Detection of Ampicillin Transposon in *Klebsiella pneumoniae*

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التحرى عن العنصر القافز ـ الأمبسلين فى *Klebsiella pneumoniae*

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

قالوا سبحانك لا علم لنا
الا ماعلمتنا
انك انت العليم الحكيم

صدق الله العظيم

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الخلاصة

تكون بكتريا ١٠ .No *Klebsiella pneumonae* غير مستقرة وراثياً وتمتلك بلازميد كبير منفرد غير اقتراني حجمه ١٢٠kbp يحتوى على جينات مقاومة الامبسلين والتتراسايكلين والستربتومايسين والكاناميسين والترايمثبرم والكلورامفينكول وحامض النالدكس وكذلك يشفر عن الصفة المخاطية في البكتريا.

تم اشتقاق أربعة مجاميع من ضروب (*K. pneumoniae I, K. pneumoniae* G, *K. pneumoniae* K, *K. pneumoniae* L) من هذه العزلة بوساطة الزرع المتكرر في وسط M_9 المزود بـ ١٥٠ مايكروغرام/مل من حامض السالسليك. تتباينت مجاميع ضروب بالنسق البلازميدي ونمط المقاومة للمضادات الحيوية مقارنة بالعزلة البرية ١٠ .No *K. pneumonae*. تكون ضرب *K.pneumoniae I* حساسة لترايمثبرم والسترتيومايسين والريفامبسين لذلك تم استعمالها كخلايا مستقبلة للبلازميد الاقتراني *R٧٥١* غير المتوافق (*Inc P*).

تمكن الامبسلين بتركيز ١٥٠ مايكروغرام/مل من حث عدم الاستقرار الوراثي للبكتريا I *K. pneumoniae* عند تكرار زرعها ٥٤٠ جيل في وسط الامبسلين. ويتأثر الاستقرار الوراثي أيضاً بانخفاض درجة الحرارة (٣٠°م). وجد بأن العزلة تقاوم تراكيز عالية أكثر من ١٠٠٠ مايكروغرام/مل من الامبسلين مقارنة بمضادات أخرى مثل الكلورامفينكول والتتراسايكلين والنوفوبايوسين والاموكسيلين. وتقترح هذه النتائج احتمال وجود عنصر قافز يشفر إلى مقاومة الامبسلين في هذه العزلة.

تم حث العنصر القافز Ap::Tn على الانتقال من البلازميد *PKPI* في خلايا المقترنة (TR_1 *K. pneumoniae*) (المحتوية على البلازميدين *PKPI* و*R٧٥١*) إلى البلازميد الاقتراني *R٧٥١* بوساطة تكرار زرعها في وسط زرعي مزود بـ ١٥٠ مايكروغرام امبسلين وفي درجة حرارة ٣٠ و ٣٧°م.

أظهر الترحيل الكهربائي في هلام الاكاروز لمحتوى DNA للخلايا الناتجة من عملية الانتقال وجود حزم بلازميدية ذات حجم إضافي مقارنة بالبلازميد الأصلي *R٧٥١* وأدنى من حجم البلازميد *pKPI*. وتقترح هذه النتائج احتمال حدوث انتقال العنصر القافز Ap-Tn من البلازميد *pKPI* إلى *R٧٥١*. تم الحصول على ثلاثة أنواع من الخلايا المقترنة *E, coli* TR_2 و TR_3 و TR_4 بعد عملية الاقتران بين الخلايا الناتجة من عملية الانتقال والخلايا المستقبلة من بكتريا *E. coli* MM٢٩٤ المقاومة للريفامبسين والخالية من البلازميدات. وأظهر الترحيل الكهربائي في هلام الاكاروز لمحتوى الـ DNA لأنواع الخلايا المقترنة بأنها تحتوي على البلازميد الاقتراني *R٧٥١* ذو حجم إضافي مقارنة بحجم البلازميد الأصلي *R٧٥١*.تبين هذة النتائج احتمال احتواء البلازميد الاقترانى على العنصر القافز المشفر لامبسلين.

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List of Abbreviations

Bla genes …….. ß-lactamase genes

ESBL …… ……... Extended spectrum ß-lactamase

Kbp ……..Kilo base pairs

IRS…… ……....Internal Resolvase sites

ICU ……. Intensive care unite

OXA. ……Oxacillin

R plasmid……Resistance plasmid

SHV……. Sulphydryl variable

TGE…… …..Transposable Genetic Elements

TEM……. Temoniera

Tn……..Transposon

s……..Transfer genes *tra* gene

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Conclusions

١. The detection of transposon encoding an extended-spectrum β-lactamase (ampicillin) on large plasmid *pKpI* in *K.pneumoniae* isolate No.١٠ from patients suffer from upper respiratory inflammation in hospital where large quantities of extended-spectrum β-lactam antibiotic are used , raises concern for the increased spread of resistance to this group of antibiotics.

٢. It has been concluded that gene responsible foe conferring resistance to ampicillin reside in a ٥.٠٠٠ bp mobile DNA segment or transposon which have ability to translocated themselves by both Intramolecular and intermolecular transposition.

٣. We deduced that transposon which conferring ampicillin resistances in *K.pneumoniae I* and transposed via co-integrate formation is belonging to Tn٣ family.

٤. Translocation of ampicillin transposon accompanied with disturb of regular or structural genes function due to insertion inactivation of this transposon to those genes.

٥. Transposition of transposon that carry ampicillin determinant was more in ٣٠C° than ٣٧C°, consequently the total change in antibiotic susceptibility pattern for *K.pneumoniae I* that grew at ٣٠C° was more than *Klebsiella pneumoniae I* that grew at ٣٧C°.

Chapter one

١.١: Introduction

Bacteria belonging to genus *Klebsiella* frequently cause human nosocomial infections. In particular, the medically most important *Klebsiella* species, *Klebsiella pneumoniae*, account for significant proportion of hospital acquired urinary tract infection, pneumonia, septicemia, and soft tissue infection (Emori and Gaynes ١٩٩٣*). K.pneumoniae* is frequently found in many different geographical locations, with beta-lactamase resistance becoming a growing problem (Matthew, ١٩٧٩).

The wide spread use of antimicrobial agents in the treatment of infections in the tropics has led to serious problems of antimicrobial resistance. The emergence and spread of antibiotic resistance in bacteria of medical importance imposes serious constraint on the option available for treatment of many infections (Nikadio, ١٩٩٨). Li and Lim (٢٠٠٠) mentioned that in the last ١٠ years, the extensive spread of multiple antibiotic –resistant *Klebsiella pneumoniae* strains, especially the extended-spectrum beta lactamase producing strains (ESBLs), has become a major threat to the ever-increasing number of immunocompromised patients; the ESBLs are usually plasmid mediated (R plasmid) offer resistance to antibiotics and are transmissible from one cell to another by direct cell contact (conjugation).

Conjugative plasmids are thought to be responsible for the rapid spread of beta-lactamase resistance in Gram –negative bacteria (Reed, ١٩٨١).

Another factor believed to be involved in the increasing of resistance is the presence of transposon carried beta-lactamase, which leads to spread of beta-lactamase resistance among different plasmids (Sander and Sander, ١٩٩٢).

Transposons are widespread in nature, having been identified in the genomes of numerous organisms, from bacteria to humans. Transposition can alter the genome functionality. It is obvious that transposition of a mobile element into particular gene inactivates it. However, insertion of a transposon can also activate the expression of neighboring normally cryptic genes (Hõrak and Kivisaar, ٢٠٠١).

Transposition can promote large DNA rearrangements including deletions, inversions and replicon fusions. Additionally, capability of transposons to transmit genetic information between cells makes transposons important tools in horizontal gene transfer. Thus, it is

evident that mobile genetic elements have important roles in genome organization and reorganization and as a consequence in the genome evolution (Moghaddam, ٢٠٠١).

Many transposons code only for factors that are needed for propagation of their DNA Using functions of the host they can spread in the genome in a replicative mode being able to overreplicate their host. Therefore, the transposons are often viewed as molecular parasites or as selfish DNA-s (Doolittle and Sapienza, ١٩٨٠, Orgel *et al*., ١٩٨٠). However, the idea that mobile elements are primarily parasitic is one-sided. Transposons often code for genes, for example antibiotic resistance conferring genes that could be useful for host under certain conditions. Really, the relationship between the transposable element and host genome may be highly variable ranging from parasitism to mutualism (Kidwell and Lisch, ٢٠٠١). Mobility of bacterial transposons is strictly regulated to low levels ($١٠^{-٢}$ to $١٠^{-٨}$ per element per generation (Kleckner, ١٩٩٠) in order to maintain the balance between their propagation and potential destructive mutagenic effects to their hosts. Actually, transposable elements stay mostly in the quiet state and translocate only in a narrow window of host cell cycle or solely in response to certain stimuli. Barbara McClintock, the discoverer of transposable elements, has suggested that transposition activity could be a response to challenges to the genome (McClintock, ١٩٨٤). Indeed, it has been shown that different stresses such as carbon starvation, temperature effects and UV light can enhance transposition of bacterial mobile elements. Moreover, it has been hypothesized that activation of transposition due to stress might serve as an adaptive response to overcome stress and to evolve the new traits (Wessler, ١٩٩٦; Capy *et al*., ٢٠٠٠).

This study included an attempt to

١- Derive variants from *K.pneumoniae* No.١٠ using salicylic acid, in order to serve as recipient host for *Inc P* plasmid R٧٥١.

٢- Induction of transposition of transposable element that confers ampicillin resistance using ampicillin stresses on *K.pneumoniae* No.١٠.

٣- Study of transposition frequency and their effect on genes that encoding antibiotic resistance in both ٣٠ and ٣٧C°.

٤- Finally isolation of ampicillin transposon on *Inc P* plasmid R٧٥١ Tp^{r}.

٢: Literature Review

٢.١: Genus *Klebsiella*:

Klebsiella is straight rod, ٠.٣-١.٠ µm in diameter and ٠.٦-٦.٠µm in length, arranged singly in pairs, or in short chains. Cells are capsulated, Gram negative non motile, facultatively anaerobic, chemoorgano-trophic, having both a respiratory and fermentative type of metabolism. Optimal temperature is ٣٧ C°.D-Glucose and other carbohydrates are catabolized with the production of acids and gas .Oxidase negative and catalese positive Indole, methyl red, Voges-proskauer, and Simmons citrate reactions vary among species .Several species hydrolyze urea .grow on KCN. H_2S is not produced .Reduces Nitrates. Most species ferment all tested carbohydrates except dulcitol and erythritol (Holt *et al.,* ١٩٩٤).

