, Kim EC. (2002). Blood stream infections by Extended-spectrum β-lactamases producing *Klebsiella pneumoniae* and *Escherichia coli* in children: Epidemiology and clinical outcome. Antimicrob Agents Chemother. 46:1481-91.
26. Jacoby GA, Medeiros AA. (1991). More extended- spectrum β-lactamases. Antimicrob Agents Chemother. 35:1967-91.

27. National Committee for Clinical Laboratory Standards. (2000). Methods For Dilution antimicrobial susceptibility test for bacteria that grow aerobically. Approved standard M7-A5 and informational supplement M100-S10. National Committee for Clinical Laboratory Standards, Wayne.

28. Luzzaro F, Docquier JD, Colinon C, Endimiani A, Lombardi G, Amicosante G, Maria Rossolini G, Toniolo A. (2004). Emergence in *Klebsiella pneumoniae* and *Enterobacter cloacae* Clinical Isolates of the VIM-4 Metallo-β-Lactamase Encoded by a Conjugative Plasmid. Antimicrob Agents Chemother. 48:-648–650.

29. Miriagou, V., E. Tzelepi, D. Gianneli, and L. S. Tzouvelekis. (2003). *Escherichia coli* with a self-transferable, multiresistant plasmid coding for metallo-β-lactamase VIM-Antimicrob Agents Chemother. 47:395–397.

30. Sirot D, Chanal C, Henquell C, Labia R, Sirot J, Culzed R. (1994). Clinical isolates from urinary tract infections in France. J Antimicrob Chemother. 34:707-14.

31. Chabia EB, Sirot D, Paul G, Labia R. (1999). Inhibitor-resistant TEM β-lactamases: Phenotypic, Genetic and Biochemical characteristics. J Antimicrob Chemother. 43:447-58.

32. Henquell C, Sirot D, Chanal C, Decpamps C, Charton P, Lafeuille B. (1994). Frequency of *Escherichia coli* isolates from urinary tract infections in France. J. Antimicrob Chemother. 34:707-14.

33. Dionisio F, Matic I, Radman M. Rodriguse OR, Taddei F. (2002). Plasmids spread very fasten Heterogeneous Bacterial communities. Genetics Society of America. 162:1525-32

34. Ochman H, Lawrence JC, Groiman EA. (2000). Bacterial gene transfer and the nature of bacterial innovation. Nature. 405:299-304.

35. Sanders KE. (1996). F-mediated conjugation, $F^+$ strains and HFr strains of *Salmonella typhimurium* and Salmonella abony. In:-Neidhardt FC, Curtiss R, Ingraham JL, Lin ECC, Low KB, Magasanik B and Rezinkoff WS. *Escherichia coli* and *Salmonella* cellular and molecular biology. ASM publication, Washington, DC. 2406-2412.

36. Leavitt A, Navon-Venezia S, Chmelnitsky I, Schwaber MJ, Carmeli Y. (2007). Emergence of KPC-2 and KPC-3 in carbapenem-resistant *Klebsiella pneumoniae* strains in an Israeli hospital. Antimicrob Agents Chemother. 51:3026–9. DOI: 10.1128/AAC.00299-07.

37. Schwaber MJ, Klarfeld-Lidji S, Navon-Venezia S, Schwartz D, Leavitt A, Carmeli Y. (2008). Predictors of carbapenem-resistant *Klebsiella pneumoniae* acquisition among hospitalized adults and effect of acquisition on mortality. Antimicrob Agents Chemother. 52:1028–1033. Medline DOI: 10.1128/AAC.01020-07.

# Determination the optimal DNA extraction method from *Streptococcus mutans* biofilm formation that isolated from patients with caries active

**Nuha J. Kandala, Ibtihal A.H.m. Al- Karaawi, and Diaa A.S. Mehdi**

*Dept. of Biotechnology/ College of Sciences/ Baghddad University –Iraq*

*Email: nuhakasto@yahoo.com*

***ABSTRACT***

Twenty five isolates related to 0ral streptococci were obtained from twenty samples taken from patients with caries active and healthy people. Nine isolates of total number of oral streptococci was identified as *Streptococcus mutans* ,while other isolates (16 isolate )were related to other species of oral streptococcus . The ability of *S.mutans* isolates to produce slime layer was detected by using tubes method and congo red method. the results indicated that five isolates were slime layer produced by tubes method while seven isolates were slime layer produced by congo red method .

Sensitivity of four selective isolates was tested against 13 Antibiotics. Results revealed that isolates showed multi resistance to antibiotics ranging between (5-8) antibiotics, and all isolates of *S.mutans* were resistant 100% to cephalexin, piperacillin, cefoixime, cefazidim and ampicillin /cloxacillin. While Amoxicillin, Penicillin ,Imipenem and Ciprofloxacin were the most effective drugs used in the present study.against the isolates.

Agarose gel electrophoresis of DNA samples of four selective isolates of *S.mutans* showed , three of isolates harbor two plasmid bands different in size and position . In this study comparative methods have been performed to analyze the efficiency for extraction and purification of total DNA from selective isolates SMP2 .The results showed that except from indirect lysis by SET and glass beads all other DNA isolates, lysis by sonication as well as enzymatic lysis were pure and in good quantity. DNA lysis by sonication was accepted instead of lysis by lysozyme as the method of choice for lysis the cell and extraction DNA from gram positive bacteria .

**الملخص باللغة العربية**

تم الحصول على 25 عزلة من المسبحيات الفموية من 20 عينة ماخوذة من اشخاص مصابين بتسوس الاسنان ومن اشخاص اصحاء . شخصت 9 عزلات على انها تعود الى النوع *Streptococcu mutan*s , في حين تعود باقي العزلات ( 16عزلة) الى الانواع الاخرى من المسبحيات الفموية. اختبرت قابلية عزلات *S. mutans* على انتاج الطبقة اللزجة باستخدام طريقتي الانابيب واكار احمر الكونغو, وبينت النتائج ان 5 عزلات كانت منتجة للطبقة اللزجة باستخدام طريقة الانابيب بينما كانت 7 عزلات منتجة للطبقة اللزجة باستخدام طريقة اكار احمر الكونغو .

أظهرت نتائج اختبار حساسيةاربع عزلات منتخبة تجاه 13 مضادا حيويا, امتلاك جميع العزلات لنمط المقاومة المتعددة للمضادات الحيوية تراوحت مابين (5–8) مضادا , وكانت جميع العزلات مقاومة 100 % لمضادات. السيفالكسين والببيراسلين والسيفوكزام والسيفازيدام ومزيج الامبسلين / الكلوكساسلين , في حين اظهرت المضادات البنسلين والاموكيسلين و الاميبينيم و السبيروفلوكساسين المستخدمة في هذه الدراسة فعالية علاجية جيدة ضد العزلات.

بينت نتائج الترحيل الكهربائي في هلام الاكاروز لعينات الـــ DNA لاربع عزلات منتخبة لبكتريا *S. mutans* امتلاك 3 عزلات الى حزمتين بلازميدية تختلف في الحجم والموقع , وتضمنت هذه الدراسةاختبار كفاءة استخلاص وتنقية الـــ DNA الكلي من العزلة المنتخبة SMP2 باستخدام عدة طرق للمقارنة , واظهرت النتائج ان التحليل باستخدام طريقة التكسير الفيزيائي للخلايا الـــ sonication والتحلل الانزيمي( باستثناء التحليل الغير مباشر باستخدام البفر SET وتحليل الخلايا باستخدام الكرات الزجاجية) اعطى نتائج جيدة من ناحية الكمية ونقاوة عالية لـــ .DNA , لذا عدت طريقة الـــ Sonication ( التكسير الفيزيائي )عوضا عن التحليل الانزيمي طريقة بديلة لتحليل الخلايا واستخلاص الـــ DNA من البكتريا الموجبة لصبغة كرام .

## *INTRODUCTION*

In the oral cavity we found few microorganisms are able to adhere to the teeth , and limited group from these are cariogenic ,which include streptococci, lactobacilli, staphylococci, corynebacteria, actinomyces species and various anaerobes in particular bacteroides (1). *Streptococcus mutans*, considered to be the primary etiological agent of human dental caries by forming biofilm (2), (3) , possesses various of abilities to colonize tooth surfaces. *S. mutans*, under certain conditions, is numerically significant in cariogenic biofilms and forms biofilms with other organisms in the oral cavity (3).

*S. mutans* can bind to salivary pellicles formed on the teeth by expresses several surface adhesions , such sucrose-dependent adherence is mediated by glucan binding proteins and water-insoluble glucans produced from sucrose by glucosyltransferase (GTF) enzymes(4). The cells of *S. mutans* are spherical or ovoid, 0.5-2.0 µm in diameter, occurring in pairs or chains when grown in liquid media, and stain gram positive, non motile, non sporeforming. Some species are encapsulated, facultative anaerobic, catalase and oxidase negative . The cell wall is composed of peptidoglycan (murein) and teichoic acids that prevent osmotic lysis of cell protoplast and confer rigidity and shape on cell. *S.mutans* has a capsule that is composed of polysaccharide, and its structural subunit is dextran glucose (5).

The cell walls of gram-positive bacteria can be efficiently broken by use of the peptidoglycan-degrading enzymes lysozyme and mutanolysin. However, to minimize the number of reagents (and possible sources of bacterial DNA) and to find a method equally efficient for cell walls of streptococci, and other bacteria that related to gram-positive (6). Attention has been paid to biofilm production in *S.mutans* . isolation , identification from patient with caries active and healthy people, dtermination the susceptibility to different antibiotics groups ,detecting the best method to analyze the efficiency for extraction and purification of total DNA from *S.mutans* (gram positive bacteria) and detection of genetic factors controlling the biofilm production in selective isolates.

## *MATERIALS AND METHODS*

### Samples Collection:

Twenty samples were collected during the period between 10 to 25 November 2010 from patients in Baghdad City hospital and from healthy people (Biotechnology Department/College of Science/Baghdad university) . These samples were distributed as follows :

1- from healthy people ( 10 samples) taken From surface of teeth.

2- From patients with caries active (10 Samples) taken from different type of caries pit, fissure and root, with ages (20-45 years).

### Bacterial Isolates:

All the samples were taken by sterile cotton swabs and dispersed in sterile tubes containing Brain Heart Infusion Broth, then incubated for 48 hrs at 37C in candle jar and each sample then streaking on the surface of selective media Mitis Salivarious agar and incubated for 48 hrs at 37C in candle jar.

Culture Media:

Culture media used in this study included (Brain Heart Infusion agar, Brain Heart Infusion Broth (Difco/USA), Mitis Salivarious agar,(Hi-Media-India ) , Blood Base agar (oxoid, England), Congo red agar, Mueller Hinton media (Hi-Media-India ), were prepared according to the instructions of the companies (5).

Rapid identification of *Streptococcus mutans* :

This technique provides a useful method for identification of *Streptococcus mutans* from other oral streptococci according to Microscopic Examination ,Cultural characteristics and biochemical test (5), (7), (8).

Antibiotic Sensitivity Test:

Thirteen various available and commonly used antibiotics discs were used in the antibiotic sensitivity test. The susceptibility of four isolates to different antimicrobials was determined by Kirby-Bauer disk diffusion method on Mueller Hinton media (9). The sensitivity and resistant were determined by measuring the diameter of inhibition zones around the antibiotic disc (10).

Detection of biofilm formation:

Two methods were performed for detection of biofilm formation in clinical isolates Congo red agar method (11) and adherence method on smooth surfaces (12)

Isolation of DNA:

The buffer and solutions used for Isolation of DNA:
Buffer solutions were prepared as described by (13) briefly.

Lysis of bacterial cells **:**

1-The pellet of bacterial cells were suspended with normal saline then subjected to sonication (25 kHz) for 2,4, 6,8, 10or 12 min (every 2 min discontinuous).This step was repeated several times according to time of cell exposure for lysis .

2- The suspension of bacterial cells with SET buffer was subjected to treat with glass beads (2.4g to 10 ml and vortexes for 15 min).

3- The suspension of bacterial cells with SET buffer was subjected to treated with lysozyme

4- The suspension of bacterial cells were subjected to treat with SET buffer only without lsyozyme or sonication and glass beads .

Total DNA Isolation (Salting out Method):

The method was used in this study described by (14) to isolate both plasmid and chromosomal DNA from selective isolate of *S. mutans* (SMP 2, SMP 1, SMP 5, SMH 11)

Agarose Gel Electrophoresis:

The method described by (14).

Estimation of the DNA concentration by the Spectrophotometer:

This method described by (15). The concentration of DNA was calculated according to the formula:-
DNA concentration (mg/ml) =O.D 260 nm X 50 X Dilution factor.

The spectrophotometer was used also to estimate the DNA purity ratio according to this formula:-
DNA purity ratio= OD260 / OD280

This ratio was used to detect nucleic acid contamination in protein preparations.

Bacterial conjugation:

*S. mutans* (SMP 2) isolate which resist to Vancomycin was selected (as donor cells ) for studying bacterial conjugation with *S. mutans* (SMH 11**)** which resist to Cefotaxim (as recipient cell ). Mating experiments were performed by using filter method as described in (16). Selective media were prepared for transconjugants selection:

- Brain heart infusion agar supplemented with 30µg / ml Vancomycin.
- Brain heart infusion agar supplemented with 30 µg / ml Cefotaxim
- Brain heart infusion agar supplemented with Vancomycin.and Cefotaxim

## *RESULTS AND DISCUSSION*

### Isolation and Identification of Streptococcus Isolates

Bacterial Isolates

Nine *S.mutans* isolates were identified according to morphology, characteristics with some biochemical tests (5), Phenotypic identification of each isolates was performed by using a commercial identification system (Api 20 system) according to the instructions of the manufactures.

### Prevalence of *Streptococcus*

It was observed in the results in table (1), that the number of *S.mutans* isolates among the total samples and isolates was nine isolates while other oral streptococcus was 16 isolate and the number of *S.mutans* isolates according to the source of isolate ( caries active ) and ( non caries active ) were 3 and 6 isolates .

As shown in the table, the percentage of *S.mutans* isolates sp. obtained from the total number of caries active in total isolates was (42.86%) which is a higher percentage comparable with the samples from not caries active (27.28%). In the study of Al-Mudallal (17) revealed that Thirty isolates (60%) from Fifty plaque samples were collected from teeth. While Al-Kaziragy (18) referred to thirteen isolates (24.52%) identified as *S.mutans* from the total number of oral streptococci while the rest isolates were related to other species of streptococci were (75.48%) .

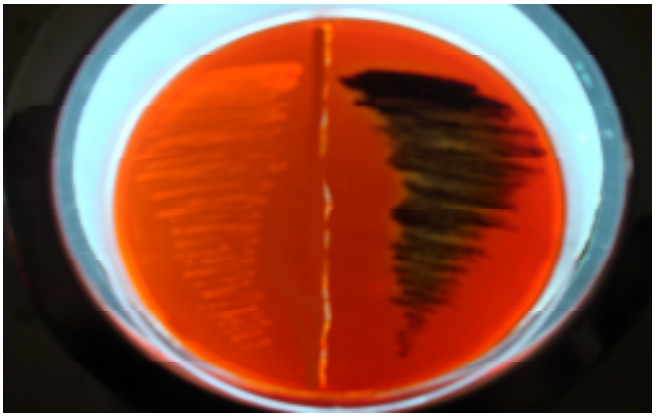
### Detection of biofilm formation

Congo red agar method It count as rapid impressible method for detecting biofilm-forming bacteria and it has many advantages the most important is the isolates still live in the medium (11).

The results showed that seven isolates (77.78%) produced biofilm; Hence the colony of the biofilm producing isolate appeared as sparkle dark colonies, while the colony of the non biofilm former two isolates appeared as sparkle red colonies (figure. 1A).while the detection of slime layer producing isolates by using christensen method indicated that five isolates (55.55%) produce slime layer but, 44.45% of isolates not able to produce slime layer (figure. 1C), .

The different in results between these two method indicated that congo red method is more efficient and sensitive for detecting the production of slime layer because its contains sucrose which is used for detection of glugan production by streptococcal species and cotain congo red which is used for shown exopolysaccharide while Christensen method is not always successful for detecting the weak slime production and variation in media many affect the result (12) .This

bacterium has evolved a biofilm lifestyle for survival and persistence in its natural environmental dental plague(19).The abilities to form tenacious biofilms and to tolerate environmental insults are considered major virulence attributes of this organism (20) . *S,mutans* is the most efficient cariogenic microorganisms ,rapidly inducing caries in human (2).



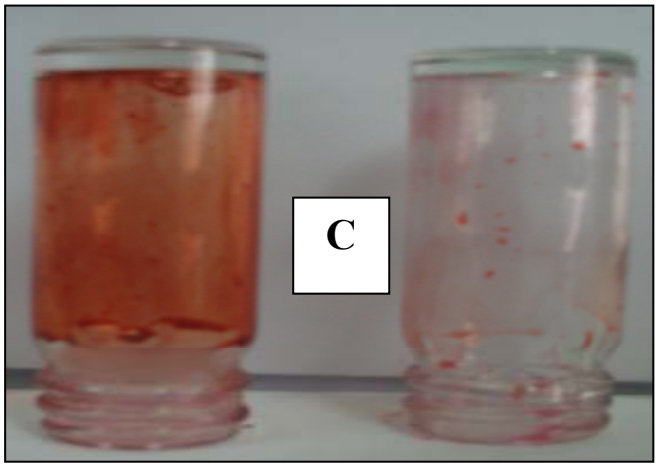



**Figure (1 ) Biofilm formation on Congo red agar after 24 hour incubation at 37C°.**

**A- Biofilm formation by *S.mutans* SMP5 isolate**
**B- Biofilm non formation by *S.mutans* SMH11 isolate**
**C- C- Adherence of *S.mutans SMP2* on smooth surfaces of test tubes**

**Table (1): Isolation source and percentage of oral *Streptococcus* spp . isolates**

| Isolation Source | Number of Samples | Number of *S.mutans* Isolates | Number of *Oral Streptococcu* Isolates |
|---|---|---|---|
| The external surface of teeth | 10 | 3 | 8 |
| caries active | 10 | 6 | 8 |
| Total | 20 | 9 | 16 |

**Antibiotic susceptibility of *S.mutans***

Four selected isolates (SMP1, SMP2 SMP5, SMH11) were tested for their sensitivity toward thirteen antibiotics discs by using disk diffusion method. All isolates were found to be resistant to at least 6 antibiotics tested. Hence all the isolates were considered to be multi drug resistant.

The results demonstrated 100 % resistance to some antibiotics related to Beta- lactam group such as cephalexin, piperacillin, cefoxime, ceftazidime, and ampicillin /cloxacillin. (Table 2) , while four isolates were sensitive to Amoxicillin, Penicillin ,Imipenem, Rifampin and Ciprofloxacin were the most effective drugs used in the present study.

In majority, our results indicates that one isolate MSH11 showed complete resistance to seven antibiotics used in this test, while SMP1,SMP2 and SMP5 showed resistance to six antibiotics used..

In another study in Iraq, *Al-Kaziragy* (18) found that all isolates of *S.mutans* appeared sensitivity to 9 antibiotics (100%) (Amoxicillin ,Tetracyclin ,Gentamicin ,Eryrthromycin, , Ciprofloxacin , Cephalothin, cefoixime, cefotaxim and cephalexin,) and resistant to Bactracin .

(21) demonstrated that *S. mutans* isolates showed sensitivity to Amoxicillin,Tetracyclin,Gentamicin and Eryrthromycin .

The mechanisms behind the increased resistance of dental plaque bacteria to these agents is due to mutations affecting the drug target of their cells, the presence of efflux pumps or to the production of modifying enzymes (22).

**Table (2): Antibiotic susceptibility test of *S.mutans* isolates**

| Antibiotic | SMP1 | SMP2 | SMP5 | SMH11 |
|---|---|---|---|---|
| Amoxicillin | S | S | S | S |
| Cefoixime | R | R | R | R |
| Cefotaxime | R | S | R | R |
| Penicillin | S | S | S | S |
| Cefazidime | R | R | R | R |
| Imipenem | S | S | S | S |
| Rifampin | S | S | S | S |
| Pipracillin | R | R | R | R |
| Amoxicillin\clavulanic acid | S | S | S | R |
| Ampicillin\cloxacillin | R | R | R | R |
| Ciprofloxacin | S | S | S | S |
| Vancomycin | S | R | S | S |
| Cephalexin | R | R | R | R |

**Isolation of Plasmid DNA**

Four isolates (SMP1, SMP2, SMP5, and SMH11) were selected and detected for their plasmid profiles.
Plasmid DNA was extracted using salting out method. The results of Figure (2) show that the three isolates SMH11,SMP2,SMP5 were similar in their plasmid profiles and the plasmids were different in size when electrophoresis was performed , while SMP1 not possesses any plasmids ,but all of them biofilm production .

These isolates were also multi drug resistance to 5 antibiotics that was used in this study especially the β-lactam antibiotics related to 3GC, such as ceftazidime ,ceftraxone and cefotaxime.
Studies world wide showed that *S. mutans* isolates harbor more than one plasmid approximately 5.6 kilobase(kb). Encoded resistance to large number of antibiotics and metal ions bacteriocin production and immunity, accessory catabolic pathways and mechanisms for conjugation-like transfer activities (5).

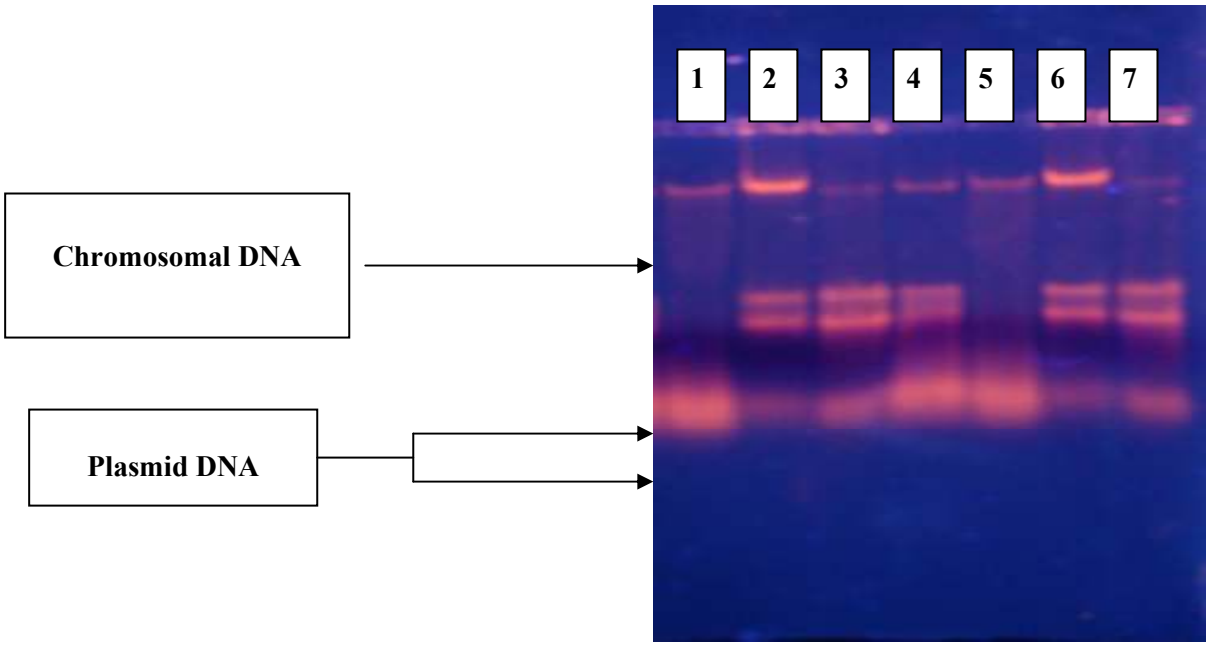



**Figure (2) : Agarose gel electrophoresis of plasmid DNA from *S.mutans spp*. isolates with agarose concentration (0.8%),voltage 5volt/cm,during 1.5hr**

**Lane 1 and 5 : plasmid profile of isolate. *S.mutans* SMP1**
**Lane 2 and 6 : plasmid profile of isolate *S.mutans* SMP2**
**Lane 3 and 7 : plasmid profile of isolate *S.mutans* SMH11**
**Lane 4 plasmid profile of isolate *S.mutans* SMP5**

**The modified direct lysis extraction method**

In this study comparative methods have been performed to analyze the efficiency for extraction and purification of total DNA from gram positive bacteria , revealing that the extraction DNA from G+ bacteria suffer from low efficiency, mainly due to incomplete cell lysis when we extracted DNA without lysis by enzyme .

This study shows how the modified direct lysis method can actually give good yield of DNA from a selective isolate SMP2. four different steps of DNA extraction modifications were tried with SMP2.

The isolated DNA was analyses on 0.8 % agarose gel (Figures 3) and (Figure 4). The lysis of isolate SMP2 was enhanced by sonication for 2, 4 ,6 min (lane 1,2,3 ) , 8 min ( lane 4,5,6 ) replicate) or 12 min ( lane 9 ) and the results showed the isolate SMP2 harbor two plasmid band by lysis with sonication for 8 min ( in different time discontinuous), which considered the best time for lysis cell comparable with results that obtained from lysis cells for 2,4 and 6 min ( lane1,2,3 )

The DNA absorbs at 260 nm and protein at 280 nm. To evaluate the purity of the extracted DNA, absorbance ratio at 260/280 nm (DNA/protein) was determined (Table 4).

In figure (5) shows the Comparison of results obtained with aliquots of SMP2 isolate processed by the lysozyme and sonication with lysis by glass beads and SET only.

The results mentioned above showed that except from indirect lysis by SET and glass beads, all other total DNA isolates, lysis by sonication as well as enzymatic lysis was pure and in good quantity. DNA lysis by sonication was accepted as the method of choice for extraction DNA from gram positive bacteria such as streptococcus as it gave maximum amount of pure intact DNA.

(23) and (24) demonstrated the problem of finding a DNA isolation procedure for clinical specimens that would produce DNA from both gram-positive and gram-negative bacteria with equal efficiencies. The cell walls of gram-positive bacteria can be efficiently broken by use of the peptidoglycan-degrading enzymes lysozyme and mutanolysin. However, to minimize the number of reagents (and possible sources of bacterial DNA) and to find a method equally efficient for cell walls of streptococci, and other bacteria that related to gram-positive we preferred the use of physical degradation method sonication, we aimed at determining a treatment time that would enhance the release of DNA from bacterial cells. In our study we succeeded in finding a DNA isolation method by sonication which released and purified bacterial DNA, especially that from streptococci, at least as efficiently as the standard method (with lysozyme) and avoided the use of toxic and explosive components.