Morphologically *Klebsiella* species simulate *Escherichia coli* except that they are non motile and possess polysaccharide capsule. They are widely distributed in nature, occurring both as commensals in human and animal intestine as well as Saprophytes in soil, water and vegetations (Chakraborty, ١٩٩٩).

The taxonomy of *Klebsiella* is characterized by nomenclature reflecting its colorful taxonomic history. Originally the medical importance of genus *Klebsiella* (family Enterobacteriaceae) led to its being subdivided into three species corresponding to disease they caused: *K.pneumoniae, K.ozaenae, and K.rhinoscleromatis* .As the taxonomy became increasingly refined due to development of new methods such as numeric taxonomy, the species classification in this genus was continually revised (Bascomb *et al.,*١٩٧١).

In the early ١٩٨٠s*, Klebsiella* isolated from the environment, which had been classified as *"Klebsiella*-like organism" were increasing being classified into provisional taxa. This group gave rise to four new species: *Klebsiella terrigena, Klebsiella orinthoinolytica, Klebsiella planticola, and Klebsiella trevisanii* (Ferragut *et al.*, ١٩٨٣). In ١٩٨٦, the last two species were combined in to one species, *Klebsiella planticola*, because of their extensive DNA sequence homology (Gavini *et al.,* ١٩٨٦).

The vast majority of *Klebsiella* infections, however, are associated with hospitalization, as opportunistic pathogens .*Klebsiella* spp. Primarily attack immunocompromised individuals who are hospitalized and suffer from severe underline disease such as diabetes mellitus or chronic pulmonary obstruction. Nosocomial *Klebsiella* infections are caused mainly by *K.pneumoniae*, the medically most important species of the genus. To a much lesser degree*, K.oxytoca* has been isolated from human clinical specimens (Horan *et al*., ١٩٨٨).

Klebsiella are fairly common cause of urinary tract infection and occasionally give rise to severe bronchopneumonia ,some times with chronic destructive lesions and multiple abscess formation in the lung(Friedländer's pneumonia).in many cases there is also bacteraemia and mortality is high (Gross *et al* .,١٩٩٢).

٢.٢: *Klebsiella pneumoniae:*

This microorganism was first isolated by Friedländer (١٨٨٣) from patients suffering from labor pneumonia. These are short plump Gram-negative bacilli non motile and capsulated. They ferment sugar (glucose, lactose, sucrose, and mannitole) with production of acid and gas and split urea by means of urease. They do not produce indole usually MR carbohydrate-rich medium. This characteristic distinguish them from other Enterobacteriaceae, except from some strains of *Enterobacter aerogenes* and *E.coli* (Holt *et al.,* ١٩٩٤)**.**

Klebsiella spp., particularly *K. pneumoniae*, is an important cause of nosocomial infection. *K.pneumoniae* infection may occur at almost all body sites but the highest incidence is found in the urinary and respiratory tracts. The main populations at risk are neonates, Immunocompromised hosts, and patients predisposed by prior surgery, diabetes, malignancy, etc. (Emori and Gaynes,١٩٩٣*)*. The member of genus *Klebsiella* especially *K.pneumoniae* have also been linked to epidemics of diarrhea, because some strains appear to have acquired plasmid from *E. coli* (that code for heat labile and heat stable enterotoxins) (Ewing, ١٩٨٦).

The wide spread use of antimicrobial agents has failed to endicate microbial infection despite their benefits. Antibiotic resistance bacteria have been a source of ever increasing therapeutic problem continued mismanaged selective pressure that has been regarded as an inevitable genetic response to antimicrobial therapy (Cohen and Auxe ١٩٩٢). The antibiotic resistant mutants that arise spontaneously are generally resistant to only one antibiotic. However, *Klebsiella* spp., exhibit simultaneous resistance to multiple drugs (Gutmann *et al.,* ١٩٨٥). The R plasmid offer resistance to antibiotics and are transmissible from one cell to another by direct cell contact. Conjugation of transferring drug resistance genetic determinants among intra and intergeneric bacterial population .A surveillance study has demonstrated the emergence of highly resistant *K.pneumoniae* in urinary and respiratory tract infections (Bonafed and Lois,١٩٩٧).

The existences of multiple antibiotics -resistant K.pneumoniae strains are notorious and have complicated therapy. Mortality rates at up to ٥٠٪ have been found in respiratory tract infection as an alternative to antibiotic treatment, prevention and / or treatment of *Klebsiella* infections by immunotherapy has received more attention in recent years (Donta *et al.,* ١٩٩٦).

٢.٣: Genetic contents *of K. pneumoniae*

The genetic material in bacteria is carried on double - stranded circular DNA molecule, folded multiple times to fit inside the cell and attached to the plasma membrane. This DNA molecule is generally referred to as the bacterial genome. It contains all the information for controlling the development and metabolic activities important for bacterial survival. The typical bacterial genome is a single chromosome, about ١.١٠٠ mm , containing up to ٤٠٠٠ kbp of DNA, folded into a tight mass, often less than ٠.٢mm in diameter, and complexed with small amounts of protein and RNA (Cano *et al*., ١٩٨٦).

Numerous bacteria contain, in addition to their genome, one or multiple copies of small self-replicating pieces of circular double stranded DNA, called plasmids. The molecular mass of the plasmid is between ١.٥ - ٣٠٠ MD and it contains ١-٣٠ kbp. Small plasmids contain only a few genes, while the larger one may consist hundreds of genes (up to ٣٠٠ genes) (Jawetz *et al.,* ١٩٩٥). After division of the bacterial cell, all the following generations will have the same plasmid profile, belonging to a same bacterial clone. The transfer of the plasmid between cells can be accomplished by conjugation or transformation. Genetic information, required for transfer by conjugation, is supplied by the so-called *tra*-genes, carried on the other side, by self-transmissible plasmids. Plasmids transferred by conjugation, continue to multiply once they have entered the new host. Some of their genes can even be transferred to the chromosome as transposons leading to the acquisition of functions not normally encoded in the chromosome (Zajcek, ١٩٩٤).

In general, the plasmid's genetic information is not necessary for the survival of the cell. However, they can supply the bacterial cells with new features, important for their survival in new and extraordinary conditions. Depending on what kind of a function the plasmid determinates, there are different types of plasmids: F-plasmids -

which contain genes for production of F-pills, Col-plasmids - determine a production of bacteriocines, Vir-plasmids - closely related with the pathogen potential of different bacteria, Ent-plasmids - with genes for exotoxine production determination. There are plasmids that often carry genetic information that makes the bacterium resistant to certain antibiotics and heavy metals, able to synthesize or break down unusual compounds, resistant to ultraviolet light or able to produce bacteriocines and toxins. For example, antibiotic resistance genes may be carried on plasmids (so called R-plasmids) and the plasmids are the structures that code for enzymes that acetylate adenylate or phosphorylate various antibiotics (Cano *et al*., ١٩٨٦).

Nowadays, nosocomial infections are an important cause of morbidity and mortality. Predominant pathogens are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus*, *E.coli*, *K.pneumoniae* and *Candida* spp (Haur *et al*., ١٩٩٦). Among Gram-negative bacilli, which comprise the majority of nosocomial pathogens, *K. pneumoniae*, has considerably big role (Horan et al., ١٩٨٨).

The genus *Klebsiella*, as a member of the family Enterobacteriaceae, encloses two medical significant species: *K.pneumoniae* (with four variations-*K.pneumoniae* var. *aerogenes, var. pneumoniae, var. rhinoscleromatis and var. ozaenae)* and *K. oxytoca. K.pneumoniae* is a member of the normal intestinal microflora and it can be found in minority as a part of the microflora of the upper respiratory tract. It becomes pathogenic only when will reach tissues outside of its normal flora site*. K. pneumoniae* often colonizes the skin and mucosae of hospitalized patients in intensive care units, with risk of invasive infection and septicemia. The colonization of the host's gastrointestinal tract by multi-antibiotic resistant *K. pneumoniae* strains is thought to be an essential step in nosocomial *K.pneumoniae* infections. The most frequent sites of clinically important infections are the urinary, billiary and lower respiratory tract, but it can also play a role in septicaemia, meningitis and osteomyelitis (Seldon *et al.,* ١٩٧١).

٢.٤:.Antimicrobial resistance:

Antimicrobial resistance is defined as “a property of bacteria that confers the capacity to inactivate or exclude antibiotics, or a mechanism that blocks the inhibitory or killing effects of antibiotics, leading to survival despite exposure to antimicrobials” (Ingli *et al.*, ١٩٩٤.).

Bacteria can exhibit resistance in different ways, antimicrobial resistance can occur as a result of random genetic mutations in bacteria, leading to variation in susceptibility within any bacterial population. Single-step mutation in the SHV-١ ß-lactamase enzyme makes SHV-٢ which rendered *K.pneumoniae* resistance to extended spectrum cephalosporins (Barthèlèmy *et al*., ١٩٨٨). More commonly, resistance is not due to a chromosomal event, but due to the presence of extrachromosomal DNA (plasmids) acquired from another bacteria. Different mechanisms that render antibiotics ineffective have evolved in bacteria. One of the most common mechanisms is the production of enzymes that degrade antibiotics. Resistance to aminoglycosides, beta-lactam (penicillin and cephalosporins), and chloramphenicol is by enzyme inactivation (Davies, ١٩٩٤). Bacteria have also developed the ability to modify their cell surfaces to have reduced affinity for antibiotics (Spratt, ١٩٩٤). Resistance to fluoroquinolones is chromosomally encoded and involves mutation in the target genes including DNA gyrase and topoisomerase (Chen and Lo, ٢٠٠٣). Active efflux of antibiotics as a resistance mechanism was first described for resistance to tetracycline in *E. coli* by McMurry *et al*., (١٩٨٠). Some types of efflux pumps are responsible for multiple antibiotic resistances. These pumps cover a relatively wide spectrum of antibiotics, chemotherapeutic agents, detergents, dyes, and other inhibitors (Nikaido, ١٩٩٨).

٢.٤.١: Beta-Lactams:

The beta-lactam-based antibiotics inhibit bacterial growth by blocking the final stage in cell wall synthesis that relates to cross-linking of peptidoglycan polymers by transpeptidation .The peptidoglycan polymer is an important constituent of the bacterial cell wall and is composed of alternating residues of N-acetylglucosamine and N-acetylmuramic acid. The N-acetylmuramic acid residues are substituted by peptide chains which are cross-linked to form a mesh-like character and provide structural integrity to the cell wall (Weidel and Pelzer, ١٩٦٤). The cross linking is carried out with the help of transpeptidases through a catalytic process. After each catalytic cycle, one free alanine amino acid is released and the transpeptidase enzyme is regenerated which can then participate in another catalytic cycle (Sawant, ٢٠٠٥).The transpeptidation step of cell-wall biosynthesis is blocked by beta-lactams. Inhibition of transpeptidase activity by penicillins occurs through the formation of covalent acyl enzyme intermediates. The transpeptidase gets depleted by irreversible binding with penicillin, which presents normal cross linking and eventually results in loss of integrity of the cell wall structure, which leads to cell death (Blumberg and Strominger, ١٩٧٤; Sawant, ٢٠٠٥).