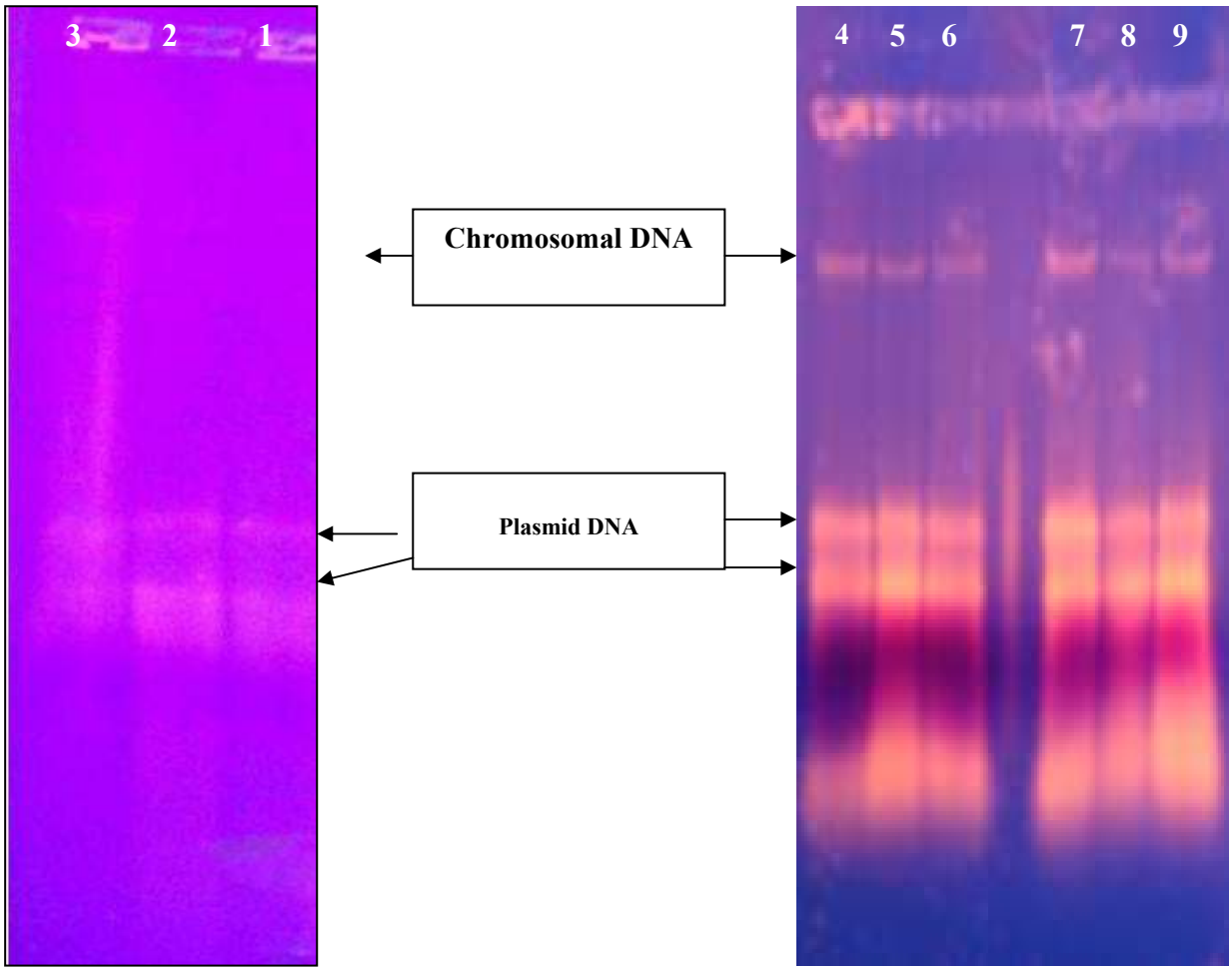



**Figure ( 3 ) :Effect of sonication in different time on detection the quantity of Total DNA isolation from** *S.mutans SMP2* isolate **by agarose gel electrophoresis ( agarose concentration (0.8%),voltage 5volt/cm,during 1.5hr): Lane 1 and 2 : plasmid profile by lysis for 2 min .**

**Lane 3 : plasmid profile by lysis for 4 min.**
**Lane 4 : plasmid profile by lysis for 8 min (discontinuous each 1 min)**
**Lane 5 : plasmid profile by lysis for 8 min (discontinuous each 2 min)**
**Lane 6 : plasmid profile by lysis for 8 min (discontinuos each 2 min**
**Lane 7 and 9 : plasmid profile by lysis for 10 min (discontinuous each 2 min)**
**Lane 8 : plasmid profile by lysis for 12min (discontinuous each 2 min**

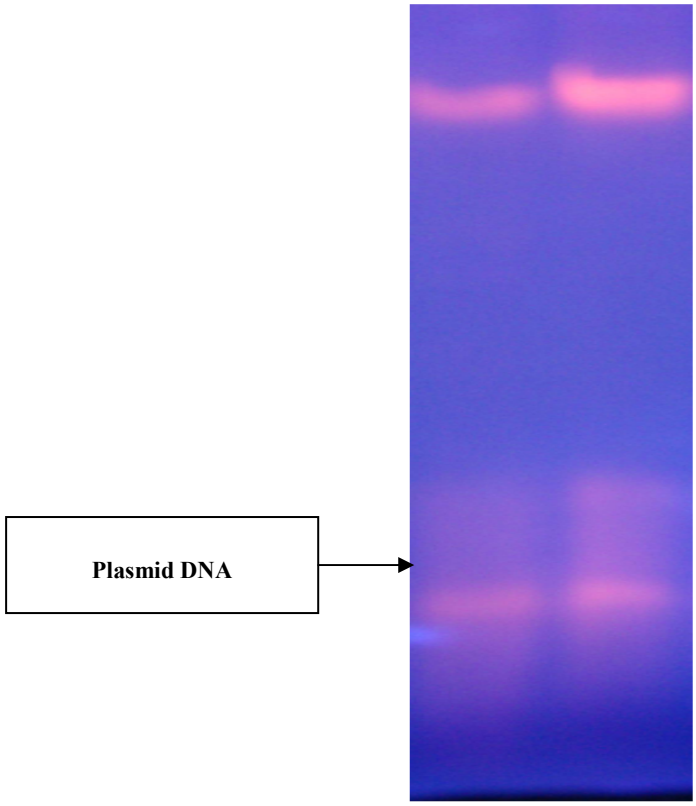



**Figure ( 4) :Effect of glass bead on lysis the cell to detect the quantity of Total DNA isolation from *S.mutans SMP2* isolate by agarose gel electrophoresis ( agarose concentration (0.8%),voltage 5volt/cm,during 1.5hr): Lane 1 and 2 : plasmid profile by lysis for 2 min .**

**Lane 1 : plasmid profile by lysis the cell with glass beads with SET**
**Lane 2 : plasmid profile by lysis the cell with glass beads without SET**

**Table (4 ) Comparative data of total DNA isolated by modified direct lysis, lysis by sonication, enzymatic lysis , lysis by glass beads and indirect lysis by SET**

| Method | A260/A280 ratio. | Yield : µg/ml |
|---|---|---|
| Lysis by sonication ( 8 min) | 1.893 | 3915 |
| Enzymatic lysis | 1.880 | 3855 |
| Lysis by glass beads | 2.04 | 1050 |
| Indirect lyses by SET | 2.1175 | 1148 |

*** the total yield, $1.8 < A260/A280 \geq 2$ indicates pure DNA**

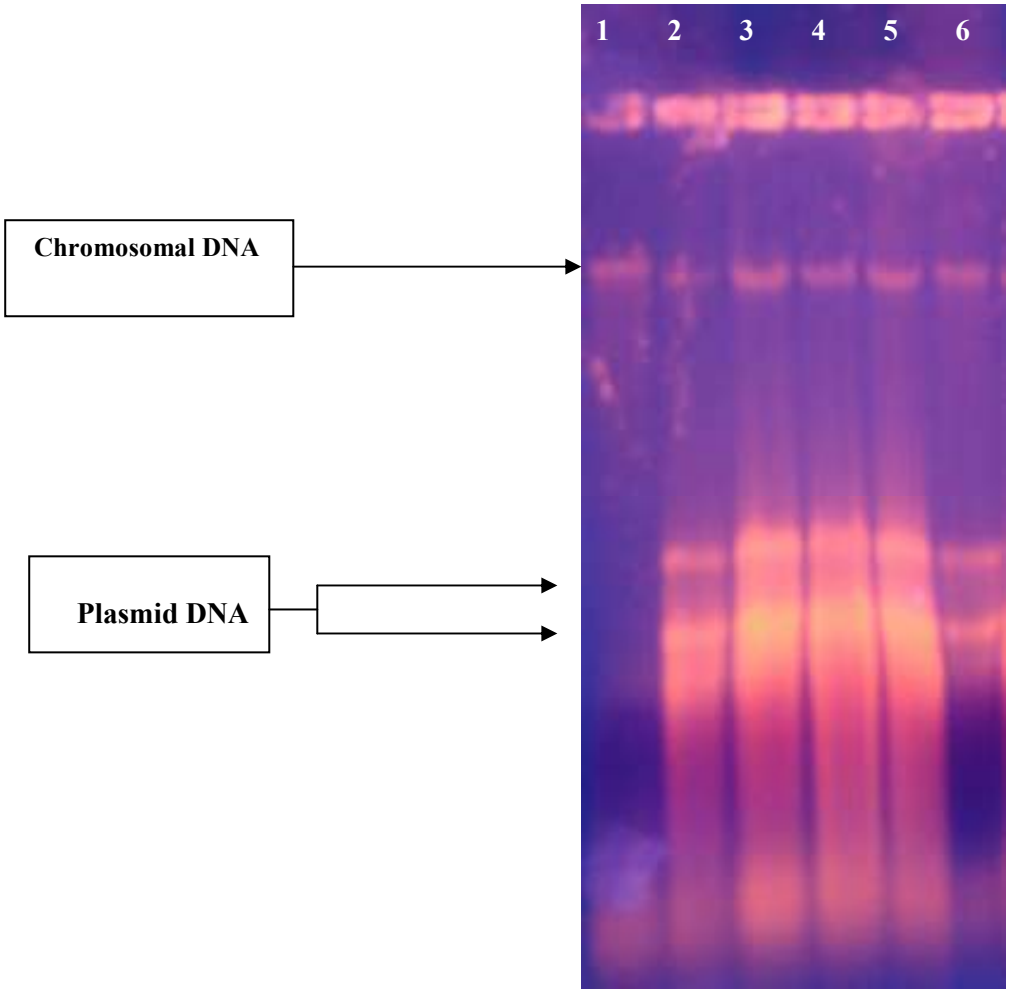



**Figure ( 5 ) : Comparison of modified method of total DNA extraction from isolates of *S.mutans* SMP2 BY agarose gel electrophoresis (with agarose concentration (0.8%),voltage 5volt/cm,during 1.5hr)**
**Lane 1 : plasmid profile lysis by SET**
**Lane 2 : plasmid profile lysis by sonication (8 min)**
**Lane 3 : plasmid profile lysis by sonication ( 10 min )**
**Lane 4 and 5 : plasmid profile lysis by sonication ( 12 min)**
**Lane 6 : plasmid profile lysis by lysozyme**

## Bacterial Conjugation

The bacterial conjugation was carried out in order to detect the role of plasmids in transferring of biofilm production as well as drug resistance.

one biofilm producing isolates ( SMP2 ) used which harbor more than one plasmid band, this isolate was considered as donor cell ( resistant to vancomycin ) and the isolate SMH11(resistant to cefotaxim) as recipient cell.

Results revealed that the conjugation between the SMH11 and SMP2 isolate was not successful. The conjugation frequency for these transconjugants was relatively very low.
The transconjugants did not expressed their antibiotic resistance because they were not able to grow in selective medium containing cefotaxim ( at final concentration of 30 µg/ml) and vancomycin (at final concentration of 30µg/ml) and not acquisition of resistance to antibiotics in recipient cell .

***REFERENCES***


1. Duailibe SA., Goncalves AG., and Ahid F J. (2007) Effect of a propolis extract on Streptococcus mutans counts in vivo. Appl. Oral. Sci. 15 (5):1-12.

2. Deng DM., Liu ML., Tencate JM., and Crielaard WC. (2007) The vicrk system of Streptococcus mutans responds to oxidative stress. Dent.Re.86(7):606-610.

3. Ahn SJ., Sang JA., Zezhang TW., Brady JB., and Robert AB. (2008) Characteristics of Biofilm Formation by Streptococcus mutans in the Presence of Saliva . Infection and Immunity. 76(9): 4259-4268.

4. Mizuho M., Yuzo T., Hideo Y., Nobuhiro H., Jun T., Haruo W. , Hidenobu S . (2006) Assessment of Genes Associated with Streptococcus mutans Biofilm Morphology. Appl. Environ. Microbiol. 72(9): 6277–6287

5. Hardie JM., and Whiley RA.(2006) The Genus Streptococcus — Oral. in : the prokaryotic,3rd ed. Springer Science, New York:ICO13-USA. 4: 76–107.

6. Rantakokko KJ., and Jalava J . (2002) Optimal DNA Isolation Method for Detection of Bacteria in Clinical Specimens by Broad-Range PCR . J. Clin. Microbiol. 40( 11).: 4211-4217,

7. Yoo SY., Park SJ. , Jeong DK., Kim K. , Lim S., Choe S., Chang Y., Park I, and Kook J. (2007) Isolation and characterization of the mutans streptococci from the dental plaques in Koreans. Microbiol. 45(3): 246-255.

8. Collee JG., Fraser AG., Marmiom BP., and Simmon AS . (1996) Practical Medical Microbiology 4th ed. Churchill Livingstone Inc. USA.

9. Bauer AW., Kirby WM., Sherries JC., and Turk MN.(1996) Antibiotic Susceptibility Testing by a Standardized Single Disc Method. Am. J. Clin. Pathol. 45: 493 – 496.

10. National Committee for Clinical Laboratory Standards (NCCLS). (2003) Performance standards for disk susceptibility tests, 8th ed. Informational Supplement Wayne.101-109

11. Freeman D., Falkiner FR., and Keane CT. (1989) New method for detecting slime production by coagulase negative staphylococci .J. Clin. Pathol.42:872-874.

12. Christensen GD., Simpson W., Bisno AL., and Beachy EH. (1982) Adherence of slime producing strain staphylococcus epidermis to smooth surfaces. Infec. and Imm. 37:318-326.

13. Sambrook J., and Russell DW. (2001) Molecular Cloning: A Laboratory Manual, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.236-239

14. Kieser T.(1995). Preparation and analysis of genomic and plasmid DNA. Johnines Center, Nerwich, NRU 7UH, UK .891-898

15. Maniatis T., Fritsch EF., and Sambrook J. (1982) Molecular cloning: : A Laboratory Manual, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y: 17.

16. Shiono A., and Ike Y. (1999) Isolation of Enterococcus faecalis clinical isolates that efficiently adhere to human carcinoma T24 cells and inhibition of adhesion by fibrenoctin and types in treatment. Infect. Immun. 67(4): 1585–1592.

17. Al-Mudallal NH. (2006) Characterization of Glucosyltransferase (GTF) of Mutans Streptococci Streptococcus sobrinus (Serotype G). Ph.D. Thesis, College of Science, Al-Nahrain University.41-43

18. Al-Kaziragy SF. (2010) The effect of Glucan binding protein on Streptococcus mutans biofilm formation and determination of chemical inhibitors. M.Sc. Thesis, Biology Department- College of Science. Baghdad University.31-35

19. Levesque CM., Voronejskaia E., Huang YC., Mair RW. , Ellen RP. and Cvitkovitch DG. (2005) Involvement of sortase anchoring of cell wall protein in biofilm formation by Streptococcus mutans. Infect. Immun. 73(6):3773-3777.

20. Ahn SJ., Wen ZT., and Burne RA. (2006) Multilevel control of competence development and stress tolerance in streptococcus mutans UA 159. Infect .Immun. 74(3): 1631-1642.

21. Jain P., and Pundir RK. (2009) Antibiotics sensitivity pattern *Streptococcus mutans* against commercially available drugs .Pharmacy Res .2(7):1250-1252.

22. Marsh PD. (2004) Dental plaque as a microbial biofilm. Caries Res. 38: 204-211.

23. Hendolin PH., Paulin L., and Ylikosk J. (2000) Clinically applicable multiplex PCR for four middle ear pathogens. J. Clin. Microbiol. 38:125-132.

24. Chaudahauri SR., Pattanayaka AK. and Thakur AR. (2006) Microbial DNA extraction from amples of varied origin . Current. Sci . 91 ( 12 ) :1697-1700

# FTIR Study of purity DNA from whole blood of α and β-Thalassemia

**Sura A. A.S. Al- Emami**

*College of Sciences / Al- Mustansirya University – Iraq*

*E-mail: sura742003@yahoo.com*

***ABSTRACT***

Thalassemia is a global public health problem. The present study aims to investigate the potential of Fourier Transform Infrared (FTIR) spectroscopy as a fast tool in the diagnosis and screening of purity DNA from whole blood of patients with α and β thalassemia. Twenty three patients with α and β-thalassemia were studied and compared to twelve healthy individuals. DNA was extracted from whole blood pool of control and patients, then it was purified using spin column of (1×6 cm) of Sephadex G-25. The FTIR results indicated presences of differences in vibration bands between α and β-thalassemias, these were : disappearance of vibration bands of streching ($CH_2$), and streching (C=O), in β-thalassemias. While disappearance of vibration band of binding (C-H) phenyl was observed in α-thalassemia. These finding could help understand possibility of FTIR to screening and diagnosis DNA thalassemia.

***Key words:*** thalassemia , DNA , purification , FTIR , spectroscopy

**الملخص باللغة العربية**

تعتبر الثلاسيميا من المشاكل الصحية العامة العالمية. تهدف الدراسة الحالية الى فحص امكانية استخدام طيف FTIRفي تشخيص DNA المنقى من دم المرضى المصابين بالثلاسيميا الفا وبيتا. تضمنت الدراسة 23 عينة من المرضى المصابين بالثلاسيميا و12 عينة من الاشخاص الاصحاء. تم استخلاص DNA وتنقيته باستخدام عمود (1x6 سم) من السفادكس G-25 .وقد اظهرت النتائج وجود اختلافات في اهتزازات الحزم لطيف FTIR لالفا–ثلاسيميا عند مقارنتها مع نظيرتها في البيتا–ثلاسيميا, حيث تبين اختفاء حزم مط (CH2)ومط (C=O) , بينما لوحظ اختفاء حزمة انحناء (C-H) لحلقة البنزين في الالفا–ثلاسيميا. هذه الاختلافات تقودنا الى إمكانية استخدام طيفFTIR في تشخيص الثلاسيميا.

## *INTRODUCTION*

Thalassemia is a global public health problem (1). It is a hereditary anemia resulting from defects in hemoglobin production (2). Structural hemoglobin (Hb) variants typically are based on a point mutation in globin gene that produce a single amino acid substitution in a globin chain (3).Thalassemia results from quantitative reductions in globin chain deletion or from mutation that adversely affect the transcription or stability of mRNA products(3).Depending on which globin chain is ineffectively synthesized, thalassemia are classified into two types : α thalassemia and β thalassemia(4),(5). The two types of thalassemia can be diagnosed by special NA testing, the most common methods used for Hb A2 determination are elution after electrophoresis , micrography on DE-52 and HPLC(6).It should be pointed out that ,even if there are definite elution times of the peaks at HPLC or specific positions of the bands at electrophoresis , the precise identification of an hemoglobin variant must be performed by more complex methods and definitely by DNA analysis of α and β globin genes(6).
Infrared(IR) spectroscopy has become an ideal complementary analytical toll to diagnose various common hematological disorders, IR spectroscopic based techniques can be used to analyze DNA alteration , secondary structural changes in proteins , and to profile cellular lipids (7).
The aim of this study was to investigate the potential of Fourier Transform Infrared(FTIR) spectroscopy as a fast tool in the diagnosis and screening of α and β thalassemia.

## *MATERIALS AND METHODS*

**Subject**: the present study includes (23) patients with thalassemia (11: α thalassemia and 12: β thalassemia) were attending Ibn-Albaladi hospital in Baghdad city. As a control 12 healthy individuals were included in this study.

**Samples:** Blood samples into EDTA tubes(2ml) were taken from healthy donors and patients with thalassemia α and β, and stored at -20 until being used.

### Methods

**DNA Extraction**: DNA was extracted from whole blood using buffer A(Tris hydrochloride, sucrose, and triton x-100), buffer B [Tris hydrochloride, EDTA, urea, and sodium dodecyl sulfate(SDS)],and buffer C (Tris hydrochloride and EDTA) with two types of solvents depending on methods of (8).

**DNA Purification**: A spin column of (1×6 cm) of Sephadex G-25 was used for purification of extracted DNA from whole blood of control and patients with thalassemia depending on methods (8).

**FTIR Spectrum**: The FTIR spectra of DNA in pre and propurification of control and patients with thalassemia α and β were studied on Shimadzu Fourier Transform Infrared Spectrophotometer (FTIR-8400S).

## *RESULTS AND DISCUSSION*

The DNA was extracted from whole blood of control and patients with thalassemia α and β by mixed 5 ml of pool whole blood with lysis buffer to lyse the cells then the fraction was suspended in a buffer containing strong protein denaturating agent SDS and urea. The dissociated DNA was extracted twice with phenol/chloroform to remove most of the proteins. The purification of DNA from the crude extract was achieved using Sephadex G-25 column to remove the contaminating proteins. The concentrations of DNA were determined by measuring the absorbance (A) at 260 nm, also the A260/A280 ratios were determined to check for protein contamination (Table 1).

**Table (1): Purity and yield of DNA purification from whole blood of control and patients with thalassemia**

| | A260(nm) | A280(nm) | A260/A280 | Yield (µg/ml) |
|---|---|---|---|---|
| Control | 1.2455 | 0.7158 | 1.74 | 24.909 |
| α-thalassemia | 1.2427 | 0.6747 | 1.84 | 24.835 |
| β-thalassemia | 1.3796 | 0.6925 | 1.99 | 27.585 |

The DNA concentrations and absorbance ratios represent the average values calculated for 12 ml blood samples from the same blood pool, extracted in a single run (9).

The results of the present study indicated that the purity of DNA of control , α-thalassemia ,and β-thalassemia were 1.74,1.84, and 1.99 respectively. These results were in agreement with the general rule that any preparation of DNA with an A260/A280 greater than approximately 1.7 is called pure(10), (11).Also these results were in agreement with the results obtained by (8) who isolated DNA from whole blood of myocardial infarction patients for studies on the apolipoprotein B gene.

The definitive diagnosis of some hemoglobinopathies and thalassemia that involve combinations of genetic defects may require DNA analysis(12). From a molecular and biochemical perspectives, IR spectroscopy has been explored for hemoglobin

reactions and structures (13).Meanwhile the scope of IR spectroscopy has been extended to the study of clinically relevant proteins leukemia cells from healthy lymphocytes detection of drug resistant cell populations in leukemia patients(14) and monitoring of glucose fluctuations in diabetic patients(15). Liu *et.al.* reported that the IR spectra revealed changes in the secondary structures of hemoglobin from beta thalassemia patients compared with that from control(16).

Fourier transform infrared spectroscopy was used in the present study to investigate it as a fast tool in the diagnosis and screening of DNA thalassemia.

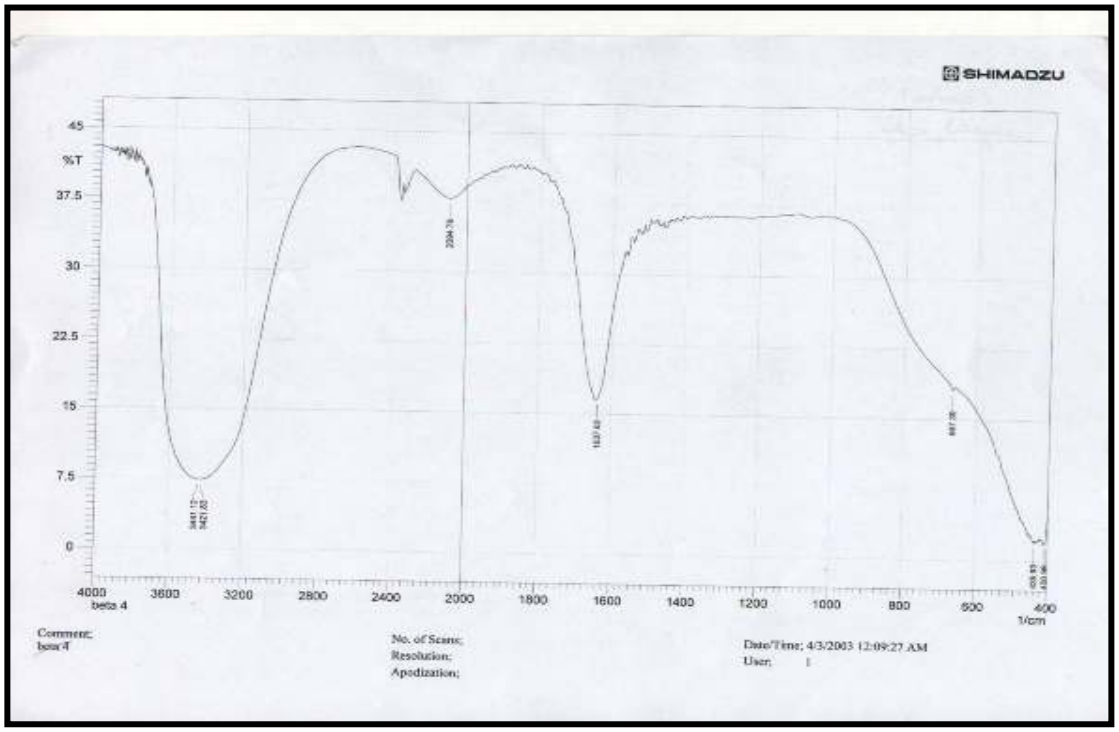
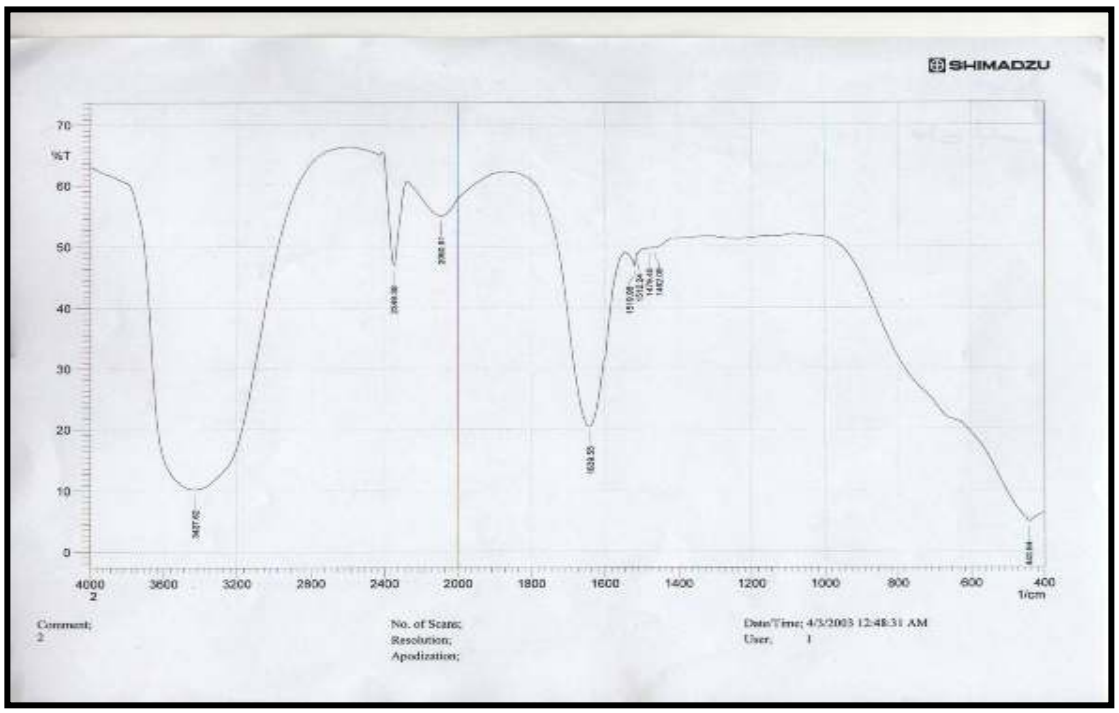



**Figure (1): FTIR spectrum of impurity DNA of control group.**




**Figure( 2): FTIR spectrum of impurity DNA of of α-thalassemia patients**

No differences in FTIR spectra were observed of impurity DNA of control (Figure 1) compared with those of α-thalassemia (Figure 2) and β-thalassemia (Figure3), while the FTIR spectra of purity DNA of control (Figure 4), α-thalassemia (Figure5) and β-thalassemia (Figure 6) indicated presence of differences in vibrations as shown in (Table 2).