The penicillins (G and V) were the first beta-lactams to be introduced and were effective against Gram-positive bacteria but had poor activity against Gram-negative bacteria. Within a few years of their introduction, penicillinase producing *Staphylococcus aureus* strains showed resistance to penicillin (Rammelkamp and Maxon, ١٩٤٢). This propelled search for new forms of beta-lactams that were not inhibited by penicillinase and had a wider spectrum of activity against both Gram-positive and Gram-negative bacteria. By the early ١٩٦٠s, semi-synthetic penicillins such as ampicillin and carbencillin were introduced. These antibiotics were more effective against Gram-negative bacteria than penicillins. In ١٩٦٥, a plasmid-borne beta-lactamase (TEM-١) was first reported in *E. coli,* which conferred resistance to ampicillin. The enzyme was rapidly disseminated among species and was spread to *P. aeruginosa* in ١٩٦٩, to *Vibrio cholerae* in ١٩٧٣, and to *Haemophilus* and *Neisseria* species in ١٩٧٤ (Matthew, ١٩٧٩).

Later on, more plasmid-borne beta-lactamases, notably SHV-١, TEM-٢, and OXA-١ were disseminated widely in the bacterial populations (Livermore ١٩٨٧ and ١٩٩٥; Sanders and Sanders, ١٩٩٢).

The first generation cephalosporins were developed with the objective of combining the broad-spectrum activity of ampicillin and achieving stability to staphylococcal penicillinase. However, like ampicillin they lacked activity against strains that had plasmids encoding TEM and SHV enzymes (Livermore, ١٩٩٥). The emergence of resistance to ampicillin and first generation cephalosporins fostered the search for newer versions of beta-lactams that were stable to beta-lactamases of Gram-negative species, especially TEM-١. This resulted in the development and introduction of second generation cephalosporins, such as cefoxitime, cefuroxime, and cefamandole. These antibiotics were able to achieve acceptable stability to TEM-١ enzymes (O'Callaghan, ١٩٧٩).

The third generation of cephalosporins included cefotaxime, ceftriaxone, ceftizoxime, ceftazidime, and cefoperazone, which were more resistant to beta-lactamases including TEM-١, TEM-٢, SHV-١, and to chromosomal Class A enzymes of *Klebsiella* spp. Activity against chromosomally encoded *AmpC*-inducible species was also observed (O'Callaghan, ١٩٧٩; Livermore, ١٩٩٥).

٢.٤.٢: *K.pneumoniae* and extended –spectrum ß-lactamase:

Clinical isolates of *K.pneumoniae* are generally resistant to a wide range of antibiotic than most *Escherichia coli* strain .They are resistant to Ampicillin and amoxicillin, but usually sensitive cephalosporins, especially the newer derivatives such as cefuroxime and cefotaxime. Resistance to chloramphenicol and tetracycline varies from strain to strain, they are often sensitive to gentamicine but transferable enzyme resistance to gentamicine and various cephalosporins has become common in strains found in some hospital (Gross, ١٩٩٢).

K.pneumoniae expressing extended spectrums ß- lactamase conferring resistance to ceftazidime and other cephalosporins and derived from TEM-١ or SHV-١ enzyme has become an increasing problem the past two decades (Jacoby and Medeiros, ١٩٩١).

The SHV-١ ß-lactamase is most commonly found in *K.pneumoniae* and responsible for up to ٢٠٪ of plasmid mediated Ampicillin resistance in this species (Bradford, ٢٠٠١).The first extended –spectrum SHV-١ enzyme was described in ١٩٨٣ in clinical isolate of *K.pneumoniae, K. ozaenae,* and *Serratia marcescens (*Knothe *et al*., ١٩٨٣).

TEM-type ß-lactamase is most often found in *K.pneumoniae* and *E. coli*. Up to ٩٠٪ of Ampicillin resistance in *E. coli* is due to production of TEM-١(Bradford, ٢٠٠١).

In ١٩٨٣, the extended-spectrum ß-lactamases (ESBLs), which confer resistance to broad-spectrum cephalosporins, were first observed in Germany (Medeiros, ١٩٩٣). The number and variety of these ß-lactamases have increased rapidly and their distribution is now worldwide. Moreover, due to the increasing use of various kinds of antibiotics, especially the third generation cephalosporins and aminoglycosides, Enterobacteriaceae, particularly *Klebsiella* species, have become resistant to broad-spectrum ß-lactams, as well as to aminoglycosides*. Klebsiella* species are now the most common Gram-negative bacteria exhibiting multiple resistances (Sirot *et al*., ١٩٩١). Many different multidrug resistance patterns of *Klebsiella* species have been documented in Europe, North-America, Africa and Asia (Arlet *et al.,* ١٩٩٠; Payne and Amyes, ١٩٩١; Bauernfeind *et al*., ١٩٩٣; Arlet *et al.,* ١٩٩٤; Urban *et al*., ١٩٩٤; Venezia *et al*., ١٩٩٥; Wallace *et al*., ١٩٩٥; Marchese *et al*., ١٩٩٦). In the United States, during the mid-١٩٨٠s, nosocomial infections due to aminoglycoside- resistant *K.pneumoniae* were prevalent in the ICUs. From ١٩٨٧ onwards, the third-generation cephalosporins were introduced in an attempt to control the spread of aminoglycoside-resistant *K. pneumoniae*. Subsequently, there was a marked increase in the incidence of cephalosporin resistance among *Klebsiella* species. In ١٩٩٠, over ٦٠٪ of *Klebsiella* isolates were resistant to both cephalosporins and aminoglycosides. Multiple antibiotic resistances in *K.pneumoniae* was also found in Australia, with an increasing resistance to a range of

unrelated antibiotics, including chloramphenicol, tetracycline, nalidixic acid, ampicillin, norfloxacin, trimethoprim and puromycin (George *et al*., ١٩٩٥).

In France, *K.pneumoniae* was also regarded as an opportunistic Gram-negative pathogen involved in outbreaks of nosocomial infections in intensive care units. About ١٥-٣٠٪ of the multiple antibiotic resistant strains of *Klebsiella pneumoniae* are also resistant to the broad-spectrum cephalosporins via plasmid-encoded ESBLs (Di-Martino *et al*., ١٩٩٧).

In Singapore, it has been observed that ٣٨٪ of all *K.pneumoniae* isolated from septicaemia patients were resistant to the third generation cephalosporins, aminoglycosides and quinolone (Inglis *et al.* ١٩٩٤). A common pattern of multiple antibiotic resistance noted in bacteria was found, namely resistance to ampicillin, ceftazidime, cefuroxime, and other third generation cephalosporins, aztreonam, gentamicin and other aminoglycosides. The bacterial species with this pattern of resistance are *Klebsiella* and other Enterobacteriaceae. From ١٩٨٦, multiple antibiotic resistances in *Klebsiella* species and the *Enterobacter* species increased significantly. They exceeded ٣٠٪ in resistance to all the commonly used antimicrobials under study (Kumarasinghe *et a*l., ١٩٩٢; Inglis *et a*l., ١٩٩٤).

٢.٥: Spread of genes conferring resistance to ß-lactam antibiotics:

Throughout the ١٩٦٠s and ١٩٧٠s there was a relentless rise in reports of resistance to ß-lactams as a consequence of the selection of bacteria that produce ß-lactamases. This increase in the level of resistance was due, at least in part, to the spread of self-transmissible plasmids that encode multiple resistance phenotypes. Furthermore, the first prokaryotic transposon carried a bla_{TEM} gene (Hedges and Jacob, ١٩٧٤). Transposons that encode extended-spectrum ß-lactamases have also been described.The occurrence of *bla* genes on mobile genetic elements undermines attempts to classify these elements by genetic location: transposons may jump between plasmids and the bacterial chromosome (Heritage *et al*., ١٩٩٢).

٢.٥.١: Plasmids:

Plasmids form a vital part of the bacterial genome and can constitute up to ١٠٪ or more of the DNA in a bacterial cell. Plasmids can transfer by a variety of means including

conjugation and transformation. Most bacteria are capable of becoming hosts to a large array of different plasmids commonly found in other strains, other species or even other genera. Plasmids thus represent a large genetic resource for diversity and adaptation of bacteria (Thorsted *et al*., ١٩٩٨).

Resistance to ß-lactam antibiotics is mediated by plasmid with variations of the SHV and TEM-type ß-lactamase genes (Barthelemy *et al.*, ١٩٨٨). Most of these plasmid-mediated ß-lactamases from *Klebsiella* species have been grouped as TEM or SHV-related ß-lactamases (Horii *et al*., ١٩٩٣). Furthermore, *K.pneumoniae* is the species in which plasmid-encoded ESBLs are commonly reported (Jacoby and Medeiros, ١٩٩١). The threat of an increase in multiple resistances to antibiotics in *K. pneumoniae* is shown by French *et a*l. (١٩٩٦) with hyperproduction of SHV-٥ ß-lactamase. Nevertheless, it has been reported that the majority of ESBL-producing isolates of *E.coli* and *Klebsiella* species are also resistant to gentamicin and other aminoglycosides (Jones *et al.,* ١٩٩٤). All these multiple resistance genes are usually carried on large transferable plasmids. Large plasmid profiles from *K.pneumoniae* associated with multiple resistances have been documented in Europe (Montgomerie *et al*., ١٩٩٣; Reed *et al.,* ١٩٩٥; Marchese *et al.,* ١٩٩٦), United States (Wallace *et al.,* ١٩٩٥; Schiappa *et al*. ١٩٩٦) and Asia (Wang *et al,.* ١٩٩٥). Prodinger *et al*. (١٩٩٦) mentioned that there was R plasmid with ٨٠ kbp responsible for conferring resistance to SHV-٥ in *K.pneumoniae* isolates. This R plasmid transfer between species of family Enterobacteriaceae in nosocomial out break stresses the need plasmid typing, especially because SHV-٥ beta-lactamase seems to regionally spread predominately via plasmid transfer. Shannan *et al* (١٩٩٨) showed that ٧٠ to ١٦٠ kbp of conjugative plasmid responsible for encoding the SHV-٤, TEM-١٥ and TEM-١٦ in K.pneumoniae. Li and Lim (٢٠٠١) showed novel large plasmid (٩٧~١٤٥Kbp) carrying multiple ß-lactamase resistance genes isolated from *K.pneumoniae* strain.