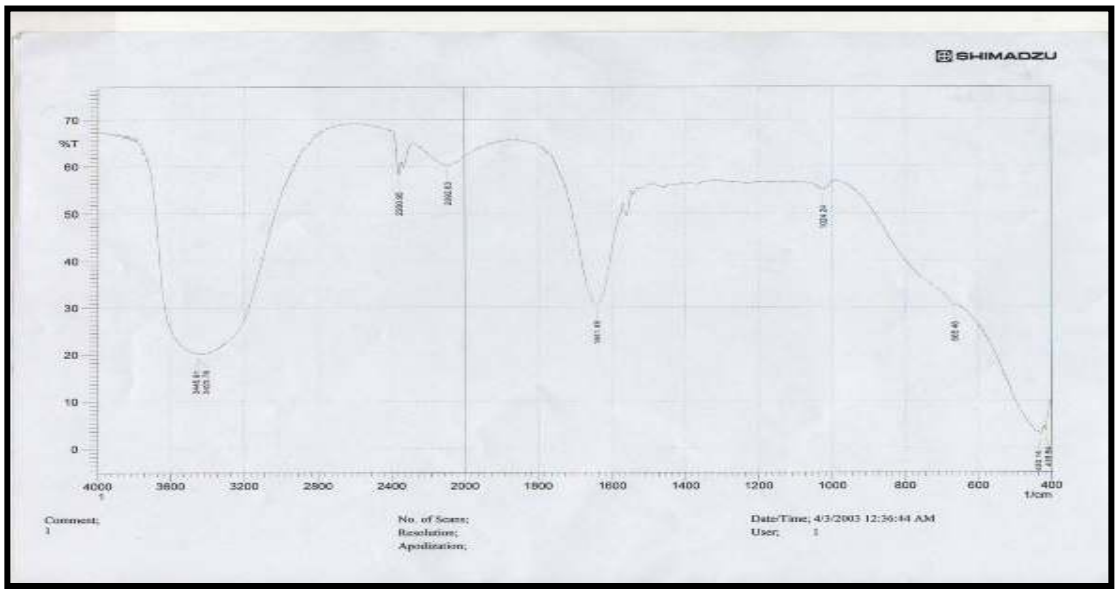
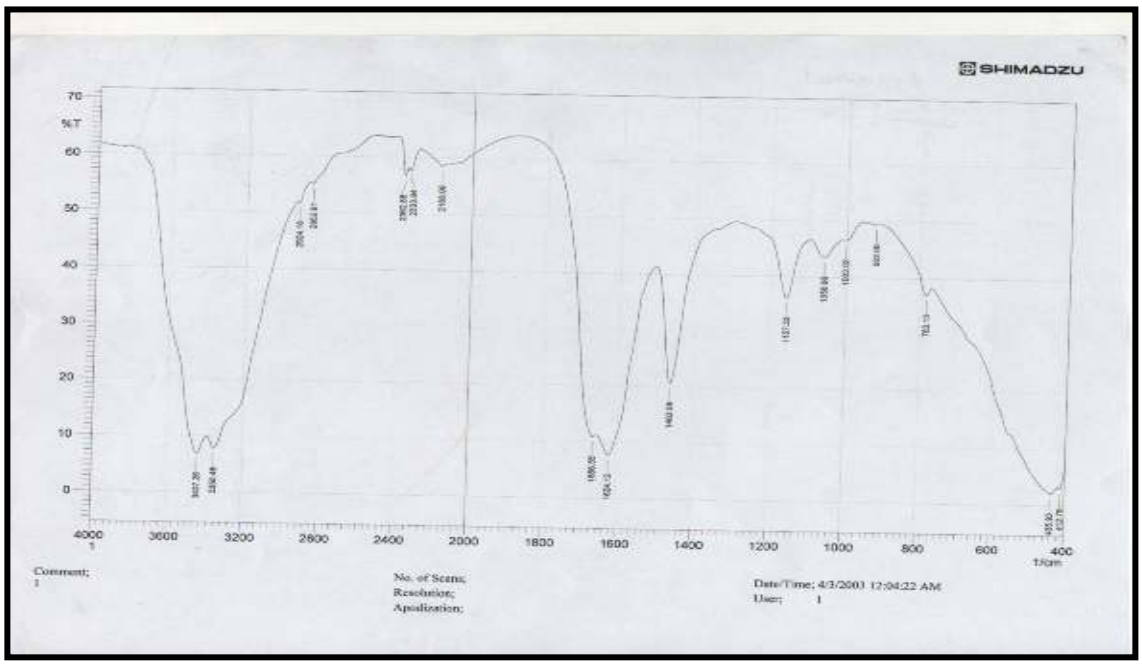



**Figure (3): FTIR spectrum of impurity DNA of of β-thalassemia patients**




**Figure( 4): FTIR spectrum of purity DNA of control group**

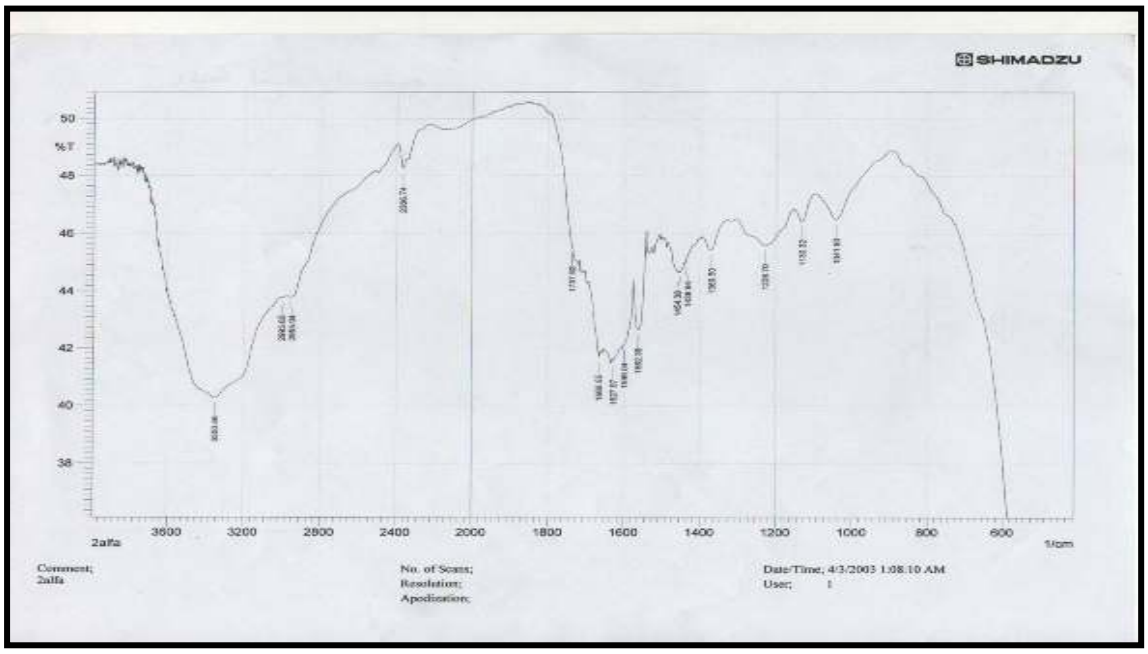
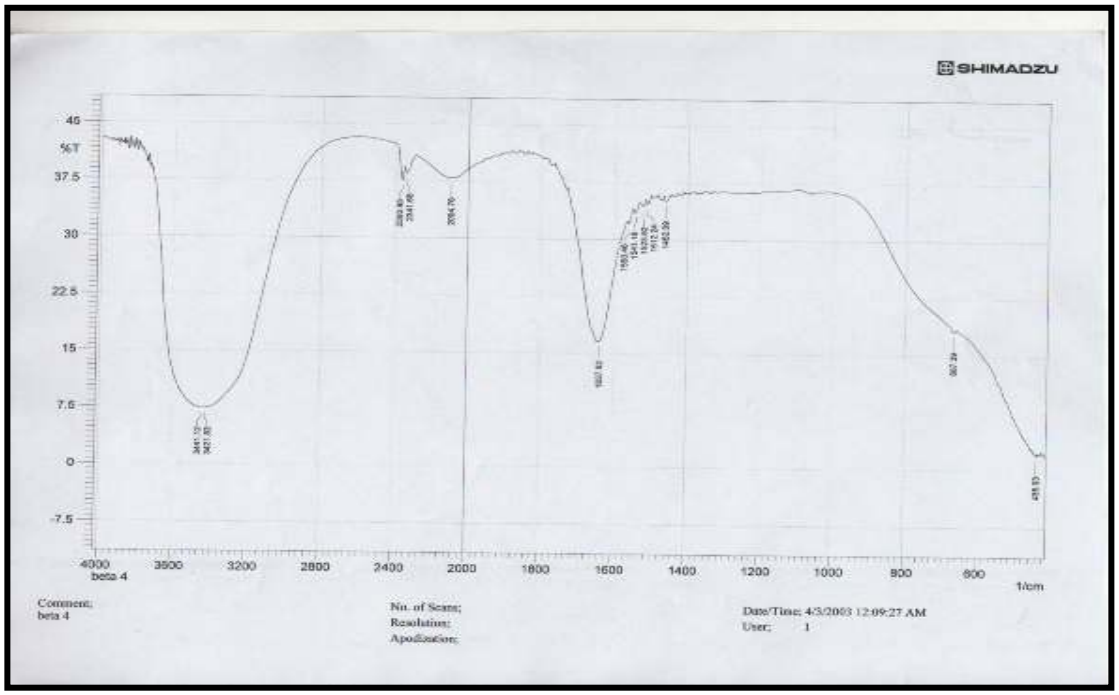



**Figure (5): FTIR spectrum of impurity DNA of α-thalassemia patients**




**Figure( 6): FTIR spectrum of impurity DNA of β-thalassemia patients**

**Table (2): FTIR spectra data of DNA control and thalassemia patients**

| Group | O-H str.cm$^{-1}$ | N-H str.cm$^{-1}$ | $CH_2$ str.cm$^{-1}$ | C=O str.cm$^{-1}$ | C-O-C str.cm$^{-1}$ | C-O str.cm$^{-1}$ | C-H phenyl bend.cm$^{-1}$ |
|---|---|---|---|---|---|---|---|
| Control | 3437 | 3350<br>1624 | 2852<br>2924 | 1666 | 1157 | 1058 | 783 |
| α-thalassemia | 3350 | 3350<br>1627<br>1592 | 2939 | 1666 | — | — | — |
| β-thalassemia | 3441 | 3421<br>1636 | — | — | — | — | 667 |

FTIR spectrum of purity DNA from whole blood of control group shown characteristic features: band at 3437 cm$^{-1}$, 3350 cm$^{-1}$ and 1624 cm$^{-1}$, 2924 cm$^{-1}$, 1666 cm$^{-1}$, 1157 cm$^{-1}$,1058 cm$^{-1}$, and 783 cm$^{-1}$ attributed to streching (O-H) , streching(N-H),streching($CH_2$),streching(C=O), streching (C-O-C), streching (C-O) ,and binding (C-H)phenyl respectively. In compared to the FTIR spectrum of purity DNA from whole blood of α-thalassemia , it have been observed disappearance of vibration bands of streching (C=O),streching(C-O-C), and binding (C-H)phenyl. Meanwhile the FTIR spectrum of purity DNA from whole blood of β-thalassemia compared with that of the control shown disappearance vibration bands of streching ($CH_2$), streching (C=O), streching(C-O-C) ,and streching(C-O) . Several differences of vibration bands of FTIR spectra were observed in the present study between α and β-thalassemias, these were

disappearance of vibration bands of streching ($CH_2$), and streching (C=O). On the other hand, disappearance of vibration band of binding (C-H) phenyl was observed in spectrum of purity DNA of α-thalassemia. These findings could help understanding the possible mechanism for screening and diagnosis thalassemia. These results were in agreement with the results obtained by (17) who demonstrated the possibility screening of β-thalassemia by FTIR (17). On the other hand, (18) reported that FTIR can be used to measure differences in protein structure which related to biological control mechanisms(18).

***REFERENCES***


1. Vichinsky EP.(2005) Changing patterns of thalassemia worldwide. Ann. NY. Acad. Sci. 1045:18-24
2. Rund D., and Rachmilewitz E.(2005) β-Thalassemia. N. Engl. J. Med. 353:1135-1146
3. Clarke GM., and Higgins TN.(2000) Laboratory investigation of hemoglobinopathies and thalassemia: review and update. Clin. Chem. 46(8):1284-1290.
4. Orathai T., Rattima J., Chanthonrot S.,and Sanguansermsri T.(2004) Diagnostic value of combined parameters for α- thalassemia screening in pregnant women. Nars. Uni. J. 12(2):19-24.
5. Ho PJ., and Thein SL.(2000) Gene regulation and deregulation. Blood Rev. 14:78-93.
6. Fucharon S., Winichagoon P., Wisedpankij R., Sae-Ngow B., Sriphanich R., Oncoung W., Muangsapaya W., Chowthaworn J, Kanokpongsakdi S., Bunyaratvej A., Piankijagum A., and **Dewaele** C.(1998) Prenatal and postnatal Diagnosis of thalassemia and hemoglopinopathies by HPLC. Clin. Chem. 44:740-748.
7. Kan ZL., Min Hs., and Henry HM.(2005) Molecular and chemical characterization of blood cells by infrared spectroscopy. Blood cells Mol. Dis. 35(3):404-412.
8. Adell K., and Ogbonna G.(1990) Rapid purification of human DNA from whole blood for potential application in clinical chemistry laboratories. Clin. Chem. 36(2):261-264.
9. Maniatis T., Fristsch EF., and Sambrook J.(1982) Molecular cloning: a laboratory manual. Cold Spring Harber, NY: Cold Spring Harber Laboratory Puplications:545.
10. Sambrook J., Fristsch EF., and Maniatis T.(1989) Molecular Cloning Edition, : Cold Spring Harber Laboratory Press, : Cold Spring Harber 2nd$^{ed}$:1-74.
11. Stffan RJ., Goksoyr J., Bej AK., and Atlas RM.(1988) Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. 54(12):2908-2915.
12. Bioshop ML., Fody EP., and Schoeff L.(2009) Clinical Chemistry Principles, Procedures, and Correlations.6$^{th}$ed. Lippincott Williams & Wilkins. Philadelphia:387.
13. Surewicz WK., and Mantsch HH.(1996) Infrared absorption methods for examining protein structure. VCH Publishers Weinheim. 135-158
14. Liu KZ, Schultz CP., Mohammad RM., Al-Katib AM., Johnston JB.,and Mantsch HH..(1998) Similarities chlordeoxyadenosin of lymphocytes from CLL patients and bryostain 1 treated WSU-CLL cells. Cancer. Lett. 127:185-193.
15. Petrich W., Dolenko B., Früh J., Ganz M., Greger H., Jacob S., Keller F., Nikulin AE., Otto M., Quarder O., Somorjai RL., Staib A., Werner G., and Wielinger H.(2000) Disease pattern recognition it with diabetes mellitus as an example. Appl. Opt. 39:3372-3379.
16. Liu KZ., Tsang KS., Li CK., Shaw RA., and Mantsch HH.(2003) Infrared spectroscopic identification of β-

thalassemia. Clin. Chem. 49:1125-1132.
17. Peng LX., Wang GW., Yao HL., Huang SS., Wang YB., Tao ZH., and Li YQ. (2009) FTIR-HTIR to identify beta- thalassemia and its mechanism study. Guang Pu Xue Yu Guang Pu Fen Xi. 29(5);1232-1236.
18. Alben JO., and Bare GH.(1978) Fourier transform infrared spectroscopic study of molecular interactions in hemoglobin. Appl. Opt.17(18):29850-29890.

# Isolation and Characterization of Phenazine Produced by SP9 Strain of *Pseudomonas aeruginosa*

**Leqaa M. aziz (1), Subhi J. Hamza (2), and Ibrahim A.K. Abdul- Rahman (3)**

*Dept. of Microbiology / College of Veterinary/ Al- Anbar University (1), (3) Dept. of Biotechnology / College of Sciences / Baghdad University- Iraq(2)*

*E-mail: dr_ibra65@yahoo.com*

***ABSTRACT***

A Pseudomonas strain, designated SP9, was isolated from clinical specimens and identified as *Pseudomonas aeruginosa* based on morphology; conventional biochemical and physiologic tests. One antimicrobial substances produced by this strain proved to be phenazine bio-product, based on TLC, HPLC and IR. For purpose of studying of the antimicrobial activity, gel filtration was employed in the purification of phenazine. The bio-product is effective against *Bacillus subtillus, Staphylococcus aureus*, *Staphylococcus epidermidis* , *Escherichia coli* and *Salmonella typhi* and *Candida albicans*.

***Key words**:* Phenazine, isolation, characterization, *Pseudomonas aeruginosa*.

**الملخص باللغة العربية**

عزلت الـــ *Pseudomonas*، SP9، من نماذج سريرية وشخصت على انها *Pseudomonas aeruginosa* اعتماداً على الاختبارات المظهرية، الفسلجية، الكيميائية الحيوية التقليدية. أنتجت هذه العزلة مادة ضد ميكروبية شخصت علـــى انها المنتج الحيوي الفينازين باستخدام التقنيات TLC، HPLC و IR. ولغرض دراسة النشاط الضد ميكروبي استخدم الترشيح الهلامي في تنقية الفينازين. وجد ان المنتوج الحيوي فعّالُ ضـــد؛ *Bacillus subtillus, Staphylococcus aureus*, *Staphylococcus epidermidis* , *Escherichia coli* *Salmonella typhi*, و *Candida albicans*.

## *INTRODUCTION*

*Pseudomonas aeruginosa* is a Gram negative bacteria that cause both acute and chronic diseases in susceptible patients population (1). *Pseudomonas aeruginosa* secrets numerous proteins and secondary metabolites, with many biological effects against pathogens which cause these diseases (20). Phenazine have been known as a groups of the most important metabolic products which produced by *Pseudomonas aeruginosa* (3). More than 100 different phenazines structural derivatives have been identified in nature, and over 6000 compounds that contain phenazine as a central moiety have been synthesized (4). These secondary metabolites are produced by a variety of bacteria, especially Pseudomonads and have been studied intensively virulence (5). Perhaps the most studied phenazine is pyocyanin which produced by *Pseudomonas aeruginosa*. Pyocyanin was isolated originally from patient wounds and subsequently demonstrated to be associated with infections caused by *Pseudomonas aeruginosa*. The presence is associated with high morbidity and mortality in Immune - compromized patients such as cystic fibrosis (6),(7). Phenazines are associated with antitumor activities (4), 8). Cells that are activity respiring, such as tumor cells, appear to be more susceptible to respiratory interference caused by phenazine compounds. Also, phenazine derivatives known to interfere with topoisomerase I and II activities in eukaryotic cells. Cancer cells, having high levels of both topoisomerases, are more susceptible to this interference. For example, active proliferation of human lymphocytes was inhibited by pyocyanin (9). The development of synthetic anticancer phenazine derivatives is an ongoing area of research aimed at combining known phenazine biological activities with increased target specificity to words cancer cells (10),(11). Phenazine compounds can be used to obtain board spectrum of antibiotic activity toward bacteria, fungi, and animal tissues (8). Phenazine derivatives were also chosen to reduce the use of chemical pesticides in agriculture (12). The studies showed that phenazine compounds also have antimicrobial activity against strains of *Bacillus subtilis*, *Candida albicans*, *Enterobacter aeruogenes*, and *Escherichia coli*. Phenazine also owing activity against *Mycobacterium tuberculosis* (13) and some plant pathogenic bacteria such as *Erwinia carotovora* and *Ralstonia solanacearum* (14).

The objectives of the present work is to isolation and identification of phenazines produced by *Pseudomonas aeruginosa* and study their activity against different microorganism.

## *MATERIALS AND METHODS*

### Production of phenazine product

*Ps. aeruginosa* were isolated by using cetrimaid agar. Cultures of *Ps. aeruginosa* were grown on King's A medium at 37°C for 11 days. 1ml of the suspension used to inoculate 100ml of Modification of Pseudomonas P medium (PsP) The composition of the modified medium is as follows: DL-alanin 2 g, Sodium citrate 10 g, Potassium sulfate 8.6 g, Potassium chloride 1.4 g, Magnesium sulfate 1.4 g, Dipotassium hydrophosphate 5 g. (15). All components were added to 1 liter of distilled water. pH was adjusted to 7.2-7.3 and sterilized by filtration through Millipore filters 0.45µm. The medium suspended as 100ml in a

250ml Erlyenmeyer flask, incubated at 37°C on rotary shaker at 110 rpm for 3 days.

**Bacterial strains**

*Bacillus subtillus*, *Candida albicans*, *Escherichia coli, Klebsiella spp., Pseudomonas aeruginosa*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Salmonella typhi*, were obtained from Baghdad university /college of science/ biotechnology department.

**Standardization of Phenazine**

Maximum absorbance of phenazine (sigma, U.S.A.) was determined by scanning of wavelength using UV scanning spectrophotometer. Chloroform was used as reference sample. Different concentration of phenazine were used (50, 100 , 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg/ml) for recording absorbance at 375 nm.

**Measurement of pigment concentration**

Dark – blue chloroform extract was measured by UV-spectrophotometic analysis at 690 nm. Pure chloroform was used as blank. Calculate of pyocyanin concentration was carried out as described by: (16)

The result multiple with molecular weight of pyocyanin (210.2) and the value in µg/ml (17).

$$\frac{\text{Luminary density of pyocyanin suspension in wave length 690 nm}}{4.3 \times 106} = \text{Pigment concentration}$$

**Extraction of phenazine**

*Ps. aeruginosa* isolate (SP9) was grown at 37 °C in PsP medium. The cultures were incubation in rotary shaker at 110 rpm for 3 days. Phenazine product was extracted employing the method described by Chancey *et al* and Kumaresan *et al* (18, 19). Five milliliters of each culture was centrifuged (5000 rpm) for 30 minutes, and the supernatant was acidified (pH 2) with concentrated HCl. Then 5 ml of benzene was added.
Samples were mixed for 1 hour and centrifuged. Four milliliters of the benzene layer was decanted and dried under air. Samples were resuspended in 1 ml of 0.1 N NaOH, and the absorbance at 375 nm was determined.

**Thin layer chromatographic method (TLC)**

The techniqe of TLC was used as described by (20) with the some modification. The sheet of silica gel 60f-254, was used for analyzing samples. Slotting line was marked 1 cm from bottom edge of the plate. A liquid of standard phenazine and sample dissolved in chloroform were spotted the plate was left few minutes to dry in dark condition before development. The TLC plates were developed in chloroform-methanol (9:1 v/v), until the solvent front reached a mark distance from the spotting line. The plate was removed and left to dry. The plate examined under UV light and calculated Rf values.

**High performance liquid chromatography (HPLC)**

Analysis was performed with a Shimadzu LC-2010 AHT liquid chromatography (Japan) with Reodyne 7125, 20µl injector, a Shimdzu SPD-2010 A UV-visible detector set at 375 nm and the column used (250×4.6 mm) C18, 5 µm particle size was used. The mobile phase was 100% acetonenitrile (CAN). The flow

rate was 0.5 ml.min$^{-1}$. The column temperature was maintained at 30 °C.

**Identification of phenazine compounds by Infra Red (IR)**

IR spectra of phenazine separated from isolate (SP9) culture was carried out by using FTIR spectrometer, Shimadzu (Japan) and compared with standard phenazine s described by Aunchalee, *et al*. (14).

**Gel filtration chromatography**

3 ml of crude phenazine was loaded into the, which packaged by sephadex G25, column. Then the column eluted with Tris buffer (0.01M with pH 7.5 contain 0.1% of Triton X100 and 0.25M sodium chloride). At flow rate of 1 ml/ 3min. In eluted fractions were detection of phenazine was carried out by employing UV-visible spectrophotometer at a wave length of 375 nm. Results were plotted as optical density and fraction number.

**Biological activity of phenazine antibiotic**

Determination of phenazine activity was done by using paper disk method: Filter paper (watman) discs 5.5 mm in diameter are prepared beforehand and sterilized by autoclave at 121°C for 15 min, then sterile paper disk was taken using forceps, and dipped in certain concentration of phenazine (50, 100 and 200 µg/ml) and placed on the surface of Moller-Hinten agar that incubated with certain microorganism. Then the plates incubated for 24 hour in 37°C. The inhibition zone around the disk was measured.

***RESULTS AND DISCUSSION***

**Identification of the isolates**

The isolates were identified as *Pseudomonas aeruginosa* using cultural and biochemical tests along with their ability to produce pyocyanin pigment .The testes were compared with schematic diagram proposed by Holt *et al* (21). Isolate SP9 was selected as the highest producer among other strains. The strain produce as much as 0.174 mg/ml.

**Separation and Identification of phenazine from cultural medium**

Separation and Identification of phenazine from cultural medium was carried as described in the material and methods. TLC was carried out using the solvent system chloroform: methanol (9:1 v/v) (see materials and methods). A band 2 with Rf 0.71 was identified as phenazine product (Fig 1). Biological activity when tested using ( 22).

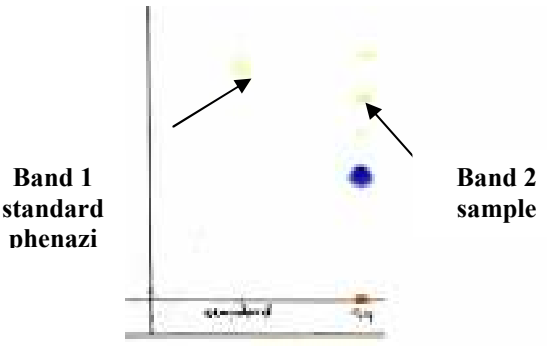



**Figure (1): Identification of phenazine by TLC**

**Identification of pheazine by HPLC**

A typical separation of phenazine was carried using HPLC system.
The presence of three peaks with a retention time of; 7.82 min, 7.95min, 8.3min in comparison with standard phenazine ( retention time of 7.9 min) were obtained. These results agree with that of Chang and Blackwood (27). The authors demonstrated the ability of *Pseudomonas aeruginosa* to produced many phenazine derivatives beside pyocyanin. Other workers reported that, strains of Pseudomonas aeruginosa can produce a variety of redox-active phenazine compounds included pyocyanin, phenazine-1-carboxylic acid (PCA), 1- hydroxy phenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN) (23, 24). Chin-A-Woeng *et al* (12) indicated that the approximate concentration of phenazine was calculated from figure 2 as 281µg/ml in comparison with
standard curve of phenazine product.

**Identification of pheazine by IR**

Further confirmation and identification of the metabolite separated from culture of SP9 was carried by IR. The spectrum shown in (Fig 3) indicate the presence of phenazine as shown in (Table 1). These results agree with that of Makarand *etal* (13).

**Purification of phenazine by Gel filtration**

Purification of phenazine was carried out by using gel filtration (sephadex G25) (25). The results of elution were shown in Figure 4. The eluents showed antimicrobial activity were pooled and concentration of phenazine was measured as descriped in the material and methods. The concentration of the phenazine was approximately 200 µg/ml.

**Antimicrobial activity of phenazine product**

Different concentrations of phenazine product were employed (50, 100 and 200 µg/ml) and tested against the following microorganisms: *Bacillus subtillus, Staphylococcus aureus* and *Staphylococcus epidermidis*, *Escherichia coli, Klebsella spp*., *Salmonella typhi, Pseudomonas aeruginosa* and *Candida albicans*. Results of the test is presented in Figure 6. phenazine product was active against all organisms except *Pseudomonas aeruginosa* and *Klebsiella spp*. The MIC of purified phenazine was found to be 200µg/ml. This concentration was more effective against *Staphylococcus aureus* and *Staphylococcus epidermidis* than *Escherichia coli* and *Salmonella typhi.*

Also antimicrobial activity against *Candida albicans* is more stronger than *Escherichia coli* and *Salmonella typhi* (26)
These results are in agreement with that results of (13). However others demonstrated that the activity of phenazine antibioties are concentration dependence (27).