٢.٥.٢: Broad host range IncP plasmid *R٧٥١*:

Plasmids of IncP group have interest because of their extraordinary large host range among gram-negative bacteria (Meyer and Shapiro, ١٩٨٠).The IncP plasmids are the most intensively studied self-transmissible plasmids (Thomas and Smith ١٩٨٧ ; Pansegrau et al ١٩٩٤). The plasmid *R٧٥١* is IncP type β ranged between ٥٢,٠٠٠ to ٥٣ Kbp.The overall G+C content of *R٧٥١* is ٦٤٪ (RP٤ approx. ٦٠٪). Certain regions average well over ٧٠٪. The *R٧٥١* genome reveals a family of repeated sequences in these regions which may form the basis of a hot spot for insertion of foreign DNA. Sequence analysis of the cryptic transposon Tn٤٣٢١ revealed that it is a composite transposon defined by inverted repeats of a ١٣٤٧ bp

IS element belonging to a recently discovered family which is distributed throughout the prokaryotes. The central unique region of Tn٤٣٢١ encodes two predicted proteins, one of which is a regulatory protein while the other is presumably responsible for an as yet unidentified phenotype (Thorsted *et al*., ١٩٩٨).

٢.٥.٣: Transposons:

Transposons, or Transposable Elements (TE), or "Jumping Genes", are lengths of DNA capable of transposition that is movement, from a plasmid to a chromosome (or another Plasmid) and vice-versa in prokaryotes, and from one part of a chromosome to another (or to another chromosome) in eukaryotes. In prokaryotic organisms such as *E.coli*, it is possible for transposons to be transferred along with plasmid or chromosomal DNA, either independently or as a cointegrate, from one organism to another during conjugation. Some plasmids, such as *pMB١*, can become mobilized during conjugation, moving along with chromosomal or other plasmid DNA, into a recipient cell, without having instigated the conjugation (Moghaddam, ٢٠٠١).

A transposon consisting of genes that code not only for its transposition, but also for unrelated activities such as antibiotic and heavy metal resistance is known as a complex transposon (Singleton and Sainsbury, ١٩٩٣). It is this additional coding that differentiates complex transposons from simple insertion sequences. Insertion sequences (IS) are TEs consisting only of the genes necessary for transposition of the sequence, using the enzyme transposase and inverted base pair repeats.

٥٣ Kbp

Fig. (٢-١) illustrates the Molecular weight of *pR٧٥١* (Meyer and Shapiro, ١٩٨٠).

Inverted repeats have the same DNA sequence at either end with opposite orientations as illustrated in fig (٢-٢) A and B below:

Genes for Transposition

٥' --TTCGAG----------CTCGAA-- ٣'

٣' --AAGCTC----------GAGCTT-- ٥'

Inverted repeats

(A)

Many transposons also have inverted repeats at either end, although some have direct repeats where the ends are identical as below:

Genes for transposition and other proteins

٥' --TTCGAG----------TTCGAG-- ٣'

٣' --AAGCTC----------AAGCTC-- ٥'

Direct repeats

(B)

Fig. (٢-٢) A&B: (The terminal repeats are important for transposition, acting as recognition points for transposase) Hettle, (١٩٩٥).

٢.٥.٣.١: **Discovery:**

Transposable elements were first suspected to exist in the ١٩٤٠'s by geneticist Barbara McClintock while carrying out experiments on maize. It was found that breakages were being

caused at a specific site on chromosome ٩ due to the insertion of new genetic material (Fedoroff, ١٩٨٥).

In prokaryotes, transposons were not discovered until the ١٩٦٠'s when a mutant *E.coli* was found that unable to ferment galactose (Gal^-). This *E.coli* was found to have DNA (insertion sequences) unrelated to the galactose gene inserted within the gene, causing its inactivation. At the same time study was being undertaken into the spread of bacterial antibiotic resistance. Sections of DNA were found with the ability to move within the genome, and between related bacterial species, that carried genes conferring antibiotic resistance. It was originally thought that these genes were rare and carried on plasmids, and it was not until ١٩٧٤ that actual antibiotic resistance transposons were discovered (Hedges and Jacob, ١٩٧٤).

٢.٥.٣.٢: Types of prokaryotic transposon:

Prokaryotic transposons fall into three groups:

Class I are mainly short insertion sequences, and carry only the genes required for the production of the enzymes responsible for their transposition. Class I elements demonstrate both basic, direct, transposition (that is transposition directly from one molecule to another without the formation of any intermediate structure, i.e. cointegrate) and cointegrate formation. In general, direct transposition is more common, type and frequency depending on the TGE. With Tn٩ direct transposition is five times more likely to occur than co integrate formation, while Tn٩٠٣ seems to use both methods with equal frequency (Kleckner, ١٩٨١). During insertion, from ٣ or ٤ to ٩ base pair repeats are made on the ends of the recipient DNA molecule, that is a short DNA sequence produced immediately before the production of DNA on the inserted transposon, joining the target DNA to the transposon.

Class I elements are further subdivided into two groups: Classes IA and IB (fig. ٢-٣). Class I A elements are the simplest, being simple insertion sequences, having only the genes for transposition and transposition regulation, i.e. IS١; IS٢; etc. Class I B are composite transposons, also containing genes coding for antibiotic resistance and other products, e.g. Tn٦٨١, genes for an enterotoxin; Tn٢٣٥٠, genes for kanamycin resistance (kan^r); Tn٢٩٠١, genes for arginine biosynthesis; etc.(Hettle, ١٩٩٥).

The Class II elements are typified by Tn٣. Class II transposons all bear a strong resemblance to one another and to the Tn٣. Transposition is via cointegrate formation followed by resolution. In all Class II elements insertion generates ٥ base pair repeats at the ends of the insertion point in the target DNA.

Class III elements are bacteriophages Mu and D١٠٨, these bacteriophages share ٩٠٪ homology in their genetic make-up (Singleton and Sainsbury, ١٩٩٣)**.** Mu (short for Mutator) is capable of causing mutations in host cells due to its ability to insert randomly in any part of the bacterial genome. Random insertion means that it can insert into a key part of a gene causing its inactivation. Unlike Class I and II elements, there are no terminal inverted repeats in Mu. There are non-symmetrical homologies between the ends and other regions within the bacteriophage, e.g. between one end of the bacteriophage and a region approximately ٨٠ bp in from the opposite end, and

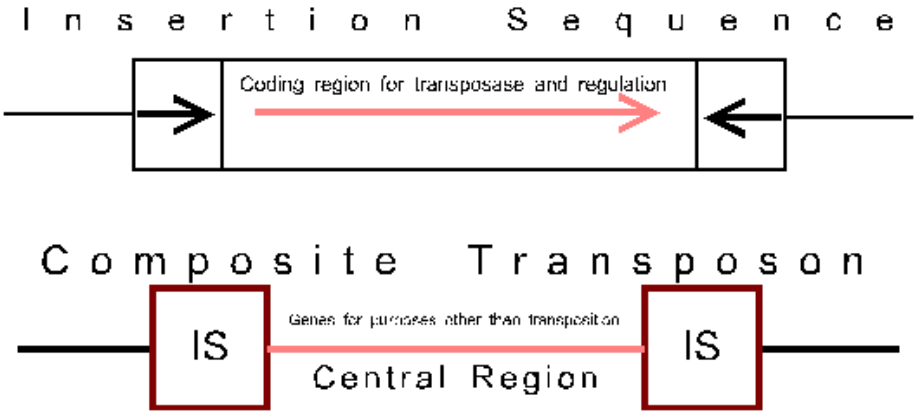

Fig. (٢-٣) Layout of Class I TGE's Class I A, top; Class I B, bottom. (Hettle, ١٩٩٥)

a sequence of ٧-٩ bp in the vicinity of one end which occurs again five times near the opposite end (Kleckner, ١٩٨١).

Replicative transposition is a compulsory part of the life cycle of Mu and D١٠٨ whether the life cycle is lysogenic or lytic (Kleckner, ١٩٨١; Bétermier *et al*, ١٩٩٢).

٢.٥.٣.٣: Transposon Tn٣ (and relatives):

Tn٣ is a ٤٩٥٧ base pair class II transposon. At each end it has identical ٣٨ base pair inverted repeats. The transposon also contains the genes for transposase (*tnp A*), resolvase (*tnp R*), and ß-lactamase (bla).

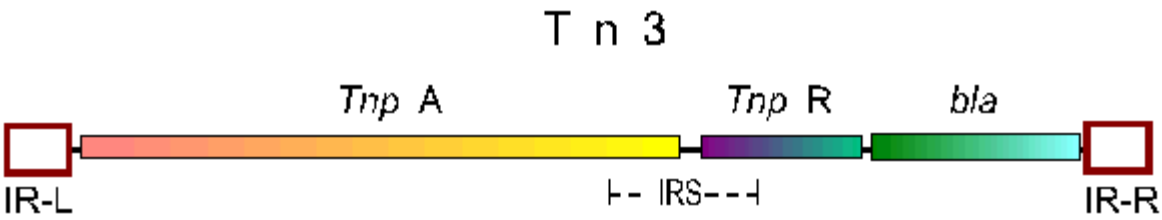

Fig. (٢-٤) The general layout of Tn٣, the area marked IRS is the internal resolution site, without this site the resolution of the co integrate can not take place. IR-L and IR-R are inverted repeats - left and right respectively. Hefforn *et al*, (١٩٧٩)

The ß-lactamase is responsible for the transposons ability to confer antibiotic resistance on the bacteria in which it is present. ß-Lactamase is able to hydrolyze the ß-lactam ring present on -lactam antibiotics such as penicillin. The hydrolysis of the ring renders the antibiotic innocuous to the organism, allowing growth and reproduction. Tn٣ provides resistance to ampicillin (Ap). Ampicillin can therefore be used in growth media to screen for the presence of Tn٣ in a bacterial colony (Kretschmer and Cohen, ١٩٧٩; Heffron *et al*, ١٩٧٩).

٢.٥.٣.٤: Mechanisms of Transposition in Tn٣:

Transposition can occur directly, by transfer of a transposable element from one plasmid to another plasmid or chromosomes with no intermediate or, via the formation of cointegrate followed by resolution. The latter always yields two transposons on separate plasmids or plasmid/chromosome. Transposition is a rare event, depending on the physicochemical conditions within the cell and the element involved, in general occurring at around $١٠^{-٤}$ - $١٠^{-٧}$ times per element per generation (Kleckner, ١٩٨١).