**Table (1): Absorbation bands of FTIR for cycle compounds of phenazine antibiotic**

| compounds | H-O | C-H aromatic | C=N | C=C aromatic | C=O | C-O | C-N | ip O-H | ip C-H aromatic | oop C-H aromatic |
|---|---|---|---|---|---|---|---|---|---|---|
| **Standard phenazine** | 3475.73 | 3059.10 | 1627.90 | 1554.63 | 1469.76 | 1323.17 | 1284.59 | 1207.44 | 856.39 | 748.38 |
| **Sample** | 3475.73 | 2958.80 | 1635.64 | 1558.48 | 1458.18 | 1361.74 | 1211.30 | 1184.29 | 852.54 | 744.52 |

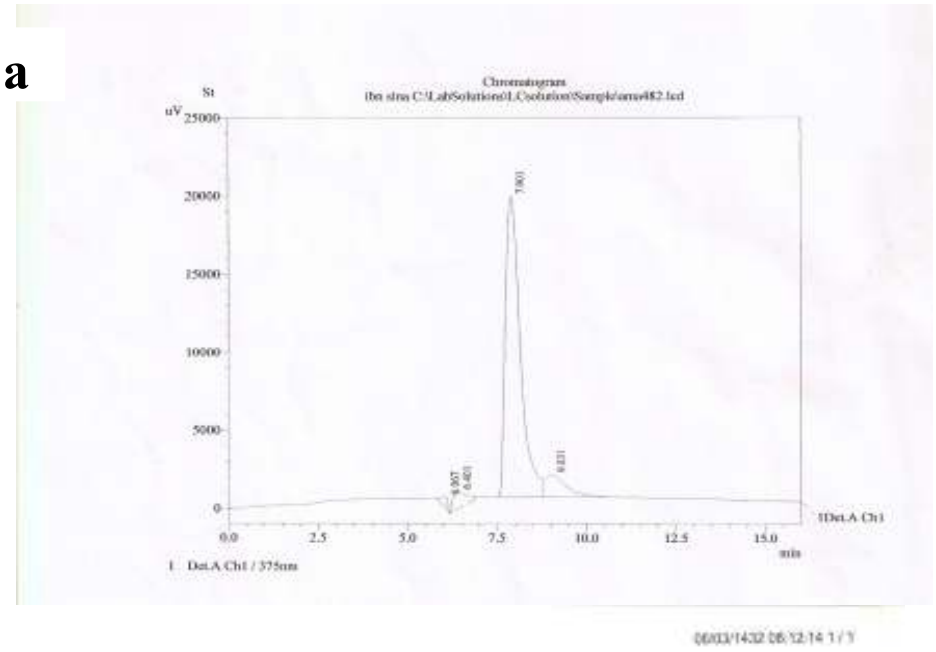
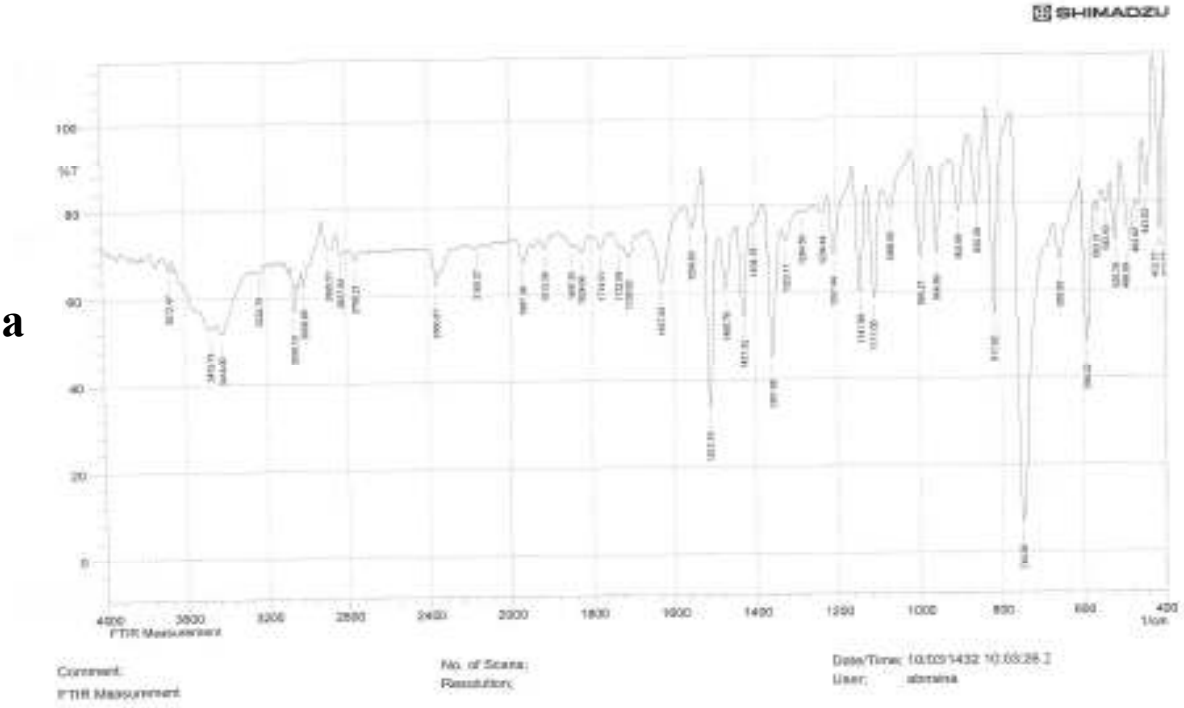



Figure (2): identification of phenazine by HPLC. a- standard phenazine. b- Sample of extract phenazine separation from SP9 isolate

a




b

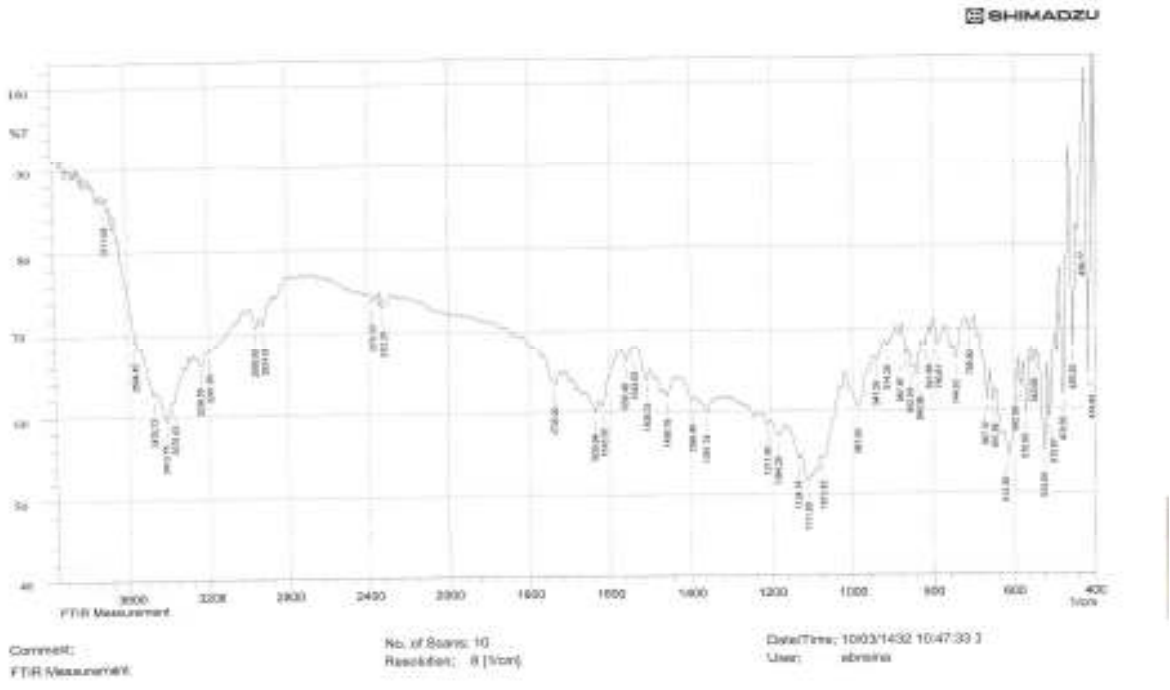



**Figure (3): FTIR measurements for: a- standard phenazine b- sample**

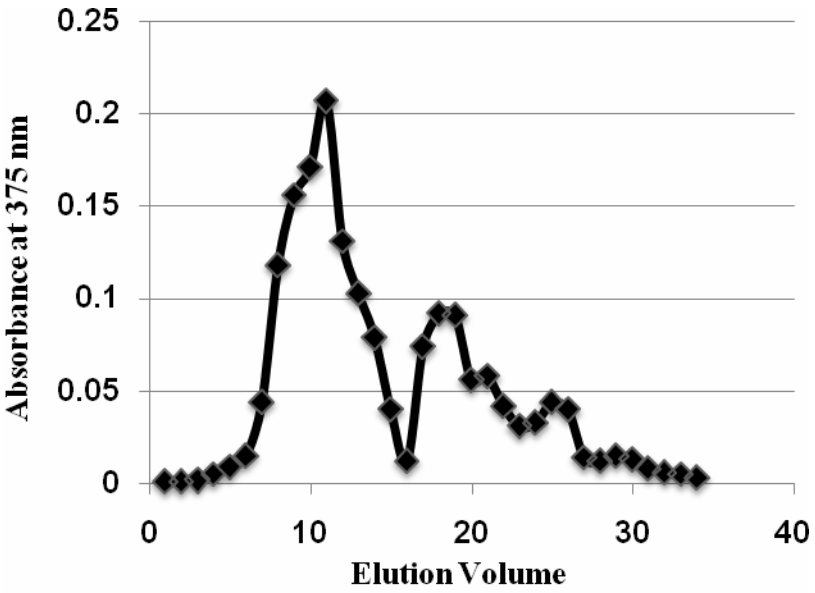



Fig (4) Gel filtaration of Phenazine extraction from SP9 isolate by sephadex G25

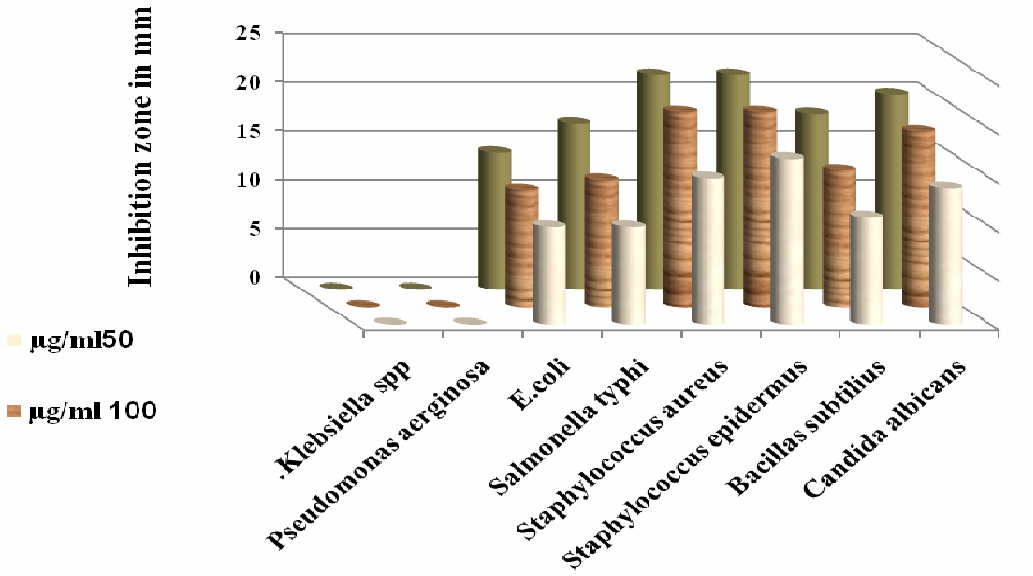



Fig (5): Antimicrobial activity of phenazine extraction from SP9 against positive and negative bacteria and yeast

***REFERENCES***


1. Wilson R., Dowling R. and Jackson A. (1996) The effects of bacterial products on air way cells and their function. Am.J.Respir.Crit.Care.Med 154:197-201.
2. Genevieve G., Sharief B., Sebastien R., Tjeerd V., Ben J. and Guigo V. (2006) Pip a novel activator of phenazine biosynthesis in *Pseudomonas chlororaphis*. J. bactriol. 188:8283-8293.
3. Collee J., Fraser A., Marmion B. and Simmons A. (1996) *Pseudomonas ,Stenotophanas, Burkholdria*. practical medical microbiology. 14th ed.
4. Mavrodi D., Blankenfeldt W., Thomashow L. and Mental M. (2006) Phenazine compounds in fluorescent *Pseudomonas* spp. Biosynthesis and regulation. Annual review of phytopathology 44:417-445.
5. Leland S. and Elizabeth A. (2010) Metabolism and function of phenazines in bacteria: impacts on the behaior of bacteria in the environment and biotechnology process. Appl. Microbiol. Biotechnol. 86:1659-1670.
6. Courtney JM.; Bradley J., McCaughan J., O'Connor TM., Shortt C., Bredin CP., Bradbury I. and Elborn JS. (2007) predictors of mortality in adults with cystic fibrosis. Pediatric pulmonology 42:525-532.
7. Murray TS., Egan M. and Kazmierczak BI. (2007) *Pseudomonas aeruginosa* chronic colonization in cystic fibrosis patient. Current opinion in pediatrics 19:83-88.
8. Laursen J. and Nielson J. (2004) Phenazine natural: biosynthesis, synthetic analogues and biological activity. Chem. Rev. 104: 1663-1685.
9. Sorensen R., Klinger J., Cash H., Chase P. and Dearborn D. (1983) In vitro inhibition of lymphocyte proliferation by Pseudomonas aeruginosa PAO1. Infec. and imm. 74:1673-1682.
10. Nakaike S., Yamagish T., Nanaumi K., Otomo S. and Tsukagoshi S. (2005) Cell-killing activity and kinetic analysis of a noval antitumor compound NC-190, a Benzo[α]phenazine derivative. Cancer sci. 83:402-409.
11. Hari N., Moorthy N., Karthikeyan C. and Trivedi P. (2009) Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. Sci. 325:1380-1384.
12. Chin-A-Woeng TFC., Bloembery GV. and Lugtenbery BJJ. (2003) phenazines and their role in biocontrol by *Pseudomonas aeruginosa*. New phytol. 157: 503-523.
13. Makarand R., Prashant DS., Bhushan L. and Sudhir B. (2007) Detection, isolation and identification of phenazine -1-carboxylic acid produced by biocontrol strains of *Pseudomonas aeruginosa*. J. Sci. indust.l res. 66:627-631.
14. Aunchalee N., Sukanya A., Chanokporn P., Paweena P., Saksit C. and Chalerm R. (2009) Synthetic, isolation of phenazine derivatives and their antimicrobial activities. Walailak. J.Sci. Tech. 6(1):78-91.
15. Ralph C. (1973) Studies on pigment production by *Pseudomonas aeruginosa*. A thesis of master. College of science. Taxas Tech university.
16. Frank LH. and DeMoss RD. (1959) On the biosynthesis of pyocyanin. J.Bacteriol. 77:776-782.
17. Al-Mahmdy L R. (2003). Isolation and identification of *Pseudomonas aeruginosa* wih the possibility of hypothetical map for its movement among the various sources. M.S.C thesis, College of science, Al-Mustansiriya university.
18. Chancey S T. , Wood D W. and Pierson III L S . (1999) Two component transcriptional regulation of N – Acyl – homoserine lactone production in *Pseudomonas aureofaciens* . Appl. Environ .Microbiol .65(6) :2294 – 2299.

19. Kumaresan K.,Subramanian M., Vaithiyanathan S., Sevagaperumal N., Gopal C. and Dilantha F. (2005) Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode. Archives of Phytopathology and Plant Protection 38(1): 69 – 76.
20. Gerene M., Shankar S., Krzysztof J., Yunxia O., George T. and Bradley E. (2003) Phenazine-1-carboxylic acid, a secondary metabolite of *Pseudomonas aeruginosa*, alters expression of immunomodulatory proteins by human air way epithelial cells. Am. J. Physiol. Lung cell Mol. Physiol. 285:584-592.
21. Holt JG., Krieg NR., Sneath PH., Staley JT. and William ST. (1994) Bergy,s Manual of Determinative Bacteriology. 9th edition.William and Wilkins Co. Baltimore. London.
22. Al-Tmemei MB. (2000) Isolation and identification of phenazine antibiotic produced by *Pseudomonas aeruginosa*. A thesis of master-college of science-Al-Nahreen university.
23. Budzikiewicz H. (1993) Secondary metabolites from fluorescent Pseudomonads. FEMS Microbiol. Rev. 104: 209-228.
24. Smirnov V. and Kiprianova E. (1990) Bacteria of *Pseudomonas* genus. Naukova Dumka. Kiev. USSR. 100-111.
25. Leisinger T., and Margraff R. (1979) Secondary metabolites of the fluorescent pseudomonads. Microbiol. Rev. 43:422-442.
26. Stephen S., Baron E., and John J. (1981) Antibiotic action of pyocyanine antimicrobial agent and chemotherapy. 20(5): 814-820
27. Chang P. and Blackwood A. (1969) Simutaneous production of three phenazine pigments by *Pseudomonas aeruginosa*. Can. J. Microbiol. 15:439-444.

# Few factors affecting the buoyancy and release of theophylline anhydrous from hydrodynamically balanced delivery system

**Nidhal K. Marei, Masar Mohsen, Mohammed Neama, and Noora Zuwar**

*College of Pharmacy / Al- Mustansirya University- Iraq*

*E-mail: dr_nidhal_khazaal@yahoo.com*

***ABSTRACT***

This study was carried out to find the effect of different polymers which are low grade of hydroxyl propyl methyl cellulose, (HPMC), sodium carboxy methyl cellulose (NaCMC), pectine , methyl cellulose (MC) and the high grade of hydroxyl propyl methyl cellulose and different amounts of these polymers on the release of theophylline using hydrodynamically balanced system as floating sustained release drug delivery system. The effect of these polymers on the lag time was studied and all the polymers gave a lag time less than 1 minute except methyl cellulose gave a longer time and changing the amount of sodium bicarbonate had impact the lag time and as the amount of sodium bicarbonate increased the lag time decreased and increasing the polymer amount led to decrease the lag time.
In all formulas, the polymer contents have been changed from 100 to 150 and 200 mg and just sodium carboxy methyl cellulose gave a significant effect as a retardant. High grade of HPMC gave highly significant effect in comparison with other polymers. The combination of carbomer with NaCMC also showed significant effect on the release of theophylline.
These findings are suggesting a promising hydrodynamically balanced delivery system for theophylline as a sustained release dosage form.

**الملخص باللغة العربية**

أجريت هذه الدراسة لايجاد تاثير تنوع البوليمرات وتركيزها وهي :المستوى القليل من الهيدروكسي بروبيل مثيل سليلوز ، و الصوديوم كاربوكسي مثيل سليلوز ، والبكتين والمثيل سليلوز ، والمستوى العالي من الهيدروكسي بروبيل مثيل سليلوز على تحرر الثيوفيلين من الحبوب العائمة باستخدام تقنية فاعلية التوازن المائي لإعطاء التأثير الطويل الأمد و كقرص عائم.
وقد تمت دراسة تأثير هذه البوليمرات على الوقت المتأخر وكل البوليمرات أعطت وقت متأخر اقل من دقيقة عدا المثيل سيليلوز أعطى وقت أطول. كما كان لتغيير تركيز بيكاربونات الصوديوم تأثيرا واضحا على الوقت المتأخر للطوفان ، فبزيادة التركيز لمادة البيكاربونات الصوديوم قل الوقت المتأخر للطوفان وأيضا زيادة كمية البوليمرات قللت الوقت المتأخر للطوفان. و قد تم تغيير تركيز البوليمرات من 100 ملغم إلى 150 و 200 ملغم وكان فقط تأثير الصوديوم كاربوكسي مثيل سليلوز واضح كمقلل لتحرر الثيوفيلين. والمستوى العالي للهايدروكسي بروبل مثيل سليلوز اعطى تأثيرا واضحا بالمقارنة مع باقي البوليمرات . مزج الكاربومير مع الصوديوم كاربوكسي مثيل سليلوز وأعطى تأثيرا عاليا لتحرر الثيوفيلين. هذه النتائج تقترح إمكانية تحضير الثيوفيلين باستخدام تقنية فاعلية التوازن المائي لتحضير الثيوفيلين كشكل دوائي طويل الامد.

## *INTRODUCTION*

Rapid and unpredictable gastrointestinal transit could result in incomplete drug release from the device above the absorption zone leading to diminished efficacy of the administered dose (1). Gastroretentive systems can remain in the gastric region for several hours and hence can significantly prolong the gastric residence time of drugs. Prolonged gastric retention improves bioavailability, reduces drug waste, and improves solubility for drugs that are less soluble in a high pH environment of small intestine (2). It has applications also for local drug delivery to the stomach and proximal small intestine (3). Therefore, different approaches have been proposed to retain the dosage form in the stomach. Those approaches include the use of high density dosage form (4); concomitant administration of drugs or excipients, which slows the motility of stomach (5); using bioadhesive or mucoadhesive dosage form (6). But the simplest and possibly the most elegant way to improve drug absorption is to hold a drug delivery system above the absorption window, because most absorption windows are located in the proximal small intestine (duodenum) and the most effective strategy will be to hold the formulation in the stomach. When a drug is formulated with gel forming hydrocolloid and carbon dioxide generating agents, it swells in the gastric fluid as it gets contact with the aqueous medium. Formation of CO2 and entrapment of that gas into the polymeric gel causes swelling of the dosage form resulting a bulk density less than 1. It then remains buoyant in the gastric fluid, resulting a prolonged gastric residence time. This floating dosage form is well known as a Hydrodynamically Balanced System (HBS) (7).

Theophylline is the most common orally administered bronchodilator. It is the standard drug substance for the treatment of bronchial asthma, chronic obstructive airway disease, status asthmatic's, bronchial emphysemas and shortness of breath caused by right heart failure due to obstructive pulmonary disease (8). Peak serum theophylline concentration occurs 1-2 h after ingestion of liquid preparations, capsules and uncoated tablets and between 4 to 12 hrs. after ingestion of sustained release preparations. Optimum serum theophylline concentration ranges from 10-20 µg /ml (9-13).

The aim of the present study was to prepare and characterize extended-release floating theophylline matrix tablets using many hydrophilic cellulose derivatives such as low and high grade of hydroxy propyl methyl cellulose, sodium carboxy methyl cellulose, pectin, methyl cellulose.
Investigations showed the effect of types and amount of different polymers on the buoyancy (lag time and floating period) and release (rate and mechanism) of theophylline from the prepared tablets. The study also involved the effect of carbomer amount (as a retardant agent) on the release of theophylline from the selected formula. The effect of sodium bicarbonate concentrations on the buoyancy of the prepared floating tablet is also studied. The results were evaluated by Kors mayer peppas equation to propose the mechanism.

## *MATERIALS AND METHODS*

### Materials:

Theophylline was a gift from AL – Hukamaa drug industry ( Mosul-Iraq) while all the polymers were purchased (high and low grade HPMC Seppic Japan), (carboxy methyl cellulose and methyl cellulose by BDH chemical LTD.Pool England),( Carbomer 940 by Goodrich, U.S.A.) (pectin from SDI –Samarra drug industry-Samarra-Iraq).

### Methods:

### Preparation of floating tablets of theophylline

The active ingredient (100 mg) and other excipients (sodium bicarbonate,PVP, magnesium stearte in addition to avicel pH 101 to keep the weight of final tablets within certain limits)were accurately weighted according to the formulations in table 1. Particular attention has been given to ensure thorough mixing and phase homogenization. The appropriate amounts of the mixture were accurately weighted in an electronic balance for the preparation of each tablet and finally compressed using 9 mm flat face punch aTDP-single punch tablet machine(Shanghai TainHe Pharmaceutical machinery Co. ltd) using direct compression method and the hardness of all tablets are fixed at 5 ton\cm$^2$. Before compression, the surfaces of the die and punch were lubricated with magnesium stearate. All the preparations were stored in airtight containers at room temperature.

### Determination of in vitro floating lag time

The in vitro buoyancy was determined by floating lag time, according to the method described by Rosa and coworkers (1994). The tablets were placed in a 100 ml beaker containing 0.1N HCl. The media was kept in a stagnant condition and the temperature was maintained at 37$^o$C. The time required for the tablet to rise to the surface and float was determined as floating lag time (14).

### Effect of types and amount of polymers:

Different types of polymers; low grade of hydroxyl propyl methyl cellulose (HPMC), sodium carboxy methyl cellulose(NaCMC), pectine , methyl cellulose (MC) and the high grade of hydroxyl propyl methyl cellulose were used. Each polymer used in different amount 100 mg, 150 mg and 200 mg to study their effect on the buoyancy ( lag time ), floating period and drug release (formula 1 to formula 15).

### Effect of sodium bicarbonate on lag time

Sodium bicarbonate amount has been changed from 25 mg in the selected formula 10 to 50 mg and 75 mg in formulas 16 and 17 respectively.

### Effect of carbomer as a retardant

Carbomer was added as a retardant with different amounts 19 mg and 28.5 mg per tablet as in formulas 18 and 19 respectively.

### Dissolution study

The release of theophylline from floating tablets was determined by using Dissolution Tester USP XXII in paddle method. The dissolution test was performed using 900ml 0.1N HCl

solution at 37°C ± 0.5°C temperature and at 50 rpm. At 15 , 30 minutes and at every 1 hour interval samples of 5ml were withdrawn from the dissolution medium and that amount was replaced with fresh medium to maintain the volume constant till the end of experiment period (8 hours). The samples were filtered and diluted to a suitable concentration with 0.1 N HCl solution. Absorbance of theophylline was measured at 273nm using a UV-165-OPC spectrophotometer (Shimadzu, Japan). Cumulative percentage drug release was calculated.

**Kinetic modeling of drug release**

To study and characterize the drug release mechanisms from formulas, the Korsmeyer –Peppas model was applied:

$M_t / M_\infty = k \times t^n$

Where Mt is the amount of released drug at time t, M∞ is the overall amount of the drug, k is the constant incorporating structural and geometric characteristics of the controlled release device, and n is the release exponent indicative of the drug release mechanism. The rate constant *k* and the diffusional exponent *n* can be obtained from the intercept and the slope of a plot of *ln*$M_t / M_\infty$ versus *ln* t respectively.

When n ≤0.45 corresponds to a Fickian diffusion, and if 0.45<n<0.89 corresponds to anomalous transport, n ≥ 0.89 indicates to a zero order or case II transport .Anomalous transport is due generally to the swelling of the system in the solvent before the release takes place in addition to polymer relaxation, while case II represents polymer relaxation (15), (16), (17).

**Statistical analysis**

Student t-test was used to determine the relation between 2 parameters.

***RESULTS AND DISCUSSION***

***In vitro buoyancy study***

Formulations were evaluated for in vitro buoyancy and all formulations had floating lag times below 1 minute except formulas 10 and 11 where methyl cellulose used and the lag time increased to 4 and 5 minute and this may be due that methyl cellulose does not swell in stomach environment as much as in the intestinal fluid (18). Similar impact had gotten with Metronidazole floating tablet using methyl cellulose (19).

Floating lag time was reduced due to increasing the amount of polymers as shown in figure 1 and table 1, this may be due to the fact that increasing the amount of polymers caused rapid formation as well as entrapment of CO2 gas into the hydrophilic polymeric gel which eventually resulted in reduction of floating lag time. From the above results formula 10 was selected to evaluate the effect of sodium bicarbonate in different amount. It was found that increasing sodium bicarbonate amount from 25 mg (formula10) to 50 mg ( formula 16) and 75 mg (formula 17) led to decrease the lag time clearly as shown in figure 8 and this may be due to formation of large quantities of bubbles inside the tablet.

**Table (1) Composition of different formulations of floating tablets**

| Formula no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Theophylline anhydrous | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Avicel PH 101 | 100 | 50 | - | 100 | 50 | - | 100 | 50 | - | 100 | 50 | - | 100 | 50 | - | 75 | 50 | 81 | 71.5 |
| NaHCO3 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 50 | 75 | 25 | 25 |
| P.V.P | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 |
| Mg. stearte | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| HPMC, low grade | 100 | 150 | 200 | - | -- | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| NaCMC | - | - | - | 100 | 150 | 200 | -- | - | - | - | - | - | - | - | - | - | - | - | - |
| pectine | - | - | - | - | -- | -- | 100 | 150 | 200 | - | - | - | - | - | - | - | - | - | - |
| Methyl cellulose | - | - | - | - | - | - | - | - | - | 100 | 150 | 200 | - | - | - | 100 | 100 | - | - |
| HPMC high grade | - | - | - | - | - | - | - | - | - | - | - | - | 100 | 150 | 200 | - | - | - | - |
| Carbomer 934 | - | - | -- | - | - | - | - | - | -- | - | - | - | - | - | - | - | - | 19 | 28.5 |
| NaCMC | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 100 | 100 |

***Effect of type and amount of polymers on release of theophylline and floating period***

Table (2) shows that changing in the amount of the polymers were studied from all the prepared formulas. This effect was shown clearly in the figures 2,3,4,5 and 6.

The release of theophylline from all prepared formulas is shown in table (2) Effect of changing the amount of polymers used is shown in figures 2-6.

The percent released of theophylline was increased abruptly at the initial level within first 30 minutes and this may be due to the presence of soluble component sodium bicarbonate in the formulations resulting in increase in drug release rate this possibly due to the formation of channels which stimulated water penetration into the inner part of the matrix and thus exposed of new surfaces of tablet matrix to the dissolution medium (20).

The effect of increasing the amount of low grade of HPMC as shown in figure -2 from 100, 150 to 200 in formulas (1,2 and 3) led to decrease in releasing theophylline 76.1%, 74% and 67.9% respectively and it was not significant ($p>0.05$) and their floating periods were 5 hours while their release stayed to the end of experiment period, and this may be due to that tablet was disintegrated to small floating pieces with gel forming as a small unit of the main tablet.

On the other hand increasing NaCMC amount as shown in figure-3 (in formulas 4,5 and 6) has a significant affect($p< 0.05$) on decreasing the release of theophylline ( 80%, 53.6% and 52.5%) respectively and the floating periods were 6 hours to formulas 4 and 5 while formula 6 was 7 hours their release stayed for the end of experiment period. This is also due to disintegration of the tablet to small floating pieces with gel forming as a small unit of the main tablet.

**Table (2): Floating lag time, period and release parameters of floating tablets of theophylline**

| Formulas | Lag time | Floating period | % release | Rate (ln k) | N |
|---|---|---|---|---|---|
| F1 | 1 sec | 5 hours | 76.1 | 4.24 | 0.034 |
| F2 | 1 sec | 5 hours | 74 | 4.14 | 0.073 |
| F3 | 1sec | 5 hours | 67.9 | 4.15 | 0.020 |
| F4 | 25 sec | 6 hours | 80 | 4.23 | 0.063 |
| F5 | 12 sec | 6 hours | 53.6 | 3.64 | 0.145 |
| F6 | 6 sec | 7 hours | 52.5 | 3.69 | 0.131 |
| F7 | 1 sec | 2 hours | 100 | 3.86 | 0.073 |
| F8 | 1 sec | 2 hours | 100 | 3.82 | 0.054 |
| F9 | 1 sec | 2 hours | 100 | 3.8 | 0.042 |
| F10 | 5 min | 4 hours | 100 | 3.70 | 0.349 |
| F11 | 4 min | 5 hours | 73 | 4.02 | 0.085 |
| F12 | 30 sec | 5 hours | 70.5 | 4.04 | 0.094 |
| F13 | 11 sec | 8 hours | 42 | 3.03 | 0.337 |
| F14 | 5 sec | 8 hours | 39.4 | 2.44 | 0.631 |
| F15 | 4 sec | 8 hours | 33.4 | 1.91 | 0.549 |
| F16 | Less than 1 sec | 2 hours | 100 | 4.11 | 0.304 |
| F17 | Less than 1 sec | 0.5 hours | 100 | --- | ------ |
| F18 | 10 sec | 8 hours | 51 | 3.65 | 0.125 |
| F19 | 10 sec | 8 hours | 48.1 | 3.30 | 0.278 |

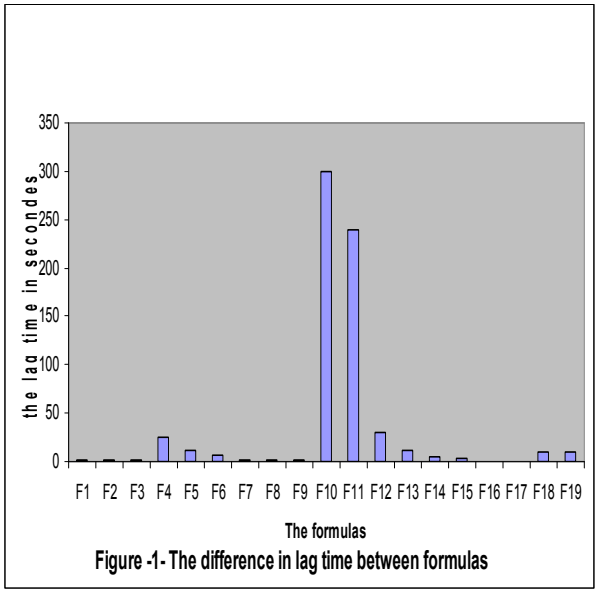



Figure -1- The difference in lag time between formulas

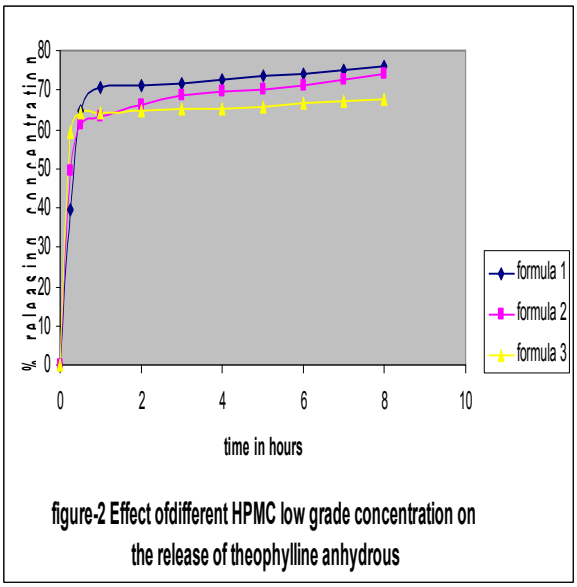



figure-2 Effect ofdifferent HPMC low grade concentration on the release of theophylline anhydrous

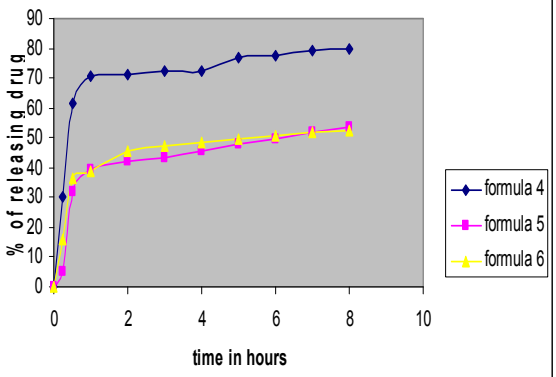



figure-3Effect of different NaCMC concentration on the release of theophylline anhydrous

Figure-4 shows the effect of increasing pectin amount which was not significant ($p>0.05$) on the release of theophylline in formulas 7, 8 and 9 and the floating of tablets were continued for 2 hours then disintegration happened and within 5 hours 100% of the drug released. This possibly due to the fact that pectine is completely soluble in water and form a viscous opalescent colloidal solution which flows readily (18).

Effect of increasing the amount of methyl cellulose is shown in figure-5 which was not significant ($p>0.05$) as in formulas (10, 11 and 12). Floating period of formulas 10 was 4 hours and after 6 hours disintegration happened to give 100% release of the drug while floating period of formulas 11 and 12 was 5 hours and they disintegrated to small floating pieces with slow down release where 73% and 70.5% release obtained after 8 hours of the run.

Effect of high grade HPMC (formulas 13, 14 and 15) is not significant ($p>0.05$) on the release of the drug. In comparison to other polymers the release has a highly significant ($p< 0.01$) effect on slowing down the release of theophylline and this could be due to that the high grade of HPMC has a greater clarity and fewer undispersed fibers which gives a prolong action of forming gel barrier and promoting floating period to 8 hours (18). This finding was in agreement with other researchers (21), ( 22), (23). The release after 8 hours of floating was 42%, 39.4% and 33.4% respectively as shown in figure-6.

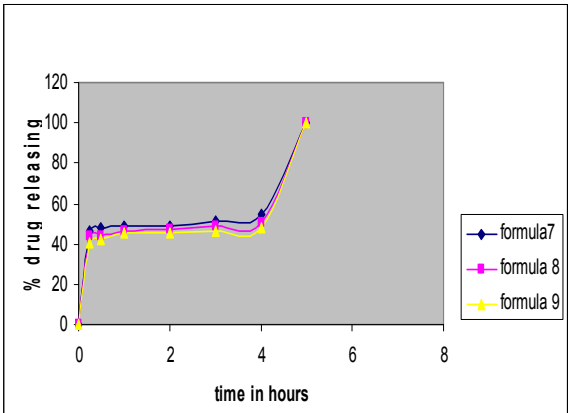



figure-4 Effect of different Pectin concentration on the release of theophylline anhydrous

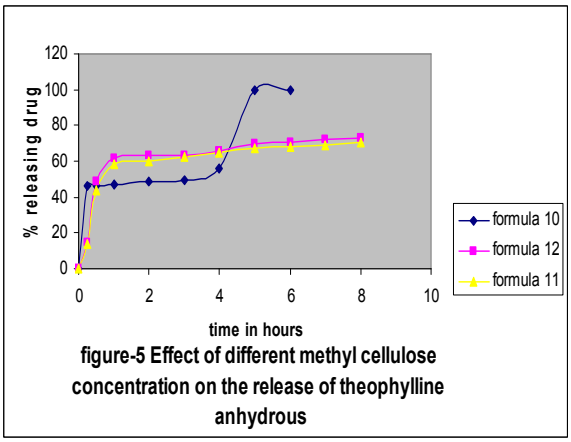



figure-5 Effect of different methyl cellulose concentration on the release of theophylline anhydrous

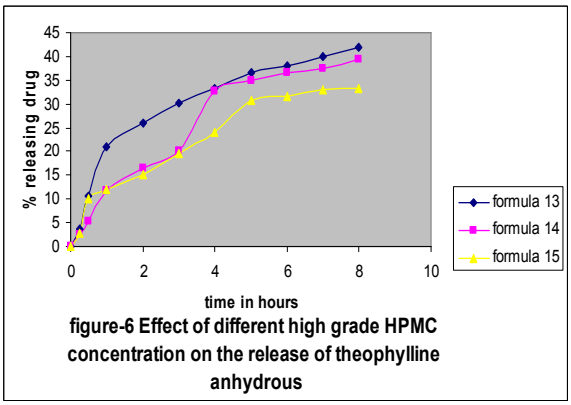



figure-6 Effect of different high grade HPMC concentration on the release of theophylline anhydrous

In corporation of carbomer as retardant together with NaCMC led to decrease the release from 80% (formula 4) to 51% (formula 18) and 48% (formula 19) with floating period 8 hours as shown in figure -7.

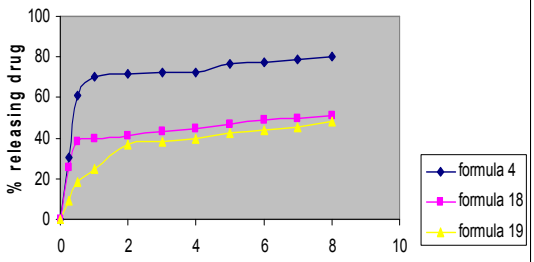



figure-7 Effect of different concentation of carbomer on the release of theophylline anhydrous on formula 4

The impact of carbomer was significant ($p<0.05$) as a retardant and delayed the release after 8 hours of floating and this slow release may be of possible rapid surface hydration, which resulted in its swelling and the consequent formation of a gel layer, this layer slowly dissolved and eroded away exposing a new gel layer and this was going slower (led to slower drug release) by increasing the concentration of the polymer (24).

So, it is observed that incorporation of carbomer causes better retardation of theophylline release than increasing amount of NaCMC in the formulas.

### *Kinetic study*

All formulas as shown in figure 8 gave a fickian diffusion according to equation Korsmeyer-Peppas equation where n values are less than 0.45 except formulas 14 and 15 which gave more than 0.45, and this is a great matching with a goal of hydrodynamically balanced floating formulation. Where, a gel barrier is formed around the tablet and once the tablet touched the dissolution media and this led to entrapment of CO2 bubbles in the tablet which is followed by floating. On the other hand, the formulas 14 and 15 showed non fickian diffusion that means anamolous transport which considered swelling of the system in the solvent before the release takes place in addition to polymer relaxation that led to retarding the release with increasing of the concentration of high grade of HPMC. Similar results were obtained for diltiazem HCL from matrix tablet containing HPMC (25).

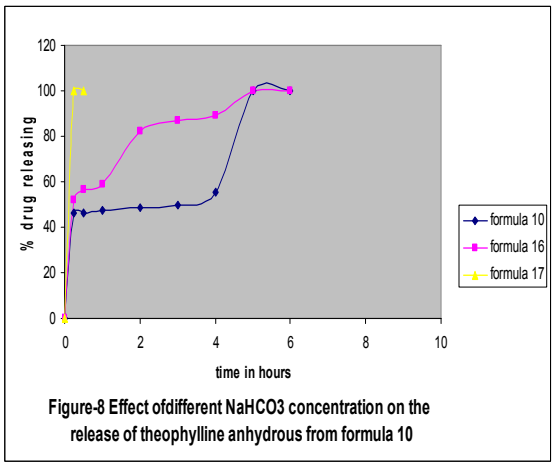



Figure-8 Effect ofdifferent NaHCO3 concentration on the release of theophylline anhydrous from formula 10

### *CONCLUSION*

From the above results, it was found that hydrodynamically balanced system formulations for theophylline showed that using high grade HPMC gave a lag time less than one minute and floating period 8 hours and slowed the release to 33.4%. Also, incorporation of carbomer with NaCMC showed short lag time, long floating period and retarding the release of drug to 48%. This suggest that the applications of hydrodynamically balanced system can be applied to obtain a promising floating sustained release tablet of theophylline.

***REFERENCES***


1. Iannuccelli V, Coppi G, Bernabei MT and Cameroni R(1998) Air compartment multiple-unit system for prolonged gastric residence. Part I. Formulation study. *Int. J. Pharm*. 174: 47-54.
2. Ponchel G., and Irache JM. (1998) Specific and nonspecific bioadhesive particulate system for oral delivery to the gastrointestinal tract. *Adv. Drug Del.Rev.* 34: 191-219.
3. Deshpande AA., Rhodes CT., Shah NH., and Malick AW. (1996) Controlled-release drug delivery systems for prolonged gastric residence: an overview. *Drug Dev.Ind. Pharm*. 22: 531-539
4. Singh BN., and Kim KH. (2000) Floating drug delivery system: An approach to oral controlled drug delivery via gastric retention. *J. Control. Release,* 63: 235-259.
5. Moes AJ. (1993) Gastroretentive dosage forms. *Crit. Rev. Ther. Drug Carrier Syst.* 10(2): 143-159.
6. Akiyama Y., Nagahara N., Nara E., Kitano M., Yamamoto I., Azuma J., and Ogawa Y. (1998) Evaluation of oral mucoadhesive microspheres in man on the basis of the pharmacokinetics of flurosemide and riboflavin, compounds with limited gastrointestinal absorption sites. *J. Pharm. Pharmacol.* 50: 159-166.
7. Uzdemir N., Ordu S., and Ozkan Y. (2000) Studies of floating dosage forms of furosemide: *in vitro* and *in vivo* evaluation of bilayer tablet formulations. *Drug Dev. Ind. Pharm.* 26: 857–866.
8. Technical Information (2009) Theophylline Anhydrous,Supersedes issue dated June- 2009-EMP 030749e-07/ 1 of 16
9. Dollery C.(1991) Therapeutic drugs. Edinburgh: Churchill Livingston; 101.
10. Reynold JE.(1991) Martindale: The extra pharmacopoeia. London: The Pharmaceutical Press:621
11. Rowland M., Tozer TN.(1996) Clinical pharmacokinetics concepts and applications. 3rd ed. New Delhi: B.I.Waverly Pvt.Ltd; 96-102
12. Satoskar RS., Bhandarkar SD., Ainapure SS.(1997) Pharmacology and pharmacotherapeutics. Revised 15th ed. Mumbai: Popular Prakashan:46-48
13. Rall TW.(1990) Drugs used in the treatment of asthma. Goodman and Gillman's the pharmacological basis of therapeutics. 8th ed. New York: Pergamon Press:618–37.
14. Rosa M., Zia H., and Rhodes T. (1994) Dosing and testing *in-vitro* of a bioadhesive and floating drug delivery system for oral application. *Int. J. Pharm*. 105: 65-7
15. Ibraheem B., AL-Laham A. (2009) Comparison Study of Carbopol and Cellulose Derivatives Polymers Effected in Sustained Drug Release from Matrix Tablets , Ara. J.pharm.,;3(9):11-24.
16. Mehrgan H., Mortazavi AS. (2005) The Release Behavior and Kinetic Evaluation of Deltiazem HCL from Various Hydrophilic and Plastic Based Matrices, Iran,J Pharm. (3):137-146.
17. EI-Samaligy SA. , Basalious EBY. (2004) Formulation and Evaluation of Diclofenac Sodium Buccoadhesive Discs, Int. J.Pharm. 286: 27-39.
18. Martindale. (1977) The Extra Pharmacopia. 27th ed. 629-631.
19. Sadiq AA.(2005) Floating Drug Delivery Systems Using Metronidazole as a model drug . University of Baghdad, college of pharmacy.
20. Sung KC., Nixon PR., Skoug JW., Ju TR., Gao P., Topp EM., and Patel MV. (1996) Effect of formulation variables on drug and polymer release from HPMC-based matrix tablets. Int. J. Pharm. 142: 53-6
21. Bravo SA., Lamas MC., and Salmon CJ. (2002) In –vitro studies of diclofinac sodium controlled release form biopolymeric hydrophilic matrices. J. Pharm. Pharmaceut.Sci. 5:213-219

22. Tiwari SB., MurthyTK., Pai MR., Mehta PR., and Chowdary PB. (2003) Controlled release formulation of tramadol hydrochloride and hydrophilic and hydrophobic matrix system.AAPS Pharm.SCi.Tech. 4: 31.
23. Mohammadi SS., Montaseri H., and Kazemi A. (2003) The effect of polymer blends on release profiles of diclofenac sodium from matrices. Eur. J. Pharm. Biopharm. 55: 351-355
24. Khan GM., and Zhu JB. (1999) Studies on drug release kinetics from Ibuprofen-carbomer hydrophilic matrix tablets: Influence of coexcipients on release rate of the drug. J. Contr. Rel. 57: 197-203.
25. Gohel MC., Amin AF., and Patrel KV.(2002) Studies in release behavior of diltiazem HCL from matrix tablet containing (hydroxypropyl) methyl cellulose and xanthan gum. Boll.Chim. Farm. 141: 21-28

# قسم الدراسات العربية

# *ARABIC SECTION*

# استعمال الكلوكان كمعدل مناعي لخمج الجروح المسببة ببكتيريا *Staphylococcus aureus*

**أليس كريكور ميلكونيان، وسن عبود، وسهاد سعد**

*قسم التقنيات الإحيائية / كلية العلوم / جامعة بغداد، بغداد- العراق*

*البريد الإليكتروني: wwassan@yahoo.com*

**الملخص باللغة العربية**

جمعت 30 عينة سريرية من اخماج الجروح لمرضى راقدين في مستشفى اليرموك التعليمي في بغداد,اظهرت نتائج الزرع الهوائي ان 15 عزلة (sw1-sw15 ) كانت تعود لبكتريا *staphylococcus aurues* بالاعتماد على نتائج الاختبارات الكيموحيوية. تم اجراء اختبار الحساسية لجميع العزلات باستخدام مضادات
[الفانكومايسين,السبروفلوكساسين,السفالكسين,الجنتامايسين ,السفتازديم,السفتركزون,النورفلوكساسين,النالدكسك اسد,الميترندازول] وقد اظهرت النتائج ان جميع العزلات كانت مقاومة لجميع انواع المضادات المستخدمة عدا العزلات [sw4,sw5,sw6] التي كانت حساسة لمضاد الفانكومايسين و العزلة[sw3 ]التي كانت متوسطة المقاومة للمضاد..تم استخدام الكلوكان كمعدل مناعي لخمج الجروح بحقنه بالغشاء البريتوني لثلاث مجاميع من الفئران وبثلاث تراكيز منفصلة[0.5،1،1.5مايكروغرام/0.1 مليلتردكستروز].تم احداث خمج الجروح لمجاميع الفئران باستخدام العزلة [sw3] التي اظهرت مقاومة لجميع انواع المضادات المستخدمة واظهرت النتائج ان افضل حالة شفاء لوحظت في المقطع النسيجي للجلد لمجاميع الفئران الممنعة بالكلوكان بتركيز [ 1مايكروغرام/0.1 مليلتر دكستروز] من الكلوكان من خلال ملاحظة ظهور خلايا الليفية [fibroblast]وتكون الاوعية الدموية في المقطع النسيجي[حالة الشفاء].

***ABSTRACT***

Thirty pus samples taken from wound infections were collected from Al-yarmook hospital in Baghdad. Fifteen isolates [sw1-sw15]and were characterized as *staphylococcus aureus* depending on the results of biochemical tests. Antibiotic susceptibility to all isolates were determined towards different antibiotics : [vancomycin ,ciprofloxacin , gentamicin , cephalexin ,ceftazidium ceftraxion,norfloxacin , naldixic acid ,metranidazol].The results showed that all isolates were resistant to the antibiotics ,except(sw4,sw5andsw6) which were sensitive to vancomycin and the isolate [sw3]which was moderately resistant to it. The glucan was used as immunomodulator and was given intraperitonially to three groups of mice in three concentrations separately [0.5,1,1.5 µg / 0.1 ml of dextrose] .The wound infection was induced in mice using the isolate sw3. The results showed that the best healing was observed with the skin tissue section for group of mice given 1 µg/0.1ml of glucan that is through the appearance of fibroblast cells and new blood vessels in section.

**المقدمة**

تنتج الجروح من تمزق الجلد و الذي يعد العائق الرئيسي لحدوث الاخماج بوساطة البكتريا الممرضة ويحدث الخمج عندما تستوطن الجروح بكتريا لها القدرة على التضاعف فضلا عن انتاجها لعوامل الفوعة التي تمكنها من التغلب على الجهاز المناعي(1).وتعد اخماج الجروح الناتجة عن الاصابات الخطرة والعمليات الجراحية من التعقيدات الشائعة التي تسبب زيادة في نسبة الامراضية و الوفيات(2) ان مصادر تلوث الجروح عديدة وتشمل المصادر الخارجية مثل الاحياء المستوطنة للجلد المحيط بالجرح والمصادر الداخلية مثل الاحياء المستوطنة للأغشية المخاطية و التي توجد بشكل نبيت طبيعي (Normal flora) (3).

تستوطن الجروح انواع بكتيرية عديدة منها *Staphylococcus aureus*: والتي تعد من الممرضات المهمة التي تتميز بقدرتها على احداث انواع مختلفة من الاصابات في مواقع متعددة من الجسم وان امراضيتها مرتبطة بقدرتها على انتاج العديد من عوامل الفوعة التي تشمل انتاج الذيفانات والانزيمات الخارج خلوية وغيرها فضلا عن امتلاكها القدرة على مقاومة مدى واسع من المضادات الحيوية(4).قسمت الجروح الى الجروح الحادة (acute wounds ) وهي الجروح الناتجة من ضرر خارجي (Extra injury ) مثل التي تكون ناتجة من العمليات الجراحية (surgical operation ) فضلا عن الجروح الناتجة من الحروق(burns) و الجروح المزمنة(chronic wounds ) وهي الجروح التي تفشل في الشفاء خلال سلسلة من الاحداث المنتظمة ضمن زمن معين(5).

ان سبب فشل علاج كثير من الاصابات البكتيرية هو ظهور المقاومة المفرطة لمدى واسع من المضادات ، لذا اتجهت الدراسات لاكتشاف مركبات لها القدرة على تثبيط هذه الاصابات او تمتلك القدرة على العمل كمساعدات (Adjuvant) للجهاز المناعي (6) ، وقد تم التعرف على كثير من هذه المواد والتي سميت بالمعدلات المناعية (Immunomodulators) وهي مركبات تؤثر في النظام المناعي فتنظم الاستجابة المناعية (Immune response) بشكل سلبي او ايجابي.

من المعدلات المناعية المعروفة الكلوكان(Glucan) المستخلص من جدار الخميرة وهومركب فعال بايولوجيا وهو بوليمر متجانس من وحدات متكررة من الكلكوز(7)، وقد أشار الكثير من الباحثين الى امتلاك الكلوكان عددا من الفعاليات البايولوجية ، منها:الفعالية المضادة للأورام (antitumor activity ) (8) ونشاطه بوصفه عاملا مساعدا(9)، وأيضا تحفيز الخلايا المساهمة في المناعة (10).

ونظرا لأهمية الكلوكان السابقة الذكر فقد هدفت الدراسة الى بيان التأثير الوقائي والعلاجي لهذا المركب وقدرته على علاج الجروح الملوثة بالبكتيريا المقاومة للمضادات الحيوية في الحيوانات المختبرية.

**المواد وطرائق العمل**

شملت الدراسة (30) عينة قيح أخذت من جروح مصابة لمرضى في مستشفى اليرموك التعليمي في بغداد ولكلا الجنسين وبأعمار مختلفة.

1. تشخيص العزلات:

تم فرز العزلات بعد نقلها الى المختبر على وسط ( staph 110 ) ووسط (mannitol salt agar) وقد تم تشخيص الأنواع البكتيرية بالاعتماد على الاختبارات الكيموحيوية الموصوفة سابقا (11)، كذلك تم استعمال نظام (API) الخاص ببكتيريا *Staphylococcus* لإجراء اختبارات تشخيصية إضافية.

2. اختبار حساسية البكتريا للمضادات الحيوية:

استخدمت المضادات الحيوية الأتية : (vancomycin30µg, gentamycin10µg, ciprofloxacin 30µg,norfloxacin10µg,cephalexin30µg, ceftrixion30µg,Ceftazidium30µg, naldixicacid30µg, metronidazol30µg) وتم اجراء اختبار الحساسية، وقياس المنطقة الخالية من النمو باستعمال المسطرة بالمليمترات وتمت مقارنة النتائج مع الجدول الخاص لمعرفة مدى مقاومة أو حساسية العزلات لذلك المضاد(12).

3. الأنموذج الحيواني:

تمت معاملة مجاميع الفئران السويسرية ذات الأوزان التي تراوحت بين ( 20–25)غراما وبعمر ( 4–8) أسابيع وتم تقسيم تلك المجاميع إلى أربعة، احتوت كل مجموعة منها ثلاثة فئران.

تم معاملة مجاميع الفئران الأربعة بتراكيز مختلفة من الكلوكان المجهز من قبل شركة (Sigma) المذاب بمحلول الدكستروزقبل التخميج ببكتيريا*Staphylococcus aureus* sw3 بفترة 24 ساعة وقبل 4 ساعات وبعد التخميج بفترة 24 ساعة (تمت عملية التخميج بعمل جرح في المنطقة الظهرية للفئران ومن ثم تم إجراء التخميج بتلويث الجرح ببكتيريا*Staphylococcus aureus* sw3 بتركيز[1× $10^8$ خلية بكتيرية /مليلتر] والتي تمثل الجرعة المسببة لظهور أعراض المرض لدى 50% من الفئران

(ID50) والتي تم تحديدها بالاعتماد على الطريقة الموصوفة سابقا(13)، أما ترتيب المجاميع فكان كما ياتي:
المجموعة الاولى:حقنت بالكلوكان في منطقة البريتون بتركيز 0.5 مايكروغرام/0.1 مليلتر من الدكستروز.
المجموعة الثانية :حقنت بالكلوكان في منطقة البريتون بتركيز 1.0 مايكروغرام/0.1 مليلتر من الدكستروز.
المجموعة الثالثة:حقنت بالكلوكان في منطقة البريتون بتركيز 1.5 مايكروغرام/0.1 مليلتر من الدكستروز.
المجموعة الرابعة (السيطرة) فقد تم حقنها في منطقة البريتون بالدكستروز( 0.1 مليلتر فقط).
وخلال تقدم التجربة تم الكشف عن استمرار تواجد بكتيريا *Staphylococcus aureus* في الجروح الملوثة بالبكتيريا وذلك من خلال أخذ مسحات مغمورة بالمحلول الملحي (normal saline) من الجروح العائدة للفئران في المجاميع الأربعة السابقة الذكر وزراعتها على وسط mannitol salt agar و ملاحظة كثافة النمو.

4. تحضير المقاطع النسيجية للجلد:

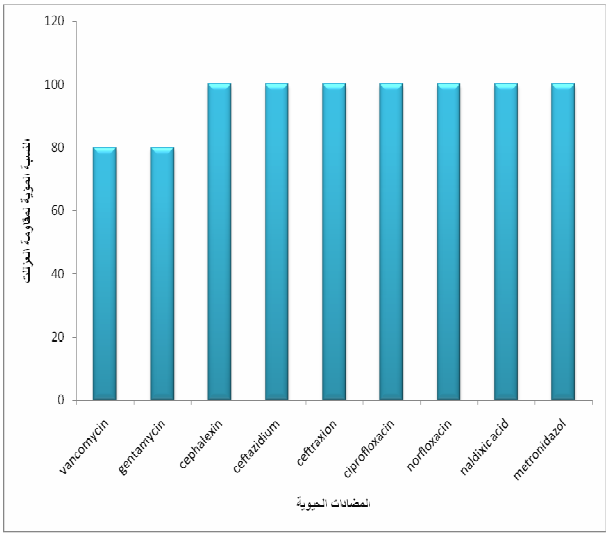
تم تحضير المقاطع النسيجية من الفئران المعاملة بتراكيز مختلفةمن الكلوكان وذلك بقتل الفئران بعد 7 أيام من التخميج ، وجرى تشريح الفئران وأخذ منطقة الجلد المحتوي على الجرح وتم تثبيتها وعملت المقاطع النسيجية للجلد المخمج (14).

**النتائج والمناقشة**

أظهرت نتائج زرع العينات في ظروف هوائية على وسط staph 110 ووسط manitol salt agar نمو 15 عينة من أصل 30 أي (50%) بينما أظهرت 15 عينة أخرى نتائج زرع سالبة.