Concentrating on Tn٣, cointegrate formation requires the action of the enzyme transposase, encoded by the *tnpA* gene, and resolution requires resolvase, encoded by the *tnpR* gene, and also the presence on the transposon of a resolution site and intact terminal inverted repeats. The terminals and the resolution site act as recognition sites and points of reference for the enzymes and are involved in binding the enzymes to the transposon; this is particularly true of the resolution site/resolvase complex (Heffron, ١٩٨٣)**.**

The mechanics of the operation for Tn٣ (and other Class II elements) can be broken down into the two steps: cointegrate formation and resolution of the cointegrate. Step ١ requires the action of transposase, the presence of intact terminal inverted repeats, and suitable insertion sites on a target DNA molecule, preferably without another intact Tn٣. Tn٣ does not show great insertion site specificity but seems to prefer "hot-spots" were insertion is more likely. Regions of high A and T concentration seem to be preferred for insertion although preference also seems to depend on other factors such as the DNA secondary structure (Heffron, ١٩٨٣**;** Davies and Hutchison, ١٩٩٥). Step ٢ requires the action of resolvase and an intact internal resolution site.

The process of transposition is still not fully understood. For Tn٣, transposition is believed to begin when transposase cleaves the target DNA at the insertion site (fig.٢-٥). The synthesis of five bp repeats by DNA polymerase I join the ends of the target molecule to the transposon and new DNA is synthesized along the lengths of the two strands of transposon DNA. Eventually, when replication is complete, there are two identical copies of the transposon which link the donor and recipient molecules as a cointegrate.

Fig (٢-٦) in previous page illustrates two possible routes for transposition. Route ١ is the obligatory pathway taken by Class II elements (e.g. Tn٣). This pathway includes cointegrate formation followed by resolution generating two copies of the transposon on two separate molecules. Route ٢ illustrates the direct insertion of a transposon from a donor into a recipient molecule, resulting in the breakdown of the donor DNA. Both of these routes are symmetrical, that is the DNA is being synthesized via replication forks from both ends of the transposon. In figure (٢-٧), the detail of replication is more clearly illustrated with the replication forks clearly shown. The full cointegrate is also illustrated in both isometric and topological view to show the relationship of the two transposons to the overall conformation of the co integrate. Resolution involves the recombination of the DNA making up the two transposons at the internal resolution site. The IRS consists of three internal sites: I, II and III. Sites II and III are used for binding the resolvase and site I is the point of cleavage.

Within the *IRS* are also sited the promoter regions for both *tnpA* (within the region of site I) and *tnpR* (between the regions of sites I and II) genes. The transposons are cleaved and

recombined in the manner illustrated at the end of route ١ in Figure (١-٦), leaving both strands with a combination of both original and new DNA, joined at the *res* site .An asymmetric model has also been proposed in which DNA synthesis is carried out only at one end of the transposon. Instead of cutting strands at both ends, the transposase cuts recipient and donor strands at one end only and a replication fork moves from one end to the other. In this model both cointegrates and simple insertions can be carried out as per the symmetrical model, resolution would be unaffected (Kleckner, ١٩٨١; Grindley and Reed, ١٩٨٥).

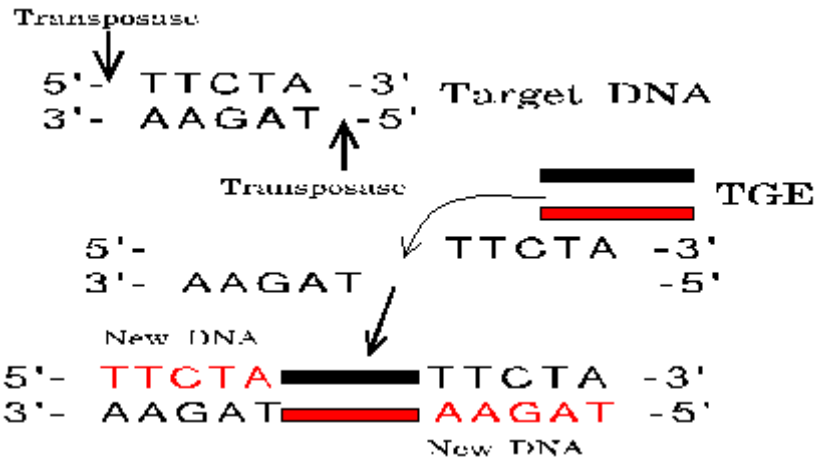

Fig. (٢-٥) Transposase Cleaves the Target DNA at the Insertion Site (Hefforn *et al*, ١٩٧٩)

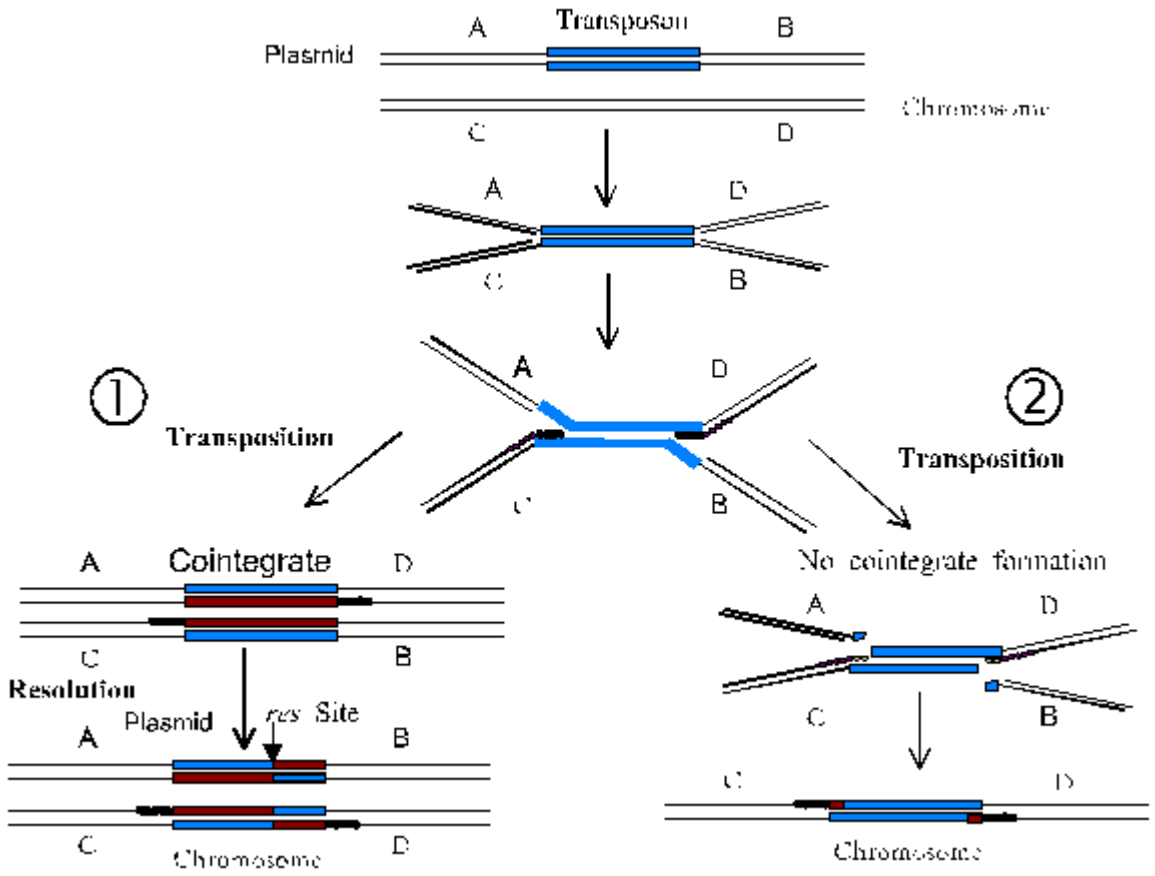

Fig. (٢-٦) Transposition Sequence Grindley and Reed, (١٩٨٥)

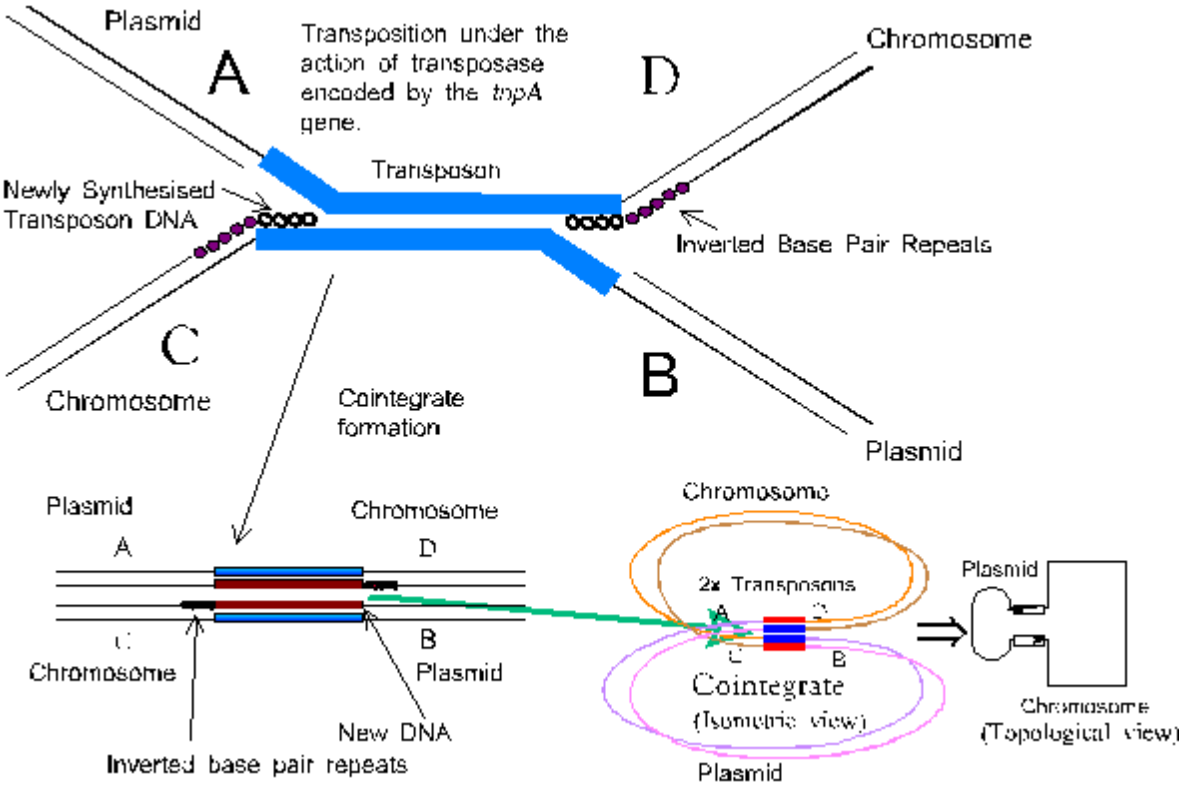

Fig. (٢-٧) Detail from Fig.(١-٥)with additional cointegrate illustration.(Grindley & Reed, ١٩٨٥)

٢.٥.٣.٥: Effects of temperature on transposition:

The rate of transposition of prokaryotic transposons has been shown to be temperature dependent (Kretschmer and Cohen, ١٩٧٩; Cornelis, ١٩٧٩).