اختبار حساسية العزلات للمضادات الحيوية:

تم اختبار حساسية عزلات بكتيريا *Staphylococcus aureus* جميعها تجاه المضادات الحيوية والتي شملت 9 مضادات ، وقد أظهرت النتائج ان عزلات بكتيريا *Staphylococcus aureus* جميعها لم تظهر حساسية تذكر و كانت مقاومة بنسبة 100% تجاه مضادات البيتالاكتام [cephalexin,ceftazidium,ceftraxion] نتيجة لامتلاكها القدرة على إنتاج إنزيمات β-lactamase والتي تعمل على حلقة البتالاكتام [β-lactam] وتحطمها وتحولها الى مضادات غير فعالة (15)، أما بالنسبة لمضادات الأمينوجليكوسيدات (Aminoglycosides) المتمثلة بالمضاد gentamycin فقد أظهرت البكتيريا حساسية قليلة بنسبة 20% وهذا يعود الى امتلاك البكتيريا الجين المسئول عن المقاومة لهذا المضاد والذي يشفر الى إحداث تحوير في موقع الهدف 30S الذي يرتبط به المضاد مسببا المقاومة (16)، أما بالنسبة لمضادات الكينولات (quinolones) [ciprofloxacin,norfloxacin,naldixic acid] فكانت البكتيريا مقاومة لها بنسبة 100% وهذا يعود الى امتلاك البكتيريا المضخات العاكسة efflux pump ) وكذلك امتلاكها للجينات المسئولة عن إحداث التغيرات في الموقع الهدف لإنزيم DNA gyrease ، مما يجعل المضادات غير فعالة أو تعود المقاومة الى الاستخدام المتكرر والعشوائي للمضادات (17).
أظهرت النتائج أن البكتيريا لم تظهر أي حساسية تذكر تجاه مضاد الميترونيدازول [ metronidazol] وكانت المقاومة 100% وهذا يعود الى امتلاك البكتيريا القدرة على إنتاج انزيم nitroreductase الذي يعمل على إحداث تغير في الموقع الهدف فسبب المقاومة (18).
اما بالنسبة لمضاد vancomycin ، فقد أظهرت العزلات البكتيرية حساسية قليلة نسبتها 20% ويعزى سبب هذه المقاومة إلى حدوث تغير في مسار تصنيع الجدار الخلوي الحساس للمضاد مؤدية إلى زيادة سمكه (19)، أو تكون المقاومة ناتجة عن انتقال جينات مشفرة لصفة المقاومة للمضاد والتي تكون محمولة على بلازميدات أو جينات قافزة من سلالات وأنواع بكتيرية أخرى إلى بكتيريا *S. aureus* مكسبة إياها صفة المقاومة لهذا المضاد خلال مدة قليلة ( الشكل 1)




**(الشكل –1)النسب المؤية لمقاومة بكتريا *Staphylococcus aureus* للمضادات الحيوية المستخدمة**

التغيرات النسيجية لجلد الفئران:

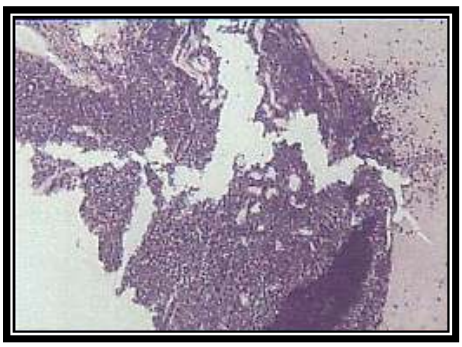
تم اختيار العزلة SW3 لإجراء التأثير الوقائي والعلاجي لمادة الكلوكان لكونها أظهرت مقاومة لجميع المضادات الحيوية المستعملة وذلك من خلال معاملة الفئران بثلاث تراكيز للكلوكان [0.5,1.0,1.5 مايكروغرام/0.1 مليتردكستروز] قبل إحداث الخمج بفترة 24 ساعة و 4 ساعات وبعد إحداث الخمج بفترة 24 ساعة ، وبعد سبعة أيام قتلت الفئران وفصل طبقة الجلد من منطقة الخمج وتم تحضير المقاطع النسيجية ، وأظهرت النتائج أن مجموعة الفئران الممنعة بتركيز [0.5 مايكروغرام /0.1 مليلتر من الدكستروز] جميعها قد هلكت أما التغيرات النسيجية للفئران المعاملة بتركيز 1.5 مايكروغرام /0.1 مليلتر دكستروز فقد تمثلت بظهور حالة التهابية (Inflammation) ؛ أي ارتشاح أعداد كبيرة من الخلايا الالتهابية ،وتكون خراجات (Abscesses) فضلا عن تنخر الأنسجة (Tissue necrosis) ، وهذا يؤكد على أن التركيزين المستخدمين لم يحفزا الجهاز المناعي بصورة جيدة لمقاومة الخمج ومن ثم حصول الاستجابة الالتهابية التي يتوسطها إفراز البادئات الالتهابية (Proinflammatory) مثل TNF من الخلايا الالتهابية والذي يكون له دور كبير في تدمير الأنسجة (الشكل2-أ) مقارنة مع مجموعة السيطرة التي تمثلت بظهور حالة الالتهاب مع تكون الخراجات وتنخر الأنسجة (الشكل2–ب) .

كما تمثلت التغيرات النسيجية لجلد الفئران المعاملة بتركيز 1.0 مايكروغرام/0.1 مليلتر من الدكستروز بظهور حالة الشفاء والتئام الجرح إذ لوحظ ارتشاح كميات كبيرة من خلايا الأرومة الليفية بالإضافة إلى ظهور ألياف الكولاجين فضلا عن ظهور الأوعية الدموية حديثة التكوين (شكل2–ج ) مقارنة بالتغيرات النسيجية لمجموعة السيطرة التي تمثلت بظهور حالة الالتهاب مع تكون خراجات وتنخر الأنسجة (شكل2–د) وهذا يتفق مع الدراسات التي أشارت الى قابلية الكلوكان على تحفيز خلايا الأرومة الليفية لإنتاج الكولاجين ومن ثم الوصول إلى حالة الشفاء(20).

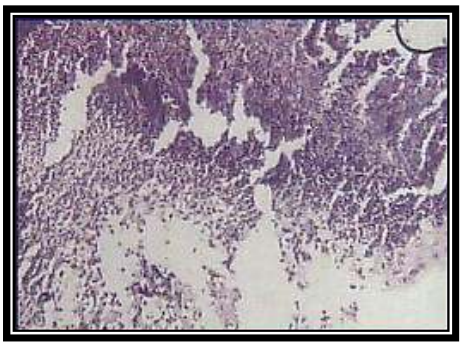
كما أشارت الكثير من الدراسات إلى قدرة الكلوكان لمنع حدوث الالتهاب فضلا عن قابليته على حماية الحيوانات المختبرية عند إعطائه قبل جرعة التحدي وهذا يعود إلى قدرة الكلوكان العالية على تحفيز الخلايا المساهمة في المناعة نتيجة لامتلاكه مستقبلات خاصة على سطح هذه الخلايا (21).

إن استخدام تراكيز عالية من الكلوكان ليس بالضرورة قادر على الإسراع بعملية الشفاء كما هو الحال مع التركيز 1.5 مايكوغرام/مليلتر إذ إن التراكيز العالية من الكلوكان تعمل على تثبيط عملية الانقسام والتكاثر للخلايا المساهمة بالمناعة، والذي يؤدي الى التقليل من قابلية المضيف على مقاومة الخمج (20)، أما نتائج عزل بكتيريا *S. aureus*، فقد كانت البكتيريا متواجدة بأعداد كبيرة في مجموعة الفئران التي استقبلت الدكستروز فقط والمجموعة التي استقبلت الكلوكان بتركيز0,5 مايكروغرام / مليلتر طوال مدة التجربة في حين كانت بأعداد متوسطة في المجموعة التي استقبلت الكلوكان بتركيز 1,5 مايكروغرام / مليلتر وأعداد قليلة جدا في الفئران التي استقبلت الكلوكان بتركيز 1 مايكروغرام/مليلتر.

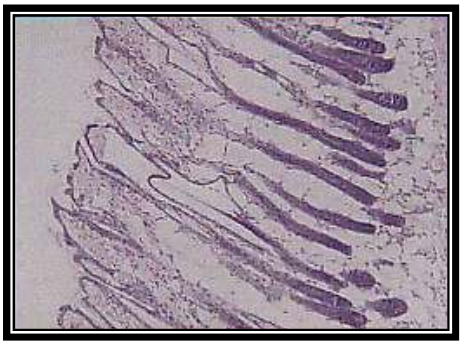
يستنتج من الدراسة أن الكلوكان كان له تأثير في تحسين الاستجابة المناعية للفئران من خلال قدرته للقضاء على الخمج و تسريع التآم الجروح من خلال تحفيز الخلايا المولدة للالياف (fibroblast).



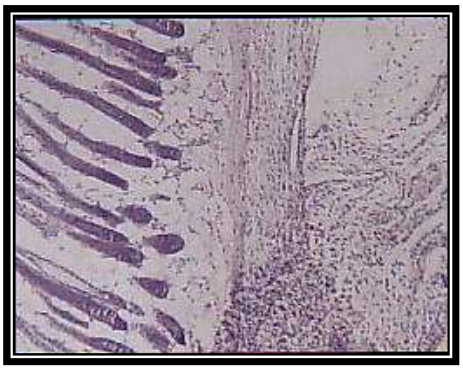
**شكل (2–أ)**



**شكل (2– ب)**



**شكل (2–ج)**



شكل (2–د)

**الشكل (2) المقاطع النسيجية لجلد الفئران المعاملة بمادة الكلوكان بتراكيز مختلفة و المصبغة بصبغة الأيوسين – الهيماتوكسلين**

**أ– مقطع عرضي لجلد الفئران المعامل بالدكستروز(سيطرة)**
**ب– لجلد الفئران المعاملة بالكلوكان بتركيز 0,5 مايكروغرام / 0.1 مليلتر دكستروز**
**ج– مقطع عرضي لجلد الفئران المعاملة بالكلوكان بتركيز 1 مايكروغرام / 0.1 مليلتر دكستروز**
**د– مقطع عرضي لجلد الفئران المعامل بالدكستروز بتركيز 1.5 مايكروغرام/0.1 مليلتر دكستروز**

**المصادر**


1. Bowler PG. (2002) wound pathophysiology, infection and therapeutic options.Anu.Med. 34(6):419-427.
2. Nichols RL. (2004) Current strategies for Prevention of Surgical site infections . curr. Infect. Dis. Rep. 6(6): 426-434
3. Kingsley A (2001) A proactive approach to wound infection . Nursing standard. 15(30): 50-58.
4. Abbott JC.(2003) Antimicrobial resistance trends:implication for treatment .J.Clin.Pharmacy. 126:118-129.
5. Marcus K.(2005) New bacteria on the block.J.Infect.Dis. 129:811-818.
6. Soltys J. and Quinn MT. (1999) Modulation of endotoxin and enterotoxin induced cytokines release y invivo treatment ith beta. (1,3)-branched beta- (1,3)- glucan. Infec. Immun., 67: 244-252.
7. Schorey JS., Lawrence C.(2008) The ability of the innate immune system to quickly recognize and respond to an invading pathogen is essential for controlling the infection .Curr.Drug.targets.9(2):123-129.
8. Brian W., Leblanc JEA., and Jonatha SR.(2006) The effect of PGG-β-glucan on neutrophil chemotaxis in vivo. J. leuko.Biol.79:1-9.
9. Plizon AC., Kaneno R., Soares AMVC., Meira DA. and Sartori A.(2003) Down-modulation of lymphoproliferation and interferon-Ỳ production by beta-glucan derived from saccharomyces cerevisiae.mem.Inst. Oswaldo.Cruz. 98(8)1083-1087.
10. Hannigan MO., Huang CK.,Wu DQ.(2004) Roles of p13k in neutrophil function .Curr.Top.Microbiol.Immunol.282:165-175.
11. Collee JG., Miles RS. and Watt B.(1996) Test for the identification of bacteria. In: Makie and Maccartney practical medical microbiology. 4th edition , Churchill livingstone. P: 131149.
12. NCCLS. (2002) Performance Standards For Antimicrobial Susceptibility Testing ; Twelfth Informational Supplement. National Committee for Clinical Laboratory Standards . 22(1): 1-130.
13. Handricks KJ., Burd TA., Anglen JO., Simpson AW., Christensen DG., and Gainor BJ.(2001) Synergy between Staphylococcus aureus and Pseudomonas aeruginosa in a rat model of complex orthopaedic wounds. J.Bone Joint. Surg. Am. 83(6):855-861.
14. Vandepitte J., Engbaek K., Piot P., Heuck CC (1991) Basic Laboratory Procedures in clinical bacteriology. World Health Organization . Geneva.
15. Galeazzi F., Blennerhassett PA., Qiu B., O'Byrne PM., and Collins SM.(1999) Cigarette smoke aggravates experimental colitis in rat .Gastroenterology.117:877-883.
16. Assadullah S., Kakru DK.,Thoker MA., Bath FA., Hussain N.,and Shah

A.(2003) Emergence of low level vancomycin resistance in MRSA.Clin.Microbiol. 21(3):196-198.
17. Hanaki H.(2004) Epidemiology and clinical effect against beta-lactam antibiotic induce.J.Antibiot.78(8):204-216..
18. Wu H., Shi XD., Wang HT., and Liu JX.(2000) Resistance of Helicobacter pylori to metronidazol ,tetracycline and amoxycilin .J.antimicrob.agent.chemother.46:121-123.
19. Michael M., and Mwangi S.(2007) Vancomycin resistant staphylococcus aureus.Encyclopedia.16(23):125-134.
20. Ishibashi KI., Miura NN., Adachi Y., Ogura N., Tamura H., Tanaka S., and Ohno N. (2002) Relationship between the physical properties of candida albicans cell wall beta-glucan and activation of leukocyte invitro. Int. Immunopharmacol. 2: 1109-1122.
21. Portera CA., Loue EJ., Memore L., zhang L., Muller AS., Browder W., and Williams DL. (1997) Effect of macrophage stimulation on collagen biocynthesis in the healing wound . Am. Surg. 63: 125-131.

# تأثير التلقيح ببكتريا Rhizobium Leguminosarum فـــي نمـــو وتكـــوين العقـــد الجذرية على نبات الباقلاءVicia Faba

**تركي مفتن سعد**

*كلية الزراعة / جامعة المثنى / العراق*

البريد الإليكتروني :*turkimuftin@yahoo.com*

**الملخص باللغة العربية**

نفذت تجربتان أحداهما مختبريه والأخرى تجربة أصص في كلية الزراعة – جامعة المثنى والتي تبعد 300 كم جنوب بغداد , للموسم الزراعـــي 2009 – 2010 بهدف معرفة أي من السلالات البكتيرية المستخدمة وهي

R405 , R417 , R450 , R467 , R483 أكثر كفاءة في عملية تثبيت النتروجين الجوي لصنفين من نبات الباقلاء (المحلي والاسباني) بعـــد أن أجريت عملية الفحص التشخيصي للتأكد من الخواص الحيوية للسلالات البكتيرية .طبقت التجربة المختبرية باستعمال التصميم التام التعشية CRD وبثلاث مكررات , في حين طبقت تجربة الأصص باستعمال تصميم القطاعات العشوائية الكاملة RCBD وبثلاث مكررات أيضا .

تضمنت التجربة المختبرية دراسة مدد التحضين ( 3 , 9 , 15 , 21) يوماً في الكثافة العددية لكل سلالة من السلالات البكتيرية في التربة وعنـــد درجة حرارة 28 م$^5$ وخلال هذه المدد حسبت الكثافة العددية للمستعمرات النامية على الوسط الغذائي باستخدام طريقة التخفيف والعد في الأطباق .

وتضمنت تجربة الأصص زراعة بذور الباقلاء للصنفين في أصص باستعمال خمس معاملات تلقيح إضافة الى معاملة النتروجين $N_1$ (100 كغم N.هـــ$^{-1}$) ومعاملة المقارنة No (بدون تلقيح بكتيري وبدون نتروجين) , وتم حساب مجموعة من القياسات والمؤشرات المتعلقة بالنبات والسلالات البكتيرية في مرحلة نمو النبات (100 – 105) يوم من الزراعة وفي نهاية الموسم تم حساب عدد القرنات. نبات$^{-1}$ وكانت النتائج كالأتي :

زيادة الكثافة العددية لبكتريا العقد الجذرية خلال مدد التحضين الأربع وكانت أعلى زيادة في مدة 15 يوم من التحضين , تفوق المعاملات الملقحة على غير الملقحة في جميع الصفات مع وجود اختلاف بين السلالات البكتيرية والأصناف المزروعة . تفوق السلالة R483 في اغلب الـــصفات المدروسة وأعطى التداخل بين السلالة R483 والصنف المحلي أعلى متوسط لعدد التفرعات . نبات$^{-1}$ , الوزن الجاف ,للمجموع الخضري , عدد العقد الجذرية , الوزن الجاف للعقد الجذرية وعدد القرنات , نبات$^{-1}$ , كما تفوقت نباتات الصنف المحلي على الصنف الاسباني في جميع الصفات المدروسة .ولأهمية محصول الباقلاء كونه من المحاصيل البقولية التي تزرع في العراق لاسيما في المنطقة الجنوبية منه تحديدا , لذا كانت هـــذه الدراسة حول نشاط بكتريا العقد الجذرية وتعايشها مع نبات الباقلاء ودورها في تثبيت النتروجين الجوي من اجل معرفة تـــأثير التلقـــيح لخمـــس سلالات مستورده من بكتريا العقد الجذرية على صنفين من نبات الباقلاء وكذلك دراسة تأثير مدد التحضين المختلفة ومعرفة المناسب منها في نمو بكتريا العقد الجذرية في التربة بالإضافة لتحديد إي من صنفي الباقلاء تعايشا مع السلالات البكتيرية

***ABSTRACT***

Two experiments were conducted . The First one is Laboratory and the second experiment in pots . The aim of this study was to determine the more efficient strain of Rhizobium (R405 ,R417 , R450 , R467 , R483) in its ability to fix nitrogen and its relation with broad bean varieties (Spanish and local).After that the identification tests were proceeded to prove the biological characteristics to these strains .

The laboratory experiment was conducted by using CRD design with three replications , and the pots experiment was conducted by using RCBD design with three replications .

The laboratory experiment to study the effects of incubation periods (3 , 9 , 15 , 21) days on numeral density of Rhizobia in soil at 28c .

The second experiment was conducted in aces at 2009 – 2010 Season in Agriculture College – AL – Muthanna University and it proceeded by culturing seeds of each varieties in aces in Five inoculation treatments with nitrogen adding treatments with nitrogen adding treatment of 100Kg .h$^{-1}$($N_1$) and control treatment(No) (Without inoculation and nitrogen adding) . Then calculation of several measures that related to the broad bean and bacterial strains had been elone in to stages of plants growth (100 – 105 days) . In the end of season , number of pods . plant$^{-1}$ was conducted the results were :

The density of nodule root bacteria was increased in forth incubation periods , and the higger density was after 15days of incubation . strain R483 gave high average of number living cells . The inoculation treatments gave high average in all measured parameters compared with non – inoculation treatment , and this relation was differing by strain of bacteria and variety of cultivated plant . strain R483 gave high results in number of branch . plant$^{-1}$ , dry weight of shoot . plant$^{-1}$ , number of nodules , dry weight of nodules .

The interaction treatment (Strain R483x local variety) was gave higher average for branch . plant , dry weight of shoot . plant$^{-1}$ , number of nodules , dry weight of nodules and number of pods . plant$^{-1}$ . The local variety was gave higher average for all parameter compared with Spanish variety .

## المقدمة

يعد النتروجين من العناصر الغذائية الضرورية التي يحتاجها النبات وبكميات كبيرة ويأتي رابعا بعد الكاربون والهيدروجين والأوكسجين (18) وتكمن أهميته كونه عنصرا أساسيا في الكثير من الفعاليات الحيوية ولدخوله في تركيب العديد من مكونات النبات مثل الحوامض النووية والبروتينات والمكونات الخلوية الأخرى .

يحتوي الغلاف الجوي على كميات كبيرة من النتروجين الجزيئي $N_2$ تصل نسبته الى 79% وان هذا المصدر النتروجين غير جاهز لمعظم أشكال الحياة في الطبيعة , ولغرض الاستفادة منة يجب كسر الآصرة الثلاثية المستقرة بين ذرتي النتروجين N≡N من خلال اختزالها وإنتاج الامونيا $NH_3$ عن طريق تفاعلات طبيعية أو اصطناعية . ومن اجل استغلال هذا المصدر اتجه علماء التقنية الحيوية الى اعتماد تقنية التثبيت الحيوي للنتروجين ( BioLogical Nitrogen Fixation) بواسطة البكتريا المثبتة للنتروجين التي يمكن أن تختزل الكثير من كميات الأسمدة النتروجينية المستعملة وتعطي تجهيز مناسبا لعنصر النتروجين للنبات وبالتالي زيادة الإنتاج وتحسين المحتوى ألبروتيني لهذه النبات في الوقت الذي تشتد حاجة سكان العالم منه للغذاء ويزداد الطلب على البروتين النباتي بسبب الزيادة الهائلة في عدد سكان العالم وارتفاع أسعار البروتينات الحيوانية .

تعتبر عملية التثبيت الحيوي للنتروجين من العمليات الحيوية المهمة التي تستطيع بواسطتها النباتات البقولية تلبية الجزء الأعظم من حاجتها لعنصر النتروجين , ولقد قدرت كمية النتروجين المثبتة حيويا بـ 114مليون طن سنويا , وما تثبته البقوليات 80 مليون طن سنويا (13) وان هذه الكمية المثبتة من النتروجين الجوي من قبل النباتات البقولية من خلال تعايشها مع بكتريا العقد الجذرية (الرايزوبيا) تسد نسبة كبيرة من حاجة النبات البقولي من عنصر النتروجين قد تصل الى أكثر من 80% (25) بالإضافة لكميات النتروجين المتبقية في التربة والممكن أن يستغلها المحصول اللاحق للمحصول ألبقولي .

وفي الدراسات الحديثة وجد أن العديد من البكتريا المثبتة للنتروجين وخاصة تلك التي تعود الى أجناس الرايزوبيا تنتج مختلف المركبات الكيميائية والهرمونات وعوامل النمو والفيتامينات تحفز بزوغ النباتات وسرعة نموها وتزيد من معدلات التركيب الضوئي وتحد من إصابتها ببعض الأمراض كونها استخدمت في المقاومة الحيوية بالإضافة الى عملها في تثبيت النتروجين الجوي (16,7)

## المواد وطرائق العمل

أجريت هاتان التجربتان في تربة أخذت من احد الحقول الزراعية القريبة من نهر الفرات في مدينة السماوه مع مراعاة أن الحقل لم يزرع بمحصول الباقلاء منذ ثلاث سنوات .

جففت التربة هوائيا ونخلت باستخدام منخل قطر فتحاته 2ملم للتجربة المختبرية و 4ملم لتجربة الأصص وقدرت بعض الصفات الفيزيائية والكيميائية والحيوية جدول (1)

1:- التجربة المختبرية :

أخذت نماذج تربة وجففت هوائيا وطحنت ثم نخلت بمنخل قطر فتحاته 2ملم ثم وزن 10 غرام من هذه التربة على أساس الوزن الجاف ثم وضعت في دوارق مخروطية الشكل 250ملم , عقمت الدوارق مع التربة والبتموس المضاف أليها بعد غلقها بسدادات قطنية ووضعت في جهاز Autoclave على درجة حرارة 121م$^5$ وضغط 15 باوند/انج$^2$ ولمدة ساعة واحدة ولفترة ثلاثة أيام . أضيف 1ملم من اللقاح البكتيري السائل للسلالات المستخدمة الى التربة لكل دورق ثم رطبت التربة بحدود $\frac{1}{3}$ بار والمحافظة على هذا المحتوى من الرطوبة الموجودة في الدورق خلال مدة البحث بالطريقة الوزنية . حضنت جميع المعاملات بالحاضنة وعند درجة 28 م$^5$ وحسبت أعداد بكتريا الرايزوبيا لكل سلالة بمدد التحضين المذكورة بطريقة التخفيف والعد بالأطباق نفذت التجربة باستعمال التصميم التام التعشية CRD وبثلاث مكررات .

**جدول (1) بعض الصفات الكيميائية والفيزيائية والحيوية للتربة المستخدمة في الدراسة**

| الصفات الكيميائية والفيزيائية | | |
|---|---|---|
| درجة التفاعل | | 7.65 |
| التوصيل الكهربائي (ديسي سيمنز . م$^{-1}$) | | 3.10 |
| المادة العضوية (غم . كغم$^{-1}$) | | 21.00 |
| النتروجين الجاهز(ملغم . كغم$^{-1}$) | | 65.00 |
| البوتاسيوم الجاهز (ملغم . كغم$^{-1}$) | | 140.00 |
| الفسفور الجاهز (ملغم . كغم$^{-1}$) | | 8.50 |
| CEC (سنتي مول . كغم$^{-1}$) | | 17.5 |
| الكلس (غم . كغم$^{-1}$) | | 24.4 |
| نسجه التربة (مزيجيه) | الرمل (غم . كغم$^{-1}$) | 360 |
| | الغرين (غم . كغم$^{-1}$) | 400 |
| | الطين (غم . كغم$^{-1}$) | 240 |
| الصفات الحيوية | | |
| البكتريا الكلية / غم تربة جافة | | $10 X4.5^7$ |
| الفطريات الكلية / غم تربة جافة | | $10X1.8^4$ |

2:- تجربة الأصص :
عقمت الأصص البلاستيكية بقطعة قطن طبي مشبعة بالكحول الاثيلي عبئت هذه الأصص بتربة مجففة ومارة من منخل قطر فتحاته 4ملم وان سعة الأصيص الواحد 5 كغم حضر لقاح سائل على وسط YMS لكل من السلالات البكتيرية الخمسة R483 , R467 , R450 , R417 , R405 وصنعت كمية من بذور صنفي الباقلاء في اللقاح السائل للسلالات الخمسة لمدة ساعة ونصف لغرض تلويثها باللقاح بعد ترطيب البذور بمحلول الصمغ العربي تركيز 4% وتركت بعض البذور من دون تلقيح آذ غطست بالوسط السائل بدون بكتريا (ماء مقطر) ولمدة ساعة ونصف ايضاً . زرعت الأصص ببذور الباقلاء ملقحة وغير الملقحة بمعدل 5 بذور لكل أصيص وسقيت بالماء وبعد مرور سبعة أيام من الإنبات خفت النباتات الى ثلاثة نباتات لكل أصيص .
تم إضافة 100 كغم نتروجين . هـ$^{-1}$ على هيئة سماد اليوريا بدفعتين الأولى عند الزراعة والثانية بعد 45 يوم من الإنبات والتي تمثل معاملة النتروجين ($N_1$) أضيف سماد سوبر فوسفات الكالسيوم الثلاثي لجميع المعاملات وبمعدل 80 كغم $P_2O_5$ هـ$^{-1}$ وسماد كبريتات البوتاسيوم بمعدل 60 كغم $K_2O$ هـ$^{-1}$ تم أجراء القياسات بعد مرحلة 100 – 105 يوم من الزراعة وفي نهاية الموسم حسبت أعداد القرنات. نبات$^{-1}$
الصفات الكيميائية : تم قياس الصفات الكيميائية الآتية

- درجة تفاعل التربة : باستعمال جهاز PH – meter كما ورد في (23)
- التوصيل الكهربائي (EC) : تم قياسه في راشح العجينة المشبعة للتربة باستخدام جهاز Conductivity Bridge (23)
- المادة العضوية : قدرت وفقا لطريقة Walkey و Black الواردة في (19)
- السعة التبادلية للايونات الموجبة (CEC) : قدرت باستعمال خلات الامونيوم وفق ماورد في (23)
- النتروجين الجاهز : قدر باستعمال جهاز كلدال وفقا للطريقة الواردة في (15)
- البوتاسيوم الجاهز : قدر وفقا للطريقة الموضحة في (15)
- الفسفور الجاهز : قدر وفقا لطريقة (28)
- الكلس : قدر وفقا للطرق الواردة في (24)

الصفات الفيزياويه :

- نسجه التربة : قدرت بطريقة الماصة Pipette الواردة في (23)
- رطوبة التربة : قدرت النسبة المؤية للرطوبة عند الشد $\frac{1}{3}$ بار في التربة باستخدام جهاز Pressure membrane وفقا للطرف الواردة في (24)

الصفات الحيوية :

- البكتريا الكلية: استعملت طريقة التخفيف والعد في بالأطباق لحساب عدد البكتريا الكلية في التربة باستعمال وسط Soil Extract Agar (12)
- الفطريات : استعملت طريقة التخفيف والعد بالإطباق باستعمال وسط Rose Bengal Agar وفقا لطريقة Martin في (23)

**النتائج والمناقشة**

1 :- التجربة المختبرية :
تبين نتائج الجدول (2) أن أعداد الخلايا الحية قد ازداد بصورة معنوية وعلى مستوى احتمال 0.05 خلال مدد التحضين الأربع (3 , 9 , 15 , 21) يوما مقارنة بالأعداد التي سجلت في بداية التجربة (الزمن صفر) وبدأت بالانخفاض قليلا في المدة الاخيره آذ بلغت الكثافة العددية لخلايا الرايزوبيا لكل من السلالات R483 , R467 , R450 , R417 , R405 خلية .غم$^{-1}$ تربة جافة على التوالي , وقد ازدادت الأعداد بعد مرور 3 أيام من التحضين لتصل الى (6.97 , 5.37 , 9.40 , 9.20 , 10.77) x$10^7$ خلية .غم$^{-1}$ تربة جافة للسلالات أنفا على التوالي , لكنها انخفضت في مدة التحضين الثانية بعد مرور 9 أيام الى( 5.73 , 10.77 , 8.20 , 4.93 , 7.67 , 8.97) x$10^7$ خلية . غم$^{-1}$ تربة جافة على التوالي , ثم ازدادت بعد مرور 15 يوم من مدة التحضين وأصبحت ( 12.27 , 7.23 , 11.23 , 11.33 , 14.83) x$10^7$ خلية . غم$^{-1}$ تربة جافة آذ بلغت أعلى معدلات لأعداد البكتريا , بينما انخفضت خلال مدة التحضين الأخيرة 21 يوما الى (12.30 , 8.63 , 10.90 , 6.40 , 8.03) x$10^7$خلية . غم$^{-1}$ تربة جافة على التوالي وبنفس الوقت بعثت أعلى بكثير مما كانت عليه في الزمن صفر . أن سبب انخفاض العدد بعد 9 أيام

من التحضين قد يعود الى موت بعض أعداد الخلايا لتناقص العناصر الغذائية , أما سبب زيادة الأعداد بعد 15 يوم فقد يعود الى تحلل الخلايا الميتة بحيث أمكن الاستفادة منها مصدرا غذائيا للخلايا الحية الأخرى وهذا ماشار أليه (21) في دراستهم للبكتريا العقدية المتخصصة على الفاصولياء ومع (4) في دراسته للبكتريا العقدية المتخصصة على الحمص . وقد أعطت السلالة R483 أعلى معدل لعدد الخلايا الحية بلغ 9.72 $10^7$x خلية . غم$^{-1}$ تربة جافة , أما بالنسبة لمدد التحضين فقد أعطت مدة التحضين 15 يوما أعلى معدل لعدد الخلايا الحية من بكتريا العقد الجذرية بلغ 11.50 $10^7$x خلية . غم$^{-1}$ تربة جافة .