Transposon Tn٣ in *E.coli* has an optimum between ٢٦C° and ٣٠C°, above ٣٠C° the rate falls of rapidly with a direct negative linear relationship between frequency and temperature,. Transposition events depend on the ability of transposase and resolvase to carry out their functions as described above (Mechanisms of transposition in prokaryotes). Changes in the physiological conditions in the cell may cause a conformational change in either the transposase or the resolvase, making them less efficient. In the case of Tn٩٥١ it is

suggested by Cornelis, (١٩٧٩), that it is the action of cointegrate formation that is influenced by the temperature variation.

٣.١: Materials and Substances:

٣.١.١: Apparatus and Tools

Table (٣-١) Apparatus and tools used during study

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

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

Spectrophotometer	BEJING RAYLEIGH ANALYTICAL, MC	China
Ultra violet transilluminator	UPLAND	U.S.A
Water bath	TAFESA	W. Germany
Medical Deep Freezer	SANYO	Japan
Millipore Filter (٠.٤٥, ٠.٢٢)	Sartorions Membrane Filter W.	Germany
Digital Camera	SONY	Japan

٣.١.٢ Materials

Table (٣- ١) Materials that used during experiments.

Material	***Company***
a- Chemical Material Agarose , ammonium chloride, Calcium chloride, chloroform, CTAB, glacial acetic acid, K_2HPO_4, low melting agar, magnesium sulphate, Na_2HPO_4, Na^{2}- EDTA, Phenol, potassium chloride anhydrate, sodium	BDH (England)

chloride, Salicylic acid, sodium hydroxide, Sodium Acetate, Sodium dodecyl sulphate, Tris base.	
b-Sugars and Amino acid D-glucose, lactose, Sucrose. Casamino acid, Thiamine, yeast extract.	BDH (England)
c-Dyes Bromothymol blue, Ethedium bromide, India ink.	BDH (England)
d-Enzymes Lysozyme Proteinase K.	CinnaGen (Iran) CinnaGen (Iran)

۳.۱.۳ Plasmids and bacterial strains

Table (۳-۳) plasmids and strains that used in experiment

plasmids and bacterial strains	***Phenotype &Genotype***	***Source***
E.coli GMP٤١٧٦ (*pR٧٥١*)	*hsd R*$^-$*, hsd M*$^-$*, recA*$^-$ *,IncP,tra*$^+$ *leu*$^-$*, pro*$^-$*, lac, gal*$^-$*,* Sm^r	Institute of genetic engineering and biotechnology for postgraduate studies Baghdad university
E. coli MM٢٩٤	*hsd R*$^-$*, hsd M*$^+$*, edn AI, pro*$^-$*, thi, , Rif*^{r}	Biology department University of Sulaimani

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

hsd M$^+$: presence of modification system

hsd M$^-$: lack of modification system

rec A$^-$: lack of recombination system

end AI : lack of end nuclease activity

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

tra$^-$: lack of tra genes.

Inc P : incompatibility P group.

pro$^-$ **, lue**$^-$ **and thi**$^-$ (proline, luecine, and thymine respectively):need proline , luecine and thymine in their growth media .

Tp : trimethoprim,

r : Resistance

٣.١.٤ Culture media and solutions

٣.١.٤.١ Buffers and solutions

a. Antibiotic solution:

Antibiotic solutions were prepared according to (Al-zaaq, ١٩٨٧ and Baron and finegold, ١٩٩٠).The antibiotic solution stocks sterilized by Millipore filter ٠.٢٢, and stored at ٤ C°.

Table (٣-٤) concentration of antibiotics that used in the culture media

Antibiotic	**Final concentration in culture media (*µg/ml*)**	**Solvent used to dissolve antibiotic**	**Manufacture Company**
Ampicillin(Ap)	١٥٠	Distilled water	Oxoid(England)
Rifampcin (Rif)	١٠٠	Aceton	Sigma(Germany)
Tetracycline (TC)	٢٠	Ethanol alcohol/water ٥٠٪	Oxoid
Kanamycin (Km)	٥٠	Distilled water	Sigma
Streptomycin (Sm)	١٠٠	Distilled water	Oxoid
Chloramphenicol (Cm)	٥٠	Aceton	Oxoid
Amoxillin (Ax)	٥٠	Distilled water	Sigma
Trimethoprim (Tp)	٥٠	Aceton	Sigma
Cefotaxime (Ctx)	٥٠	Distilled water	Sigma

b- ***Saline EDTA:***

The buffer was prepared by dissolving ٠.١٥ mol of sodium chloride, ٠.١ mol of EDTA in distilled water and the pH was adjusted to ٨ with ١ N NaOH. Then volume of solution was

adjusted to one liter with distilled water. The solution was sterilized by autoclave (Murmur, ١٩٦١).

c- Lysozyme solution:

This solution was prepared freshly by dissolving ١٠ mg of Lysozyme in ٢٠٠ µl STET buffer (Sambrook *et al.,* ١٩٨٩).

d- SDS solution

This solution was prepared by dissolving ٥٠mmol of Tris-base in ٧٠ml of distilled water then the PH was adjusted to ١٢.٦ with ١.٢ ml of ٢N NaOH .The volume was completed to١٠٠ml with distilled water. The solution was sterilized by filtration and stored at ٤C° (Kado and Liu, ١٩٨٩).

e- TE buffer

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

f- EST buffer

The buffer was prepared by dissolving ٠.٠٥٠ M of Tris-base, ٠.٠٠٥ M of EDTA and ٠.٠٥٠ M of NaCl in distilled water then the pH adjusted to ٨. The volume of the solution was completed to one liter with distilled water and the solution was sterilized by autoclave (Sambrook *et al.,* ١٩٨٩).

g- EDTA-Sodium acetate

The Solution was prepared by dissolving ٣ mol of sodium acetate, ٠.٠٠١mol of EDTA in distilled water. The pH was adjusted to ٧ with acetic acid, and then the volume of the

solution was completed to one liter with distilled water. The solution was sterilized by autoclave (Sambrook *et al.,* ١٩٨١٩).

h- chloroform- isoamyl Alcohol mixture

This solution was prepared in a ratio of ٢٤:١ and stored in bottle at ٤C° (Sambrook *et al.,* ١٩٨٩).

i- **Phenol solution**

The solution was prepared by melting the phenol at of ٦٨C°, and then ٠.١٪ of antioxidant ٨-hydroxyquinoline was added to it, un equal volume of buffer solution of ٠.٥ mol Tris-HCl at pH ٨ was added to the mixture and mixed well by mechanical vortex and then the solution left to set at room temperature. The upper aqueous layer was separated from the lower layer. The upper layer was discard by pasture pipette and then an equal volume of ٠.١ molar Tris-HCl pH ٨ was added to the phenol and this process was repeated until the pH of phenol become more than ٧.٨. The phenol solution was stored under thin layer of ٠.١ molar Tris solution in a dark bottle at ٤C° (Sambrook *et al.,* ١٩٨٩).

j- Phenol: chloroform: isoamyl alcohol mixture

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

k- TBE buffer

The solution was prepared by dissolving ٠.٠٨٩ mole of Tris-base, ٠.٠٨٩ mole of boric acid and ٠.٠٠٢ mole Na_2-EDTA in distilled water. The pH of the solution was adjusted to ٨ with ١ N NaOH and the volume of solution was completed to one liter with distilled water. The solution was sterilized by autoclave (Sambrook *et al.,* ١٩٨٩).

l- Ethidium bromide dye:

The dye solution was prepared by dissolving ٠.٥ mg of Ethedium bromide in ١٠ ml distilled water, and the solution was stored in dark bottle at room temperature (Sambrook *et al.,* ١٩٨٩).

m--Extraction solution:

Solution I was prepared by mixing ٥٠ mM glucose, ٢٥ mM Tris -Cl (pH ٨) and١٠ mM EDTA (pH ٨).The solution was prepared in batches of ~١٠٠ ml then the solution was sterilized by autoclave and stored at ٤C°.

Solution II was prepared by mixing ٠.٢ N NaOH (freshly diluted from ١٠ N stocks) and١٪ SDS. **Solution III** (٣M Sodium acetate) pH ٤.٨: It was prepared by mixing ٦٠ ml of ٥ M potassium acetate, ١١.٥ ml glacial acetic acid and ٢٨.٥ ml of distilled water (Sambrook *et al.,* ١٩٨٩).

n- Salicylic acid solution

A stock solution of concentration ٢٠mg/ml of was prepared by dissolving ١gm of the salicylic acid in ٥٠ml distilled water and sterilized by Millipore filter ٠.٤٥µm (Domenico *et al.,* ١٩٨٩).

o -Normal saline

It was prepared by dissolving ٠.١٥ moles from sodium chloride and ٠.١ mole of $KH_٢PO_٤$ in distilled water, the pH was adjusted to ٧.٢ by adding ١ N NaOH . Then the volume was completed to one liter by adding distilled water, and then sterilized by autoclave.

p- CTAB solution:

The solution was prepared by mixing ١% CTAB in ٥٠ mM Tris–١٠ mM EDTA, and then the pH was adjusted to ٨.٠ (Corinaldesi *et al., ٢٠٠٤).*

٣.١.٤.٢ Culture media

A-Ready culture media

Table (٣-٥) Ready culture media used during experiments

The following media were prepared as recommended by the manufacturing company and autoclaved

Culture media	Manufacture company
Brain-Heart infusion agar	(BHF), (Mast diagnostics company; U.K).
Brain-Heart infusion broth	(BHI), (Mast diagnostics company; U.K).
Eosin methylene blue agar (EMB),	(Difco U.S.A).
MacConKey agar	(Difco-U.S.A).
Muller-Hinton agar	(Difco-U.S.A).
Nutrient agar	(Mast diagnostics U.K).
Nutrient broth	(Mast diagnostics U.K).

B-Prepared culture media

١- Minimum medium M٩

It was prepared by dissolving ٩ gm of $Na_٢HPO_٤$, ٣ gm of $KH_٢PO_٤$, ٠.٥ gm of sodium chloride, and ١ gm of $NH_٤Cl$ in ٩٨٠ ml deionized water at pH ٧.٤ then sterilized by autoclave. After that ٢ ml of ١ M $MgSO_٤$ solution, ١٠ ml of ٢٠٪ glucose solution, ٦ml of Casamino acid solution at the final concentration (٥mg/ml) and thiamine (٥٠mg/ml) was sterilized separately by filtration and added to the medium. When the medium was used as solid medium ٢% agar would be added to it (Sambrook *et al.,* ١٩٨٩).

٢-SOC liquid medium

It was prepared by dissolving ٢ gm of trypton, ٠.٥ gm yeast extract, and ٠.٠٠٥ gm sodium chloride in ٩٠ ml distilled water and then ١ ml of ٠.٢٥ M of potassium chloride was added, the pH of the solution was adjusted to ٧ and the volume completed to ١٠٠ ml. Before using, ٠.٥ ml of ٢ M magnesium chloride, and ١ ml of ٠.٢٥ molar of glucose previously been sterilized by filtration was added when the medium used as solid culture, ٢% of agar would be added (Sambrook *et al.,* ١٩٨٩).

٣- Salicylic acid medium

The medium prepared by adding salicylic acid at concentration of ١٥٠µg/ml to the $M_٩$ medium (Al-Jlawi, ٢٠٠٠).

٤-Luria broth

It was prepared by dissolving١٠ gm of trypton, ٥gm yeast extract, and ١٠ gm sodium chloride in ٩٥٠ ml distilled water. The pH of medium was adjusted to ٧ then the volume was completed to one liter, and then sterilized by autoclave (Sambrook *et al.,* ١٩٨٩).

٣.٢. Methods

٣.٢.١: Sterilization:

٣.٢.١.١: Dry sterilization:

Glasswares were sterilized using oven instrument at ١٨٠ C° for two hr.

٣.٢.١.٢: Autoclaving:

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

٣.٢.٢: pH of culture media:

The hydrogen ion concentration (pH) for all culture media was adjusted to (٧.٢ – ٧.٤) using pH meter.

٣.٢-٣: Identification of *K.pneumoniae*:

٣-٢.٣.١: Microscopical examination (Gram staining film):

Smears were prepared by taken a small amount of isolated colony from a bacterial culture on a glass slide by mixing a colony of tested bacterium with a drop of distilled water, stained with Gram stain and examined using oil immersion objective lens under ١٠٠X power.

٣-٢--٣-٢: Cultural characteristics:

The morphological characteristics of isolated colonies were carefully studied with the aid of a lens ١٠X lens.

Depending on above two criteria the cultures were primarily classified as pure or mixed cultures.

٣.٢.٤: Antibiotic resistance

A-Antibiotic sensitivity test (Antibiotic susceptibility test by disk-diffusion method (Kirby Bauer test) :

A single colony of bacterial strain was transferred to a fresh test tube containing ٥ ml Brain-Heart infusion broth then incubated for ٢٤ hours at ٣٧ C°. After that ٠.١ ml of the inoculum was transferred to a Muller-Hinton agar plate, streaked by a sterile straight wire all over the surface of the medium three times. After ١٠-١٥ min, antibiotic discs were plated on the medium then incubated at ٣٧ C° for ٢٤ hours. After incubation the inhibition zone

diameters were determined in millimeters (mm), comparison was done with standard inhibition zone (Mahon and Manuselis, ٢٠٠٠).

Table (٣-٦) Antibiotic disc used in antibiotic susceptibility test.

Antibiotic disc	**Concentration (*µg/disc)***	**Manufacture company**
Amoxicillin (Ax)	٢٥	Jovet
Ampicillin (Amp)	١٠	Oxoid
Cefotaxime (CTM)	٣٠	Oxoid
Chloramphinicol (C)	٣٠	Oxoid
Nalidixic acid (NA)	٣٠	Oxoid
Neomycin (N)	٣٠	Oxoid
Novobiocin (NV)	٣٠	Oxoid
Rifampicin (RA)	٥	Oxoid
Streptomycin (S)	١٠	Oxoid
Tetracycline (TE)	٣٠	Oxoid
Trimethoprim (TEM)	٥	Oxoid

B- Antibiotic resistance test in solid cultures media

The Mullar-Hinton agar was prepared and sterilized by autoclave, cooled to ٥٠ C°. Antibiotic solution in appropriated concentration was added to the cooled culture medium,

poured into sterilized Petri dish and left to solidify, then. The bacterial isolates were cultured by picking and patching method. The result were recorded by presence (+) or absence of growth (-) (Nassif *et al*., ١٩٨٩).

٣.٢.٥: Detection of presence of plasmid DNA

٣.٢.٥.١. Plasmid DNA Extraction

A-Extraction of large plasmid

Alkaline lysis method of Kado and Liu, ١٩٨١ was used to extract large plasmids and the salting out method was used to prepare total DNA (Pospiech and Neuman, ١٩٩٥) as below:

١. A single colony of bacterial isolate was incubated into LB agar plates that containing ١٥٠ µg/ml of ampicillin. Then the plates were incubated at ٣٧ C° for ٢٤ hr.
٢. The bacterial cells were collected by sterilized loop and transfer to polypropylene tube with plastic cap containing ٥ ml of STE buffer.
٣. The bacterial cells were precipitated by centrifugation at ٣٠٠٠ xg for ١٥ min. the supernatant was discarded and the pellet was resuspended by ٥ ml of STE buffer.
٤. Aliquot of ٦٠٠ µl of ٢٥ % SDS solution and ١٢٠ µl of proteinase K (٢٠mg/ml) were added to the bacterial suspension and incubated in water bath at ٥٥ C° for ٢ hr.
٥. Aliquot of ٢ ml of ٥ M NaCl solution was added to the lysate, then mixed by inversion, and left to cool to ٣٧ C°.
٦. Equal volume of phenol-chloroform-isoamyl alcohol mixture (٢٤:٢٤:١) was added to the lysate then mixed gently by inversion for ٣٠ min until an emulsion formed.
٧. The mixture was separate by centrifugation at ١٢٠٠٠ rpm for ١٠ min at ٤ C°. The aqueous phase was transferred by pipette to a fresh tube. (The steps ٦ and ٧ were repeated until no protein was visible at the interphase of the organic and aqueous phases).
٨. Equal volume of chloroform was added to the aqueous phase that contains the nucleic acid and step ٧ was repeated.
٩. Aliquot of ٠.٦ % (v/v) isopropanole was added to the extract and mixed by inversion then the DNA was spooled onto a sealed pasture pipette rinsed in ٧٠ % cold ethanol then dissolved in ١-٢ ml TE buffer pH ٨.

B- Plasmid extraction by alkaline lysis:

١. Single colony of the bacterial isolate was grown in ١٠ ml of LB broth containing ٥٠ mg/ml ampicillin and incubated at ٣٧ C° for ٢٤ hr. with shaking. Bacterial cells were harvested by centrifugation at ١٠٠٠٠ rpm for ١٠ min.

٢. The pellet resuspended in ١٠ ml of EST/saline buffer.

٣. The suspension centrifuged at ١٠٠٠٠ rpm for ١٠min. (the step ٢, ٣ repeated twice)

٤. The pellet was resuspended in ٠.٢ ml of solution I, then transferred to sterile eppendorf tube and omitted for ٥ minutes at room temperature.

٥. Aliquot of ٠.٤ ml of solution II was added to the mixture and then the eppendorf tube was gently inverted for many times and omitted in ice bath for ١٥ min.

٦. Aliquot of ٠.٣ ml of cold solution III was added to the mixture above and the eppendorf tube was inverted for many times before putting on ice bath for ٥ min.

٧. The mixture was centrifuged at ١٤٠٠٠ rpm for ٥ min.

٨. Aliquot of ٠.٥ ml of the supernatant was transferred to the new sterile eppendorf, then an equal volume of phenol-chloroform-isopropanole solution was added and mixed very well then the solution objected to centrifugation as previous step.

٩. The upper layer was transferred to the new sterile eppendorf, and both the middle and lower layer omitted .this step was repeated till the protein was excluded from the solution.

١٠. Aliquot of ٣ M of cold sodium acetate in ratio of ٠.١ volumes was added to the supernatant that contain plasmid DNA and mixed very well.

١١. Double volume of cold absolute ethanol was added to the suspension and mixed gently and then left at -١٠ C° for two hr.

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

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

C- Determination of DNA concentration by spectrophotometer:

Concentration of prepared DNA was estimated using spectrophotometer by applied this equation (Sambrook *et al.,* ١٩٨٩):-

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

٣.٢.٥.٢: Gel electrophoresis

According to method of Sambrook *et al.*(١٩٨٩).,agarose gel were prepared by dissolving ٠.٧ gm agarose powder in ١٠٠ ml of ١ X Tris-borate buffer ,heated to boiling in water bath . The gel was cooled to ٦٠ C° then ١٠٠ µl of Ethedium bromide was added to final concentration of ٠.٥ µg/ml and mixed thoroughly .The edge of the a lean, dry glass plate was sealed with tape so as to form a mold and the mold set on horizontal section of the bench . The comb was placed above the plate. The gel was poured into the mold (١٠×٢٠ cm). After that the gel was allowed completely to set for ٣٠-٤٥ min at room temperature, the comb and surrounding former cover were removed and the gel soaked in a gel tank containing (١ X) Tris-borate buffer.١٥ µl of DNA samples were mixed with ٣µl of loading buffer dye, Finally ١٠µl of this mixture was loaded into the wells, then the gel tank were closed by lid and electrical leads was attached ١-٥ v/cm was applied for ١-٢ hr. After that the electric current turned off and removes the leads and the lid from the gel tank. The gel was examined by UV-light and photographed the gel.