أن زيادة أعداد الخلايا بزيادة فترات التحضين يعزى الى قابلية الخلايا للانقسام والتكاثر باستمرار مدد التحضين لاسيما زمن جيل البكتريا *Rhizobum* قصير من اجل الانقسام والتكاثر . وتظهر نتائج جدول (2) فروق معنوية عند مستوى احتمال 0.05 للتداخل بين السلالات البكتيرية ومدد التحضين فقد أعطى التداخل بين السلالة R483 ومدد التحضين 15 يوم أعلى متوسط لأعداد بكتريا العقد الجذرية بلغ 14.86 $10^7$x خلية . غم$^{-1}$ تربة جافة .

**جدول(2) تأثير مدة التحضين في أعداد الخلايا الحية لسلالات مختلفة من بكتريا الرايزوبيا**

| السلالة | مدة التحضين (يوم) | | | | | معدل تأثير السلالة |
|---|---|---|---|---|---|---|
| | 0 | 3 | 9 | 15 | 21 | |
| R405 | 1.56 | 6.97 | 5.73 | 12.27 | 8.03 | 6.91 |
| R417 | 1.06 | 5.37 | 4.93 | 7.83 | 6.40 | 5.12 |
| R450 | 1.49 | 9.40 | 8.20 | 11.23 | 10.90 | 8.24 |
| R467 | 1.61 | 9.20 | 7.67 | 11.33 | 8.63 | 7.69 |
| R483 | 1.72 | 10.77 | 8.97 | 14.83 | 12.30 | 9.72 |
| معدل مدة التحضين | 1.49 | 8.34 | 7.10 | 11.50 | 9.25 | |
| L . S . D 0.05 للسلالة = 0.791 , مدة التحضين = 0.791 , للتداخل = 1.769 | | | | | | |

2:- تجربة الأصص

أ – أعداد العقد الجذرية (عقدة . نبات$^{-1}$)

أدت إضافة اللقاح البكتيري الى زيادة معنوية عند مستوى احتمال 0.05 في معدل عدد العقد الجذرية لكل نبات من 9.00 عقدة . نبات$^{-1}$ في معاملة المقارنة الى 69.00,42.50,35.00,5767,29,67 عقدة . نبات$^{-1}$ في معاملات التلقيح بالسلالات , R483 R467 , R450 , R417 , R405 بينما سجلت معاملة النتروجين اقل معدل لعدد العقد الجذرية بلغ 4.00 عقدة . نبات$^{-1}$ (جدول 3) وذلك لان الكميات العالية من النتروجين تؤدي الى تقليل أعداد العقد الجذرية وأوزانها (5) وان أعداد العقد الجذرية المتكونة نتيجة إضافة لقاحات بكتيرية للنباتات البقولية يتناسب عكسيا مع مستوى النتروجين الجاهز في التربة (20) كما تفوق الصنف المحلي 42.29 عقدة . نبات$^{-1}$ بينما سجل الصنف الاسباني 28.24 عقدة . نبات$^{-1}$ مما بشير الى التوافق العالي بين السلالات البكتيرية المستخدمة والصنف المحلي بالإضافة الى تأقلمها لظروف المنطقة كما يلاحظ وجود تأثير معنوي على مستوى احتمال 0.05 للتداخل بين المعاملات والأصناف وسجلت معاملة التداخل بين السلالة R483 والصنف المحلي أعلى المتوسطات لهذه الصفة.

لقد احتوت النباتات غير الملقحة والتي أضيف لها السماد النتروجيني بكمية 100 كغم N .هـ$^{-1}$ على اقل معدل من العقد الجذرية وللصنفين ويمكن أن يفسر ذلك الى أن عملية تثبيت النتروجين الجوي تحتاج الى صرف طاقة وان إضافة النتروجين الى المحصول البقولي تعني زيادة توافر العنصر بصوره جاهزة ما يجعل النبات يستغني عند تكوين العقد الجذرية (7) بالإضافة الى انخفاض تكوين هذه العقد مع زيادة تجهيز النتروجين (17) وربما يعزى سبب توافق السلالتين R483 , R467 الى كفاءتهما العالية لإنتاج اكبر عدد من الخلايا الحية مقارنة ببقية السلالات خلال مدد التحضين المختلفة الأمر الذي انعكس على زيادة عدد العقد الجذرية للنبات من خلال زيادة أعداد البكتريا التي لها القابلية على إصابة واختراق الجذور وتكوين العقد الجذرية وهذا ماتوصل أليه (27 , 10 , 11)

**جدول(3) تأثير التلقيح البكتيري في أعداد العقد الجذرية (عقدة . نبات$^{-1}$) لنبات الباقلاء**

| المعاملات | الأصناف | | معدل المعاملات |
|---|---|---|---|
| | المحلي | الاسباني | |
| معاملة المقارنة No | 11.00 | 7.00 | 9.00 |
| R405 | 21.00 | 38.33 | 29.67 |
| R417 | 72.00 | 43.33 | 57.67 |
| R450 | 58.00 | 12.00 | 35.00 |
| R467 | 48.00 | 37.00 | 42.50 |
| R483 | 83.00 | 55.00 | 69.00 |
| معاملة النتروجين $N_1$ | 3.00 | 5.0 | 4.00 |
| معدل الأصناف | 42.29 | 28.24 | |
| L . S . D 0.05 للاصناف = 1.367 , للمعاملات = 0.731 , للتداخل = 1.933 | | | |

ب – أوزان العقد الجذرية (ملغم . نبات$^{-1}$)

تشير نتائج الجدول (4) الى أن التلقيح البكتيري باستعمال سلالات الرايزوبيا أدى الى زيادة معدل الوزن الجاف للعقد الجذرية المتكونة على جذور النباتات عند مرحلة 50% تزهير بصوره معنوية قياسا مع عدم استخدام التلقيح البكتيري , وقد تفوقت السلالات R417 , R450 , R467, R483 حيث بلغت متوسطات الأوزان الجافة للعقد الجذرية 136.0 , 123.5 , 156.0 , 190.0 ملغم . نبات$^{-1}$ على التوالي بينما أدت إضافة النتروجين الى تقليل معدل الوزن الجاف للعقد الجذرية حيث بلغ 30.0 ملغم . نبات$^{-1}$ وكما بينت النتائج وجود فروقات معنوية بين الصنف المحلي والصنف الاسباني في هذه الصفة فقد تفوق الصنف المحلي على الصنف الاسباني واعطي معدل وزن للعقد الجذرية مقداره 154.1 ملغم . نبات$^{-1}$ , وسجل التداخل بين الأصناف والسلالات 272.0 ملغم . نبات$^{-1}$ للسلالة R483 والصنف المحلي مقارنة بالتداخل بين معاملة المقارنة والصنف المحلي آذ سجلت وزن جاف للعقد الجذرية بلغ 22.0 ملغم . نبات$^{-1}$.

أن هذه الزيادة للعقد الجذرية للنباتات الملقحة تعود الى أن التلقيح البكتيري يؤدي الى زيادة أعداد البكتريا في التربة ثم أمكانية حصول عملية الاصابة وتكوين العقد الجذرية وزيادة حجمها ووزنها وهذا ماشار أليه (1'28)

**جدول(4) تأثير التلقيح البكتيري في أوزان العقد الجذرية (ملغم . نبات$^{-1}$) لنبات الباقلاء**

| المعاملات | الأصناف | | معدل المعاملات |
|---|---|---|---|
| | المحلي | الاسباني | |
| معاملة المقارنة No | 118.0 | 67.0 | 92.5 |
| R405 | 90.0 | 82.0 | 86.0 |
| R417 | 180.0 | 92.0 | 136.0 |
| R450 | 211.0 | 36.0 | 123.5 |
| R467 | 186.0 | 126.0 | 156.0 |
| R483 | 272.0 | 109.0 | 190.5 |
| معاملة النتروجين $N_1$ | 22.0 | 38.0 | 30.0 |
| معدل الأصناف | 154.1 | 78.6 | |
| L . S . D . 0.05 للأصناف = 29.19 , للمعاملات = 15.60 , التداخل = 41.27 | | | |

ج – تركيز النتروجين في المجموع الخضري (%)

أدى التلقيح البكتيري بالسلالات الخمس الى حصول زيادة معنوية في معدل تركيز النتروجين في المجموع الخضري لصنفي نبات الباقلاء آذ بلغ معدل تركيز النتروجين 3.162 ,2.772, 2.955, 3.146 , 3.068% للنباتات الملقحة بالسلالات R450 , R467 , R483 , R405 , R417 على التوالي قياسا بمعاملة المقارنة التي بلغ فيها تركيز النتروجين 2.358% أما معاملة النتروجين فقد أعطت أعلى تركيز للنتروجين بلغ 3.650% جدول (5) أن زيادة أعداد البكتريا عند التلقيح البكتيري وكفاءتها في أحداث الإصابة على الجذور يؤدي الى زيادة كمية النتروجين المثبتة حيويا ومن ثم زيادة تركيز النتروجين في النبات وهذه النتائج تتفق مع ماتوصل أليه (4) آذ أدى التلقيح البكتيري الى زيادة محتوى نبات الحمص من النتروجين , كما أنها تتفق مع ماتوصل أليه (6) في دراسته للتلقيح البكتيري لنبات ألماش و (9) في دراسة لاستجابة نبات الباقلاء للتلقيح بالسلالات البكتيرية ,ويمكن الاضافه الى أن تلقيح نباتات الباقلاء بسلالات الرايزومينا أدى الى تكوين العقد الجذرية جدول(3) وهي بمثابة مصانع تعمل على تثبيت النتروجين وتحويله من الصورة غير الجاهزة للنباتات الى الصورة التي يمتصها النبات أي أنها أدت الى تراكم النتروجين في النبات وهذا ما أشار أليه (2) آذ أدى تلقيح بذور فستق الحقل ببكتريا العقد الجذرية الى زيادة مستوى النبات من النتروجين.

وتبين النتائج ايضاً وجود فروقات معنوية بين الأصناف حيث سجل الصنف المحلي تركيز للنتروجين 3.174% في حين سجل الصنف الاسباني 2.856% .

وقد كان للتداخل بين المعاملات والأصناف تأثير معنوي في زيادة تركيز النتروجين في النبات اذ كان أعلى تركيز 3.970% عند تسميد النبات الصنف المحلي بينما أعطت معاملة المقارنة مع الصنف الاسباني اقل معدل بلغ 2.220% .

**جدول (5) تأثير التلقيح البكتيري في تركيز النتروجين للمجموع الخضري (%) لنبات الباقلاء**

| المعاملات | الأصناف | | معدل المعاملات |
|---|---|---|---|
| | المحلي | الاسباني | |
| معاملة المقارنة No | 2.497 | 2.220 | 2.358 |
| R405 | 3.203 | 3.120 | 3.162 |
| R417 | 2.940 | 2.603 | 2.772 |
| R450 | 3.080 | 2.830 | 2.955 |
| R467 | 3.250 | 3.030 | 3.140 |
| R483 | 3.277 | 2.860 | 3.068 |
| معاملة النتروجين $N_1$ | 3.970 | 3.330 | 3.650 |
| معدل الأصناف | 3.174 | 2.856 | |
| L . S . D . 0.05 للاصناف = 0.05523 , للمعاملات = 0.02952 , التداخل = 0.07811 | | | |

د – عدد التفرعات في النبات:

نتائج الجدول (6) تؤكد أن التلقيح البكتيري أدى الى زيادة في عدد التفرعات قياسا بمعاملة المقارنة آذ سجلت النباتات الملقحة بالسلالات R405 , R417 , R450 , R467 , R483 تفوقا معنويا فقد بلغت متوسطات عدد التفرعات لهذه النباتات 3.00 , 3.83 , 4.00 , 417 , 5.00 فرع . نبات$^{-1}$ على التوالي في حين سجلت معاملة المقارنة اقل متوسط لعدد التفرعات بلغ 2.50 فرع . نبات$^{-1}$ من جانب أخر حافظت السلالة R483 على تفوقها عن بقية السلالات البكتيرية واقترابها من معاملة النتروجين في عدد التفرعات , آذ لم تختلف معنويا عن معاملة السماد النتروجين $N_1$ وأشارت معاملات التداخل بين السلالات البكتيرية والأصناف الى وجود فروق معنوية حيث أعطت معاملة التداخل بين السلالة R483 والصنف المحلي أعلى متوسط لعدد التفرعات بلغ 5.67 فرع . نبات$^{-1}$ في حين سجلت معاملة المقارنة مع الصنف الاسباني اقل متوسط لعدد التفرعات بلغ 2.33 فرع . نبات$^{-1}$ وهذا مايؤكد أن نشاط وملائمة السلالة البكتيرية لصنف معين تتوقف على مدى انتشار ونشاط السلالة البكتيرية ومدى ملائمتها وتوافقها مع ذلك الصنف المزروع (22) لذلك نجد بان السلالات البكتيرية قيد الدراسة قد تباينت في قيمها لهذه الصفة بالنسبة للصنف الواحد.

**جدول (6) تأثير التلقيح البكتيري في عدد تفرعات نبات الباقلاء (فرع . نبات$^{-1}$)**

| المعاملات | الأصناف | | معدل المعاملات |
|---|---|---|---|
| | المحلي | الاسباني | |
| معاملة المقارنة No | 2.67 | 2.33 | 2.50 |
| R405 | 3.33 | 2.67 | 3.00 |
| R417 | 4.67 | 3.00 | 3.83 |
| R450 | 4.67 | 3.33 | 4.00 |
| R467 | 4.33 | 4.00 | 4.17 |
| R483 | 5.67 | 4.33 | 5.00 |
| معاملة النتروجين $N_1$ | 5.00 | 4.67 | 4.83 |
| معدل الأصناف | 4.33 | 3.67 | |
| L . S . D . 0.05 للأصناف = 0.931 , للمعاملات = 0.497 , التداخل = 1.316 | | | |

هـ – الوزن الجاف للجزء الخضري(غم . نبات$^{-1}$):

توضح نتائج (7) أن الوزن الجاف للمجموع الخضري لنبات الباقلاء قد تأثر معنويا بالسلالات البكتيرية المختلفة ( R450 , R467 , R483 R405 , R417,) التي سجلت (11.32,11.30,9.80,9.47,9.01) غم . نبات$^{-1}$ على التوالي في حين أعطت معاملة المقارنة 6.91 غم . نبات$^{-1}$ , أما معاملة النتروجين فسجلت 12.78 غم . نبات$^{-1}$ وهذا يتفق مع ماأشار إليه (14)الى أن استخدام البكتريا المثبتة للنتروجين يؤدي الى زيادة تراكم النتروجين المثبت وتحويله الى أحماض امينية ومن ثم الى مركبات بروتينية يستفيد منها النبات في تكوين الأنسجة المختلفة مما يؤدي الى تحسين النمو وزيادة المجموع الخضري ولم نلاحظ وجود اختلافات معنوية بين الصنفين المحلي والاسباني لصفة الوزن الجاف للمجموع الخضري . أما التداخل بين المعاملات والأصناف فقد أدى الى حصول زيادة معنوية لاسيما في المعاملات الملقحة ,فقد أعطت نباتات الصنف المحلي الملقحة بالسلالة البكتيرية R467 وزنا جاف متفوقا على جميع المعاملات الملقحة بلغ 12.72 غم . نبات$^{-1}$ مقارنة بنباتات الصنف الاسباني غير الملقحة بالبكتريا التي أعطت وزن جاف للمجموع الخضري بلغ 6.12 غم . نبات$^{-1}$ أما معاملة التداخل بين النتروجين والصنف المحلي سجلت أعلى وزن جاف بلغ 13.14 غم . نبات$^{-1}$ لكنها لم تختلف معنويا عن معاملة التداخل بين السلالة R467 والصنف المحلي ومعاملة التداخل بين السلالة R483 والصنف المحلي.

أن زيادة نسبة النتروجين الجوي من خلال العلاقة التعايشيه بين البكتريا وجذور النبات تؤدي الى زيادة نمو النبات من خلال زيادة تراكم المادة الجافة فضلا عن زيادة التفرعات نبات$^{-1}$ (جدول 6) الأمر الذي أدى الى زيادة الوزن الجاف للمجموع الخضري (8 , 3 , 10 , 6)

**جدول (7) تأثير التلقيح البكتيري في الوزن الجاف للمجموع الخضري (غم . نبات$^{-1}$) لنبات الباقلاء**

| المعاملات | الأصناف | | معدل المعاملات |
|---|---|---|---|
| | المحلي | الاسباني | |
| معاملة المقارنة No | 7.70 | 6.12 | 6.91 |
| R405 | 9.44 | 8.57 | 9.01 |
| R417 | 10.72 | 8.22 | 9.47 |
| R450 | 10.44 | 9.16 | 9.80 |
| R467 | 12.72 | 9.88 | 11.30 |
| R483 | 12.01 | 10.63 | 11.32 |
| معاملة النتروجين $N_1$ | 13.14 | 12.42 | 12.78 |
| معدل الأصناف | 10.88 | 9.29 | |
| L . S . D . 0.05 للأصناف = 1.367 , للمعاملات = 0.731 , التداخل = 1.933 | | | |

**المصادر**


1.الأمين , صادق صاحب هادي ( 1999) تأثير محتوى التربة من الطين في نشاط بكتريا اللقاح العقدية . رسالة ماجستير . كلية الزراعة . جامعة بغداد

2 . البلداوي , سلمان برهان عبد الحسين (2004) تأثير التلقيح ببكتريا الرايزوبيا في نمو وحاصل فستق الحقل . مجلة الزراعة العراقية . مجلد 9 , 77 - 85

3 . التميمي . جميل ياسين علي الكهف (1998) دراسة العوامل المؤثرة في التثبيت البيولوجي للنتروجين الجوي في نباتات الخضر البقولية . أطروحة دكتوراه . كلية الزراعة . جامعة بغداد .

4 . الكرطاني , رحيم هادي عبد الله (2005) تأثير الحديد والفسفور في كفاءة بكتريا الرايزوبيا وفي نمو وحاصل الحمص . رسالة ماجستير . كلية الزراعة . جامعة بغداد

5 . النعيمي , سعد الله نجم عبد الله (2000) مبادئ تغذية النبات . وزارة التعليم العالي والبحث العلمي . دار الكتب للطباعة والنشر . جامعة الموصل .

6 . حسن . علاء عيدان (2004) تأثير الملوحة في كفاءة بكتريا ال .Bradyrhizobium Sp في نبات الماش . أطروحة دكتوراه . كلية الزراعة . جامعة بغداد .

7 . سعد , تركي مفتن ( 1997) استجابة الحمص والعدس للتلقيح بسلالات مختلفة من بكتريا العقد الجذرية Rhizobium أطروحة دكتوراه . كلية الزراعة . جامعة بغداد .

8 . عبد الرضا , حسن علي (1997) تأثير الحديد والمولبيدنيوم في كفاءة بكتريا الرايزوبيا وفي نمو وحاصل فول الصويا . أطروحة دكتوراه . كلية الزراعة . جامعة بغداد .

9 . محمد , هناء حسن واحمد علي الجنقه (2007) تأثير التلقيح البكتيري على حاصل ونوعية الفول Vicia Faba . مجلة جامعية سبها للبحوث والعلوم التطبيقية . المجلد 6 . العدد(2) :5-14 ليبيا

10 . يوسف , أمل نعوم و سغد، تركي مفتن(1999) دور التسميد الحيوي بالبكتريا العقدية في تحسين ونمو وإنتاجية محصول ألماش (Vigna radiate L .) والحنطة (Triticum aestivum L.) الذي يعقبه . مجلة الزراعة العراقية . مجلد 4 (2) : 130 – 119

11. يوسف , امل نعوم وعبد الحميد إبراهيم صباح ورعد حبيب علي (2001) استجابة نباتات ألجت (Medicago sativa L.) المزروعة في تربة متأثرة بالملوحة للتلقيح بالبكتريا العقدية (Rhizobium meliloti) . المجلة العراقية لعلوم التربة . مجلد 1 .عدد (1) :180 -187

12 . Allen , O N . (1953) Experiments in Soil bacteriology Burgress Publishing Co . Minneapolis , Minnesota .76-79

13. Beck , D P ,Materon LA, and Afandi F. (1993) Practical Rhizobium – Legume technology manual . Technical Manual No. 19 . ICARDA . Aleppo , Syria .29

14. Bashan , Y .; Gina H., and Luz E. (2004) Azospirillum plant relation ship : physiological , molecular , agricw tural and environmental advance (1997 – 2003) . National Research Council . Canada .101-111

15. Black C A . (1965) Methods of Soil analysis . Agron . j . Publisher Madison , Wisconsin . USA : 79-86

16. Dakora F D . (2003) Defining new roles For plant and rhizobial molecules in sole and mixed plant cultures in volving symbiotic Legumes . New physiologist . 158(1) : 39 – 49 .

17. Franco C A . (1977) Contribution of the Legume – Rhizobium symbiosis to the ecosystem and Food production in exploiting the Legume – Rhizobium in tropical agriculture (Vincet , J . M ; A . S. Whiteny and J . Bose , eds .) 235 – 237 . University of Hawaii . USA .

18. Hopkins W G . (1999) Introduction to plant physiology . John wiely and sons . Inc .296-299

19. Jackson M L . (1958) Soil chemical analysis . Premtce Hall – Inc . Englewood Cliffs , N . J .90-93

20 . Longnecker N . ; Brennan R and Robson A. (1998) Lupin nutrition . In : Lupinsas crop plant , Biology , production and Utilization Gladstones , J . J . ; C . Atkins and J . Hamblin (eds) . CAB International New York , 121 – 148 .

21. Abdel – Hamid MS; Selim SH A; Ragab AA and Saleh EA (2002) Inoculation time as aprime Factor affecting successful nodulation of common pea (Phaseolus Unlgarius L

improving producitivity . In : Nitrogen Fixation by legumes in Mediterranean agriculture . (Beck , D . P . Mteron Eds ) . Martinus Nijh off Publication: 11 – 23 .

26. Simmon T . ; Sindelarova M and Kalalova S. (1992) Reaction of three Soybean (Gly cine max) cultivars to inoculation with different strain of Bradyrhizobium japonicum . Scientia Agriculture Bohemosolvaca . 24 (2) 133 – 140 .

27. Terry R E . and Jolley VD. (1994) Nitrogenase activity in required for the activation of iron stress response in iron – inefficient T203 Soybean J . of plant Nutrition . 17(8) : 1417 – 1428 .

28. Watanabe F S . and Olsen SR. (1965) Test of ascorbic acid method for determining phosphorus in water and $NaHco_3$ extract from soil . soil . sci . soc . Am . Proc 29 : 377 – 378

.) Arab univ . J . Agric . Sci Ain shams Univ . ; Cairo , 10(2) : 221 – 241

22. Mckerise B D . (1997) Summary and future Prospects For the improvement of forage Legumes using biotechnology . In : Biotechnology and the improvement of forage legumes Mckerise B . D . and B . C . E . Brown (eds): 427 – 435 . CAB International .

23 . Page A L . Miller RH, and Kenney DR. (1982) Method of soil analysis – Part 2 . (chemical and Microbiological properties ). 2nd ed . Am . Agron . Inc . Publisher . Madison , Wisconsin , USA:371-375 .

24. Richard L A . (1954) Diagnosis and improvement of saline and alkaline soils , U . S . Dept . Agr . H .b . no . 60: 36-41

25. Saxena M C (1988) Food Legumes in the Mediterranean type of environment and ICARDA'S in

# إنتاج رقائق محمصه غنية بالألياف الغذائية من تمر الزهدي (Date Flakes)

**محمد مؤيد محمد، سندس حميد أحمد وفرح داود سالم**

*دائرة البحوث الزراعية / وزارة العلوم والتكنولوجيا – بغداد– العراق*

*البريد الاليكتروني: hams_63@yahoo.com*

**الملخص باللغة العربية**

تم تصنيع رقائق تمر محمصه ذات لون بني فاتح من عجينة التمر الغنية بالألياف الغذائية ( Date Dietary Fiber paste) بعد غسلها بكمية معينه من الماء ثم معاملتها بكميه قليله من النشا وتحميصها. تحتوي هذه الرقائق على 80.0% ألياف غذائية, 9.2% بروتين, 1.6% أملاح معدنية, 1.1% سكريات مختزله, 6.1% رطوبه و2 % نشا. يمكن استخدام هذا المنتج كوجبة إفطار جيدة بعد إضافة الحليب والسكر , بالاضافه إلى إمكانية استخدامه في معالجة مرضى الإمساك.

***ABSTRACT***

Date flakes have been manufactured in our laboratories form the paste of the dates, which is rich in Dietary Fiber. The paste was first washed with water then treated with small amounts of starch then Toasted. The final product consist of 80% Dietary fiber, 9.2% protein, 1.6% minerals, 1.1% reducing sugar, 6.1% moisture, and 2% starch. This product could be used as a good breakfast meal with milk and sugar, also to treat patients suffer from constipation.

**المقدمة**

لقد ازداد إقبال الناس في الآونة الأخيرة على تناول المواد الغذائية ألحاويه على نسبة عاليه من الألياف الغذائية وهذا يرجع إلى زيادة الوعي الصحي والاهتمام بالرشاقة حيث أكدت الدراسات إن هناك علاقة عكسية واضحة بين انخفاض نسبة الألياف في المواد الغذائية ألمتناوله من جهة وظهور بعض الأمراض من جهة أخرى كالإمساك والبواسير وسرطان القولون والسمنة وغيرها (1, 2, 3).

تتواجد الألياف بنسب مختلفة في الغذاء الذي نتناوله وهي مواد ضرورية جدا للإنسان وتعطي سعرات حرارية واطئه لكونها غير قابلة للهضم من قبل أنزيمات الجهاز الهضمي للإنسان (4 ,5, 6). وهي عبارة عن مواد كاربوهيدراتيه معقدة يمكن تقسيمها إلى نوعين الذائبة وغير الذائبة (4 ,5, 7).

تشمل الألياف الذائبة (Soluble fiber) على مواد بكتينيه وأصباغ ومواد شبه سليلوزيه (Hemicellulose) (14, 15), وهي تذوب في الماء لتكون مادة هلامية تعمل على خفض نسبة الكوليسترول في الدم وكذلك تقلل من امتصاص السكريات في الأمعاء الدقيقة وبذلك فهي نافعة لمرضى السكر وهي تتواجد في الهرطمان, البزاليا, الباقلاء, التفاح,الحمضيات,الجزر والشعير (8, 9, 10).

أما الألياف الغير ذائبة (Insoluble fiber) فتشتمل على السليلوز واللكنين ومواد شبه سليلوزيه وهي متواجدة في الحنطة, الخضراوات والمكسرات ومن فوائدها زيادة حجم الغذاء في الأمعاء وتقلل من زمن مروره فيها وبذلك تمنع الإمساك , وقد فسر الباحثون بان بقاء الغذاء في الأمعاء لفترة قصيرة يقلل من مدة تعرض خلايا الأمعاء إلى المواد السامة ومنها مسببات السرطان (Carcinogens) إن وجدت وبذلك تقلل من احتمالية الاصابه بسرطان القولون (11, 12, 13).

إن التمور ألعراقيه وخاصة تمر ألزهدي التجاري يحتوي على نسبة جيدة من الألياف الغذائية حوالي (8%) (14), وان الألياف الناتجة بعد عملية استخلاص السكريات في مصانع الكحول والخل والدبس غير مستغله وتذهب هدراً كعلف حيواني. ونظراً لاحتواء الألياف على نسب لابأس بها من البروتين والأملاح المعدنية والفيتامينات وانطلاقاً من مبدأ إيجاد منافذ جديدة واقتصاديه لاستخدامات التمور خدمه لاقتصادنا الوطني نشأت فكرة تصنيع رقائق التمر المحمصة الغنية بالألياف الغذائية.

**المواد وطرق العمل**

1-تحضير عجينة التمر:

تم عزل النوى من 300غم تمر زهدي (بعد غسله جيدا بالماء) واظيف الماء بنسبة (1:3) ماء : تمر, وأجريت عملية الطبخ لمدة 40 دقيقه باستعمال خلاط كهربائي وحمام مائي بدرجة حرارة 80 م.

رشح الخليط باستخدام قمع بخنر مجهز بقماش (خام اسمر) تحت ضغط مخلخل. وتم غسل الألياف بكمية من الماء (1 لتر) للتخلص من اكبر كميه من السكريات, يبخر الراشح للحصول على الدبس. وفرشت عجينه الألياف (المتبقية في قمع الترشيح) في أواني زجاجيه وتم تجفيفها في فرن كهربائي لمدة ساعة ونصف بدرجة حرارة 120 م. تم وزن الألياف الجافة الناتجة من استخدام 300غم تمر زهدي. كما تم حساب نسبة الرطوبة ومكونات هذه الألياف من بروتين وأملاح وسكريات مختزله باستخدام جهاز كلدال وجهاز الامتصاص الذري ( Atomic Absorption), وجهاز كروماتوغراف الغاز السائل G.L.C. على التوالي(15).

إذا قدرت الرطوبة باستعمال فرن كهربائي نوع (Heraeus) وكانت درجة حرارة التجفيف المستعملة 75-80م لمدة اربع ساعات, اذ تم وزن النموذج قبل التجفيف وبعد التجفيف ،(15) وطبقت المعادلة التالية:

$$\text{الرطوبة الكلية \%} = \frac{\text{وزن النموذج بعد التجفيف}}{\text{وزن النموذج قبل التجفيف}} \times 100$$

قدر البروتين بطريقة كلدال وذلك بأخذ 0.2 غم من رقائق التمر وتنقل العينة إلى دورق هضم زجاجي ذي عنق طويل (Digestion flask) سعة 500-800مل , يوزن 18غم من كبريتات الصوديوم مع 1غم من كبريتات النحاس البلورية مع 25 مل من حامض الكبريتيك المركز ثم توضع في الدورق ويسخن المزيج في دورق الهضم تحت نقطة غليان الحامض حتى تختفي الرغوة. ونستمر بالهضم وعلى سرعة متوسطه لحين صفاء المحلول مشيراً إلى نهاية عملية التأكسد حيث تحتاج عملية الهضم من 1.5- 2 ساعة بعدها يستمر التسخين لمدة ساعة أضافية وبعد صفاء المحلول يبرد الدورق إلى اقل من 25م° ثم يضاف إلية 200مل من الماء المقطر بعدها يضاف 70- 75 مل من محلول هيدروكسيد الصوديوم المركز على الجدار الداخلي للدورق مع اضافة كمية قليلة من مسحوق الزنك او السلينيوم ويتم توصيل دورق التقطير إلى المكثف ويبدأ التقطير لحين خروج الامونيا من دورق التقطير وانتقالها إلى دورق الاستلام إذ ان أول 150 مل من المحلول المقطر يحتوي على جميع الامونيا المقطرة وبعد الانتهاء من التقطير يسحح المحلول في دورق الاستلام إلى نقطة النهاية بوساطة محلول هيدروكسيد الصوديوم القياسي (0.1 عياري)

بعد إضافة دليل الأحمر المثيلي إلى المحلول (15،17).
وبنفس الوقت يتم عمل نموذجاً خالياً من الكيمياويات (Blank) مع نفس طريقة العمل الأنفة ذكراً وبعدها تطبق المعادلة التالية:

**كمية النيتروجين %=**

**(مل حامض للعينة- مل حامض للخالي) × العيارية × ملي مكافئ النتروجين**

**وزن النموذج (غم)**

**كمية البروتين % = كمية النتروجين (%) × معامل البروتين للغذاء**

قدرت الأملاح المعدنية باستعمال جهاز الـ Atomic Absorption حيث طحن نموذج رقائق التمر باستعمال مطحنة كهربائية ووزن مقدار 0.5- 1غم منه بعدها يضاف اليه خليط من كل الحوامض المركزة التالية :
100مل من $HNO_3$ و 15مل من $H_2SO_4$ و 40 مل $HCLO_4$ و تسخن على درجة حرارة 120- 200م باستعمال (Hot Plate) لحين الحصول على لون رائق ثم يؤخذ المتبقي ويركز إلى 2مل بعدها ينقل كميا إلى دورق حجمي سعة 50مل ثم التحليل باستعمال جهاز الامتصاص الذري (18).
بالنسبة للسكريات قدرت باستعمال جهاز كروموتوغراف الغاز السائل G.L.C. ( Gas Liquid chromatography) نوع Packard 419 أمريكي الصنع مثبت علية كاشف التأين الحراري Flame Ionization Detector (FID) وعمود فصل زجاجي بطول 210سم وقطر 2ملم معبأ بمادة فصل 3% OV-17 ، ومحمله عليه مادة كروموسورب بقطر حبيبة 80-100 مايكرومتر، وكانت حرارة الفرن الابتدائية 160م° ترتفع بمعدل 10م° / دقيقة لتصل الى حرارة نهائية 240م°، اما حرارة الحقن والكاشف فكانتا 270م°. واستخدم الهليوم كغاز ناقل بسرعة جريان 30مل/ دقيقة. اما الهيدروجين والهواء فقد استخدما بسرعة جريان 30 و300 مل/ دقيقة على التوالي للحصول على اللهب. تم حقن 1 مايكروليتر من نموذج رقائق التمر المحمصة بعد طحنها واذابتها بمذيب الميثانول لتقدير السكريات ( 19 ).

1- تصنيع رقائق التمر المحمصة Date (Flakes):

تم خلط عجينة الألياف الرطبة الناتجة من 300غم تمر زهدي بـ 5غم نشا, وفرشها في أواني زجاجيه وتقطيعها إلى أشكال ثم تحميصها في فرن كهربائي بدرجة حرارة 120 م ولمدة ساعة ونصف.

2- التقويم الحسي للمنتوج (Taste panel):

اجري التقويم الحسي وفقاً لاستمارة التقويم الحسي لنماذج الاغذية من قبل عشرة مقومين طبقاً للبيانات المذكورة والدرجات المحددة في استمارة التقويم ( 16 ).جدول (1)

**النتائج والمناقشة**

تحتوي التمور ألعراقيه (وخاصة الصنف ألزهدي الذي يشكل 60-70% من إنتاج تمور العراق) على نسبة جيدة من الألياف الغذائية إذ قدرت نسبتها في عجينة التمر 8% ( 14 ), فضلاً عن احتوائها على كميه من البروتين والفيتامينات وألاملاح المعدنية جدول (2), (3) وتجدر الإشارة إلى إن هذه النسب تتغير حسب كفاءة الاستخلاص والتجفيف ولغرض الاستفادة من هذه الألياف واستخدامها للاستهلاك البشري فقد تم إجراء تجارب مختبريه متعددة لغرض الحصول على منتوج ذو طعم مستساغ ومشابه للمنتجات الأجنبية التي تصنع من الحبوب. ولغرض الحصول على عجينة متجانسة سهله التحميص والقشط فقد توصلنا إلى إمكانية إضافة نشا بمقدار 5غم إلى عجينة الألياف الناتجة من 300غم تمر زهدي مع الخلط الجيد ومن ثم فرشها في أواني زجاجية وتحميصها.
الجدول (2) يبين التركيب الكيمائي لرقائق التمر المحمصة علما بان كمية الألياف الغذائية الجافة الناتجة من 300غم تمر زهدي كانت 29غم. الجدول رقم (5) يوضح بعض منتجات الإفطار الأجنبية ومحتواها من الألياف والبروتين والأملاح المعدنية والسكريات والدهون (19،20). وعلى وجه ألمقارنه فان المنتوج (Fiber one) يحتوي على اكبر كمية من الألياف 12غم في الاونس الواحد من المنتوج (الاونس الواحد يعادل 28.3 غم). والمنتوج (All-Bran) يحتوي على 9غم ألياف و5غم سكر. أما المنتوج (Cheerios) فنلاحظ احتوائه على 2غم ألياف و330ملغم أملاح معدنية, بالاضافه إلى 2غم دهون. وبالنسبة للمنتوج (Familia Swiss Birchermuesli) فنلاحظ احتوائه على كمية قليله من الألياف 4غم وكمية عالية من السكر 6غم. إن النسبة العالية المتبقية في جميع هذه المنتجات هو محتواها من مادة النشا والتي لم يتم التطرق إليها, ويلاحظ من الجداول (3) و(4) و(5) والشكلين (1) و(2) بان رقائق التمر المحمصة يمكن إن تضاهي المنتجات الأجنبية من حيث كمية الألياف الغذائية والأملاح المعدنية. كما إن النسبة القليلة المتواجدة فيه من السكريات هي سكريات مختزله سهلة الهضم. يمكن تدعيم هذا المنتوج بالفيتامينات ومضادات الأكسدة كما هو الحال في المنتجات ألمشابهه.

**جدول ( 1 ): نتائج التقويم الحسي لرقائق التمر المحمصة. ***

| الصفة | درجة الاساس % | رقائق التمر المحمصة |
|---|---|---|
| انتظام الشكل | 25 | 21 |
| اللون | 25 | 20 |
| الرائحة والطعم | 25 | 22 |
| النسجة | 25 | 20 |
| المجموع | 100 | 83 |

*** الارقام في الجدول تمثل معدل عناصر النوعية لنموذج رقـــائق التمر المحمصة لعشرة مقومين**

**جدول (2): التركيب الكيميائي لرقائق التمر ألمحمصه الناتجة من تمر الزهدي**

| المكونات | النسبة المئوية % |
|---|---|
| ألياف غذائية | 80 |
| بروتين | 9.2 |
| أملاح معدنية | 1.6 |
| سكريات مختزله | 1.1 |
| رطوبة | 6.1 |

**جدول (3): مكونات الألياف الغذائية من الأملاح المعدنية باستعمال جهاز الامتصاص الذري**

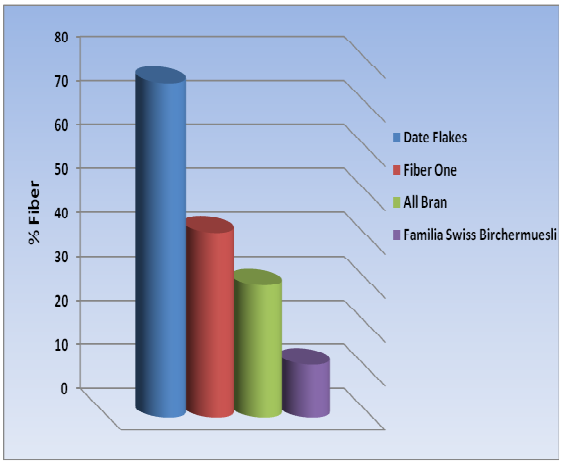
| الأملاح المعدنية | ملغم /100غم |
|---|---|
| Fe | 280 |
| Ca | 330 |
| Zn | 200 |
| Mn | 260 |
| Cu | 170 |
| Cr | 80 |
| Na | 125 |
| K | 87 |
| Mg | 49 |
| Sn | 25 |
| المجموع | 1.6غم |

**جدول (4): كمية الألياف والمكونات الأخرى في 28.3 غم من رقائق التمر ألمحمصه**

| المكونات | غم/28.3 غم منتوج |
|---|---|
| ألياف غذائية | 22.64 |
| بروتين | 2.60 |
| أملاح معدنية | 0.45 |
| سكريات مختزله | 0.31 |
| رطوبة | 1.72 |
| نشا | 0.56 |

**جدول (5): بعض منتجات الإفطار الأجنبية ومكوناتها في (28.3 غم منتوج) أي مايعادل (1 أونس) مقارنة برقائق التمر المحمصة (21).**

| اسم المنتوج | دهون غم | أملاح معدنية ملغم | سكريات غم | بروتين غم | ألياف غم |
|---|---|---|---|---|---|
| Fiber one | 1 | 220 | 2 | 3 | 12 |
| Cheerios | 2 | 330 | 1 | 4 | 2 |
| Familia Swiss Birchermuesli | 2 | 60 | 6 | 3 | 4 |
| Special (Kelloggs) | 5 | 230 | 3 | 6 | Trace |
| Familia Genuine Swiss Muesli | 4 | 2 | 7 | 3 | 3 |
| All-Bran (Kelloggs) | 1 | 270 | 5 | 4 | 9 |
| Date flakes | – | 450 | 0.31 | 2.60 | 22.64 |




**شكل(1): النسبة المئوية للألياف الغذائية في بعض منتجات الإفطار الاجنبيه مقارنة برقائق التمر ألمحمصه**

**شكل(2): رقائق التمر ألمحمصه الناتجة من تمر الزهدي**

يوصي المعهد الوطني الامريكي للسرطان(22) بضرورة تناول كمية من الألياف الغذائية تتراوح بين 20 إلى 35 غم للفرد في اليوم, إن هذه الكميه يمكن الحصول عليها بتناول كوبين من منتوج رقائق التمر المحمص بحدود (30غم من المنتوج النهائي) كوجبة إفطار مع الحليب كما إن الأملاح المعدنية المتواجدة في هذه الوجبة تقع ضمن الحدود المسموح بها (23).

من الفحوصات التي تجرى عادة على المنتوجات ألمشابهه لمنتوجنا هي احتمالية بقاء أجزاء من المنتوج بعد تناوله أو التصاق بعضه على الأسنان ( Tooth pack and Residual particles) التي تتصف بها بعض المنتجات الرديئة (21).

فقد تم اختبار المنتوج من قبل عشرة أشخاص (Taste panel) جدول ( 1 ) واتفق الجميع على عدم بقاء أي من أجزائه في الفم أو التصاقه في الأسنان، ولغرض بيان تأثير الخزن فقد تم حفظ المنتوج في أكياس من السيلوفين محكمه الغلق في المختبر ولمدة ستة أشهر ولم يلاحظ اي تغيرات في الطعم والرائحة واللون ومع ذلك فيمكن معاملة هذا المنتوج كباقي المنتجات الأخرى المشابهة بإضافة مضادات ألأكسدة أو استخدام غاز خامل كغاز النتروجين في الحفظ.

يمكن إنتاج رقائق التمر ألمحمصه على نطاق تجاري وبأشكال مختلفة وذلك بغسل التمر جيداً وإجراء عملية الطبخ وعزل النوى باستخدام (Destoner) والترشيح باستخدام مرشح أولي (Decantor) ثم تنقل الألياف إلى خزان فيه خلاط ويضاف له كميه من الماء ويرشح مرة أخرى باستخدام مرشح ثاني وتنقل الألياف إلى حوض يتم إضافة النشا مع الخلط الجيد وتصنع بحيث يمكن دفعها إلى اسطوانات معدنية ساخنة لغرض التحميص وإعطائها الشكل المطلوب. تكمن اقتصادية الإنتاج بالحصول على الدبس من تبخير وتركيز المستخلص السكري أو الحصول على السكر السائل بعد ترويق المستخلص السكري وإمراره على المبادلات ألايونيه ثم تبخير وتركيز المستخلص السكري أيضا (14) والحصول على منتوج رقائق التمر المحمصة بالحالتين واستخدام النوى بعد طحنه كعلف حيواني حيث يمكن خلطه مع باقي الأعلاف بنسب محددة.

**المصادر**


1. Weickert, M. D. and Preiffer, A. F. (2008). Metabolic effects of dietary fiber consumption and prepention of diabetes. J. Nutur., 138(3): 439-442.
2. Rodriguez-Cabezas, M. E.; Galvez, J. and Camuesco, D. (2003). Intestinal anti-inflammatary activity of dietary fiber. 22(5): 463-471.
3. Salvin, J. L. (2008). Health implication of dietary fiber, Jounal of the American Dietectic Association. 108: 1716-1722.
4. Mcintosh, M. and Miller, C. A. (2001). Diet containing food rich in soluble and insoluble fiber improves glycemic control and reduces hyperlipidemia among patients with type 2 diabetes. Mellitus. Nutr. Rev., 59(2): 52-55.
5. Southgate, D. A. T. (1982). Definitions and terminology of dietary fiber in Health and

Disease. Ed. G. Vohouny and D. Kritchevsky. Plenum press New York.

6. Reiser, S. (1987). Metabolic effects of dietary pectin's related to Human Health. Food Technology, 41(2): 91-94.
7. Schneeman, B. O. (1986). Dietary fiber and chemical properties. Method of analysis, and physiological effects, Food Technology, 40(2): 104-110.
8. Venn, B. J. and Mann, J. I. (2004). Cereal grains, legumes, and diabetes. Eur. J. Clin. Nutr., 58(11): 1443-1461.
9. Lee, Y. P.; Puddey, I. B. and Hodgson, J. M. (2008). Protein, fiber and blood pressures potential benefit of legumes. Clin. Exp. Pharmol. Physiol., 35(4): 463-466.
10. Pereira, M. A. and Ludwig, D. S. (2001). Dietary fiber and body-weight regulation, observations and mechanisms. Pediatr Clin. North. Am., 48(4): 969-980.
11. Fuchs, C. S. (1999). Dietary fiber and the risk of colorectal cancer and adenoma in women. N. Engl. J. Med., 340(3): 169-176.
12. Davy, B. M. and Melby, C. L. (2003). The effect of fiber rich carbohydrates on feature of sydrom. J. Am. Diet. Asso., 103(1): 86-96.
13. Park, Y. (2005). Dietary fiber intake and risk of colorectal cancer JAMA. 294: 2849-2854.
14. Al-Hakim, M. (1997). Semipilot plant for the production of ligid sugar from Zahdi Dates. Iraqi patent, No. 2627.

15. باسل كامل دلالي وصادق حسن الحكيم . (1987), تحليل الأغذية – العراق – جامعة الموصل.

16. المحمد، نعيم ثاني (1986). مباديء الاحصاء، وزارة التعليم العالي والبحث العلمي – مكاتب دار الكتب للطباعة والنشر – العراق – جامعة الموصل.

17. Schneeman, B. O. (1987). Soluble vs Insoluble fiber, Different Physiological Responses, Food Technology, 41(2): 860-881.
18. C. Ray Campbell and C. Owen plank. (1998). Hand book of reference methods for plant analysis Edited by : Yash P. Kalra Boca Raton London New York Washington, D. C.:37-45.
19. Jaddou, H. and AL-Hakim, M. (1980). ((Gas liquid chromatography of trimethylsilyl derivatives of sugar from Iraqi dates)), Journal of Agriculture Food Chemistry., 28:1208-1214.
20. Institute of Medicine. (2005). Dietary references in takes for energy, carbohydrates, fiber, fatty acids, cholesterol, protein, amino acid. Washington, D. C. National Academies press. P: 347.
21. Consumer Reports (USA). (1986). On Ready-to-eat cereals, We looked at 59 cereals and turned up big differences in fiber, protein sugar sodium and fat. 628-630.
22. American Cancer Society. (1989). Special report on nutrition and cancer cause and prevention.
23. Toma, R. B. and Curtis, D. J. (1986). Dietary fiber, effect on mineral and bioavilability, Food Technology, 40(2): 111-114.

# INSTRUCTIONS FOR AUTHORS

## *INTERNATIONAL JOURNAL of Science and Technology (IJST)*

### Aims and Scope

***International Journal of Sciences and Technology*** **(IJST)** is an international scope journal. The journal is edited by an international recognized Editorial Board. It is published online and printing hard copies every three months.

IJST publishes original research papers on all aspects of science and technology including scientific disciplines of Microbiology, biotechnology, cell biology, ecology, entomology, environmental science, forestry, genomics, horticulture, animal science, plant sciences, proteomics, agricultural and food science, biostatistics, biological sciences and bioengineering, computer science and engineering and water resources.

Authors are required to sign a Copyright Form granting the publishing rights for all papers accepted for publication. Production will not start until we have received of a signed Copyright Form available at http://www.icast-jo.com

### General Instructions

IJST will publish original full papers, short research communications, and letters to the editor. Full papers should be concise without compromising clarity and completeness, and should generally occupy no more than 10 published pages. Short research communications should not be more than 5 printed pages (excluding references and abstract). Results and Discussion section should be combined followed by conclusion. Materials and Methods will remain as a separate section.

### Submission of Manuscripts

Authors are required to submit their articles to IJST online for quick and more efficient processing at http://www.ijst-jo.com

Prior to submission, Authors may contact the editors to inquire about the suitability of their work at: info.icast@yahoo.com

## Preparation of Manuscripts

### ***Language***

Papers must be written in English and in Arabic, in both cases abstract in second language require. Authors whose native language is not English are strongly advised to have their manuscripts checked by an English-speaking colleague prior to submission. Manuscripts that are deficient in this respect may be returned to the author for revision before scientific review.

### Presentation of Manuscripts

* Title
* Author names and addresses
* Abstracts (Not more than 250 words)
* Key words
* Introduction
* Materials and Methods
* Results and Discussions
* References (Use numbering in the text instead of full references.
Give full references at the end of the manuscript
* The photographs should be of high quality resolution
* Graphs should be in clearly visible form so that it may become easy to redraw

The manuscript should be prepared using Microsoft Word with the following layout.

1. Manuscript should contain title page, abstract, main body, and references.
2. Tables should be added after references and each new table should be on a separate page.
3. Figures should follow the tables, putting each figure on a separate page ensuring that the figure is at least the size it will be in the final printed document. Number each figure outside the boundary of figure. Resolution of the figures should be at least 400 pixels/cm (1000 pixels/in).
4. Number manuscript pages consecutively and activate line numbering.

5. The manuscript should be double-spaced. The beginning of each new paragraph must be clearly indicated by indentation. Left-justify the text and turn off automatic hyphenation. Use carriage returns only to end headings and paragraphs. Artificial word breaks at the end of lines must be avoided. Do not insert spaces before punctuation.
6. Please use standard fonts such as Normal Arial. Use consistent notations and spellings
7. Please follow internationally accepted rules and conventions for gene and protein names, units, for symbols, and for capitalization in text, tables, and figures.

**Title Page**

The title page should include a concise and informative title, author names in full, and affiliations. The name of the corresponding author as well as his/her mailing address, telephone and fax numbers, and e-mail address should be provided in a footnote.

**Abstract**

The abstract should be one paragraph, no longer than 250 words. No references should be cited in the abstract. Abbreviations should be avoided, but if they have to be used, they must be defined the first time they appear. A list of keywords (up to six) must be included after the abstract for indexing purposes. Words that appear in the title should not be repeated in the keywords.

**General Arrangement of Text**

The text should be divided into sections with the headings: Introduction, Materials and Methods, Results, and Discussion. Subheadings within sections except introduction can be used to clarify their contents. Introduction and Discussion sections may contain present tense to convey generally accepted information. Materials and Methods and Results are normally written in the past tense.

*1. Introduction*

The introduction should define the problem and provide sufficient information to explain the background but there is usually no need for a comprehensive literature survey. The objectives should be stated but it should not contain a summary of the results.

*2. Materials and Methods*

Sufficient detail must be provided to allow the work to be repeated. This section should contain the experimental protocols and the origin of materials, tissue, cell lines, or organisms.

*3. Results*

The Results section should be in logical order presenting the experimental results. Please do not include any interpretations, inferences, arguments or speculations in this section.

*4. Discussion*

The authors should interpret their results clearly and suggest what they might mean in a larger context. Please do not repeat the information provided in the Results section.

**Acknowledgements**

Assistance received from funding agencies and colleagues should be acknowledged in this section.

**References**

Published or “in press” articles may be included in the reference list. Unpublished studies should be referred to as such or as a personal communication in the text. Citations of references should use square brackets, e.g. [1,3,5-7]. The lists of references, tables or figures should be numbered consecutively staring from 1. The references should be contain the last names and initials of up to four authors, year of publication, title of the paper, and the title of the journal. These should be followed by the volume and page numbers. References to books should include the title of the book, the year of publication, the publishing company and the place of publication. *Some examples are given below.*

*Smith PF, Patel KR, Al-Shammari AJN. 1980. An Alde hydro-Phosphoglycolipid from Acholeplasma granularum. Biochem.Biophys. Acta 617: 419-429*

*Sambrook J, Russell DW. 2001. Molecular Cloning: A Laboratory Manual, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*

*McCarthy, AJ. 1989. Thermomonospora. In: Bergey's Manual of Systematic Bacteriology (ed. Williams ST, Sharpe ME, Holt JG), Vol. 4, pp. 2552-2572. Williams and Wilkins, Baltimore, MD.*

If web site references are used, the URL should be included next to information in the text. Please check the references carefully for accuracy, missing information, and punctuations.

*Example*

Department of Health: The Interdepartmental Working Group on Tuberculosis 1999. The Prevention and Control of Tuberc1ilosis in the United Kingdom [Online] [accessed 2000 September]. Available from URL http://www.doh.gov.ukltbguide1.htm

**Tables and Figures**

Tabulation and illustration should not be used for points that can be adequately and concisely described in the text. Tables and figures should be understandable on their own without reference to the text. Explanatory footnotes should be related to the legend or table using superscript lower-case letters. All abbreviations should be defined after the footnotes below the table or by reference to a previous table in the same paper.

**Review Process**

The Journals aim at rapid publication of research results while maintaining a rigorous peer review process. The editors will provide an initial response to all pre-submission enquiries within a week. In addition, they will make every effort to give authors a decision following peer review within four to eight weeks of an article's submission. If the review process takes longer due to special circumstances, authors will be notified promptly by e-mail. Accepted articles will be published online within two weeks. Suggestions by the editors about revision do not imply that a revised version will necessarily be accepted. If minor revision is required, authors should return a revised version in two weeks. If major revision is required, authors should return a revised version within three months. You must clearly indicate the changes that have been made. Revised manuscripts that are not resubmitted within the indicated time frames will be treated as a newly submitted manuscript. Authors who disagree with Editor's decisions may contact the Chief Editor for final decision. The Editor and the Publisher

are not responsible for the scientific content and statements of the authors of accepted papers.

**Proofs**

Manuscripts should be checked carefully before submission since substantial alterations will not be permitted at the 'proof' stage. The editors reserve the right to make minor alterations to the text without altering the scientific content. Corrections should be sent to publisher in one communication. If corrections are not received within 48 hours, publisher reserves the right to proceed with publication.

**Reprints**

Reprints may be ordered in quantities of 30 using the form supplied with the proofs.

**Publication Charges**

Because the IJST is auto- self dependent, we will ask the author to pay US$200.00 as publication charge.

**Ethical Issues**

It is assumed all authors have approved the submitted manuscript, which is the responsibility of corresponding author. If accepted, the article must not be published elsewhere in any form, without the consent of the editors and publisher.

**Reproducing Published Articles**

Individuals wishing to reproduce figures, tables and excerpts of text from articles published in IJST for non-commercial purposes may do so providing the original publication is acknowledged accordingly and the authors' approval is obtained, and in this case no special permission is needed from either the publisher or the editors. Authors may also include the article in a thesis without special permission. In all other cases, permissions may be sought directly from IJST.