٣.٢.٦: Recovery of Plasmid DNA from the gel

٠.٨ % of low melting agarose gel was prepared as above then, the samples of DNA and molecular weight marker (Lambda DNA cut by *EcoR*I and *HindIII*) was electrophorized at ١٠٠ V for ١ hr. until the bromophenol blue reached the bottom of the gel. The gel was examined by UV-light and photographed it. Then the gel was placed in petredish lid on the transilluminator and razor blade was used to dissect the band. The band was trimmed as much as possible. Once we had removed the rest of the gel, it was rotated at ٩٠˚ so that it was lied flat on the Petri plate, the band was put in clean, labeled microcenterfuge tube then, the agarose was melted in ٥٥ C˚ for ٥ min. after that the DNA was precipitated adding ١ volume of a cetyl trimethyl ammonium bromide (CTAB) solution (١٪ CTAB in ٥٠ mM Tris–١٠ mM EDTA, pH ٨.٠). Samples were incubated at ٦٥°C for ٣٠ min and then centrifuged at ٥,٠٠٠ rpm for ١٠ min at ٤°C. The supernatants were discharged, and each pellet was resuspended in high-salt TE buffer (١٠ mM Tris-HCl, ٠.١ mM EDTA, ١ M NaCl; pH ٨.٠). Then ٠.٦ volume of cold isopropanol was added to each sample and the samples were incubated for ١ h on ice and centrifuged at ١٠,٠٠٠ rpm for ١٥ min at ٤°C. The pellets were resuspended in ١٠ mM Tris-HCl–٠.١ mM EDTA (pH ٨.٠), an equal volume of equilibrated phenol-

chloroform-isoamyl alcohol (٢٥:٢٤:١, vol/vol/vol) was added to each preparation, and the preparations were centrifuged at ١٠,٠٠٠ rpm for ٥ min. Each supernatant was mixed with an equal volume of chloroform isoamyl alcohol (٢٤:١, vol/vol) and centrifuged again. The supernatant was then precipitated with cold ethanol (final concentration, ٧٠٪) and sodium chloride (final concentration, ٠.٢ M), incubated at -٢٠°C for ١ h, and centrifuged at ١٠,٠٠٠ rpm for ١٥ min. Finally, the pellet was washed two times with ethanol, dried, and resuspended in TE buffer. The purity of DNA was checked by determining the ratio of absorbance at ٢٦٠ nm to absorbance at ٢٨٠ nm (Corinaldesi *et a*l., ٢٠٠٤).

٢.٢.٧: Generating of variant derivatives by Salicylic acid

K.pneumoniae No. ١٠ was subjected to successive subcultring for (٥٤٠) generation (٢٠ min/ generation) on supported M٩ medium containing ١٥٠µg/ml of salicylic acid at ٣٧ Cº. Antibiotic resistance marker of the last subculture (١٠th) detected on Mullar-Hinton agar supplemented with the antibiotics like ampicillin, tetracycline, vancomycin, kanamycin, nalidixic acid, neomycin, trimethoprim, and rifampcin and DNA content of resulting cells were analyzed (Al-Jlawi, ٢٠٠٢).

٢.٢.٨: Genetic stability detection y of *K.pneumoniae I* variant

K. pneumoniae I variant was subjected to successive subcultring for (٥٤٠) generation (٢٠ min/ generation) on Mullar-Hinton agar at both ٣٠Cº and ٣٧ Cº. Antibiotic resistance marker of the last subculture (١٠th) detected on Mullar-Hinton agar supplemented with the antibiotics like ampicillin, tetracycline, vancomycin, kanamycin, nalidixic acid, neomycin, trimethoprim, and rifampcin and DNA content of resulting cells were analyzed.

٢.٢.٩: Detection of type of antibiotic encoded by transposon

K.pneumoniae I variant was subjected to successive subcultring on Mullar-Hinton agar containing increasing concentration of antibiotics like ampicillin, amoxillin , tetracycline, novobiocin, kanamycin, nalidixic acid, , and Chloramphenicol incubated at ٣٧Cº

٢.٢.١٠: Detection of transposition of ampcillin resistance

The detection of transposition of ampcillin was performed according to the method of Ruben et al. (١٩٧٦) with some modification. Conjugative Inc P plasmid *R٧٥١* carrying trimethoprim gene was introduced into *K.pneumoniae I* variant which harbored large plasmid *pKPI* (that carry ampcillin transposon) by conjugation. Then the transconjugant selected on Mullar-Hinton agar supplemented with ١٥٠µg/ml of ampcillin, ٥٠ µg/ml trimethoprim and ١٠٠ µg/ml of rifampcin .the transconjugant that contain both plasmid (*R٧٥١* plasmid and *pKPI*) were subjected to successive subculturing for about generation (٣٠min/generation) on Mullar-Hinton agar containing ١٥٠µg/ml of ampcillin at both ٣٠Cº and ٣٧ Cº. After that antibiotic resistance marker of the last subculture (١٠th) detected on Mullar-Hinton agar supplemented with the studies antibiotics and the DNA contents of the resulting cells were analyzed.

٢.٢.١١: Bacterial conjugation.

Day one:

١- A single colony of donor strain was inoculated in to ٥٠ ml LB broth containing appropriated antibiotic.

٢- A single colony of *E.coli* MM٢٩٤ rif^r recipient was inoculated in to ٥٠ ml LB broth containing ١٠٠ µg/ml of rifampcin.

٣- The cultures were incubated overnight at ٣٧C° for ١٨ hr. with vigorous shaking or until OD ٥٩٠≈ ٠.٥٤.

Day two: Surface mating

١- Aliquot of ٥×١٠^٧ of donor cell and ١×١٠^٨ recipients were mixed in sterile eppendorf.

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

٣- The LB agar plates which contain Millipore filter were incubated at ٣٧C° for ١-٢ hr.

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

٥- Serial ١/١٠ dilution was made to $١٠^{-٨}$ of the mating mixture.

٦- Aliquot of ٠.١ ml of dilution $١٠^{-١}$ to $١٠^{-٦}$ was spread on LB agar containing rifampcin (١٠٠µl/ml) and trimethoprim (٥٠µl/ml).

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

٨- Aliquot of ٠.١ ml of dilution $١٠^{-٦}$ and $١٠^{-٨}$ was spread on LB agar containing rifampcin (١٠٠µl/ml) to estimate the number of recipient cells in the mating.

٩- All plates were incubated at ٣٧C˚.

Control:

A- Aliquot of ٠.١ ml of donor cells was transferred to rifampcin plate.

B- Aliquot of ٠.١ ml of recipient cell *Escherichia coli* MM٢٩٤ rif^r were transferred to Plates containing antibiotic of donor strain.

٩- All plates were incubated at ٣٧C˚.

Day three

The colonies that had grown on the selection plate was counted and the number of bacteria per ml in mating mixture exhibiting the phenotype rifampcin (total recipient) and Rif^r, Tp^r (transconjugant)was calculated (O' Connell, ١٩٨٤)

- The frequency of conjugation was calculated as below:

No. Of transconjugants cells/ml

Tranconjugant cells per Recipient cells= ——————————————————

No. of recipients cells /ml

٣.٢.١٢: Transformation

٣.٢.١٢.١: Preparation of competent cells

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

٢- Aliquot of ٠.٣ ml of starting culture was inoculated in to ٦٠ ml of fresh nutrient broth medium and the cells were grown at ٣٧C° for ١-٢hr.

٣- The culture was chilled on ice for ٥-٧ minutes; cell suspension was centrifuged at ٨٠٠٠ rpm for١٥min in a sterile ١٠٠ml glass centrifuge tube.

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

٥- The cell suspension was placed on ice, and then centrifuged at ٨٠٠٠ rpm for ١٥ min.

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

٣.٢.١٢.٢: DNA up takes

١- For each ٢٠٠ µl of competent cells in a transformation tube ١٠µl of a DNA solution was added and incubated on ice bath for ٣٠min.

٢- The competent cells were heat- shocked by placing the tube in a ٤٢C° water bath for ٩٠ Sec. and then ٣ min. at ice bath.

٣- Aliquot of ٨٠٠ µl of pre wormed (٣٧C°) SOC medium was added to the suspension and Incubated at ٣٧C° with moderate agitation for ٦٠ min.

٤- Aliquot of ٠.١ ml cells were spread quickly and gently on selective plated, SOC agar plates by spreading.

٥-The plates were incubated upside down at ٣٧C° over night.

٦- The transformants colonies were calculated as below:

$$\text{Transformant frequency} = \frac{\text{No. of transformants cells/Concentration of DNA (µg/ml)}}{\text{Competent cells}}$$

٧- Co-transfer for transformant cells were made on different plates containing different type of antibiotics (Sambrook *et al.* ١٩٨٩).

٤. Results and Discussion

٤.١. Re-identification of *K.pneumoniae* No.١٠ Isolate

K.pneumoniae No.١٠ had previously been isolated by (Al-Jlawi, ٢٠٠٠), which re-identified by using api ٢٠ E. as in Fig (٤-١).The isolate was resistant to most antibiotics including ampicillin,

tetracycline, streptomycin, kanamycin, trimethoprim, Chloramphenicol, and nalidixic acid The resistance of K.pneumoniae No.١٠ is mainly by large plasmid pKP about ١٢٠ kbp (Al-Jlawi, ٢٠٠٠).

The wide spread use of β-lactamase groups of antibiotic against bacterial infection and continued mismanaged selective pressure has contributed toward the emergence of extended spectrum-β-lactamase resistance bacteria (Cohen, ١٩٩٢). It demonstrated that β-lactamase which was associated with high level ampicillin resistance in *K.pneumoniae* strains are mainly produced by large plasmid pK١ (Livermore, ١٩٩٥).Philippon *et al*. (١٩٨٩) indicated that one large plasmid encoding multidrug resistance genes usually carry one β-lactamase encoded gene, which is probably a mutant of the TEM-١ gene to code for enzyme capable of hydrolyzing the extended –spectrum ß-lactam. Genes specifying resistance to β-lactamase may be included in transposable elements it has been reported that each ß-lactamase reside in a ٥,٠٠٠ bp DNA sequence called the ampicillin transposon (TnA).Tn١, Tn٣,Tn٢٦٠١, and Tn٢٦٠٢ have been identified on Rp٤, R١, Rms٢١٢, and Rms٣٢٥, respectively (Hedges and Jacob, ١٩٧٤; Kopeco and Cohen, ١٩٧٥; Yamamoto *et al., ١٩٨٠)*.These TnA are very similar in size , base sequence homology as observed by heteroduplex formation, restriction endonuclease cleavage site, and possession of short inverted repeat sequence at both end (Hefforn *et al*., ١٩٧٥). Gene specifying resistance to ß-lactamase may be including in transposable elements consequently, these experiment were performed to determine whether *K.pneumoniae* No.١٠ mega plasmid *pKP* harboring a transposon encoding ampicillin resistance.

***Klebsiella pneumoniae* (٥٢١٥٧٧٣) [٪٩٧.٧] accuracy of diagnosis.**

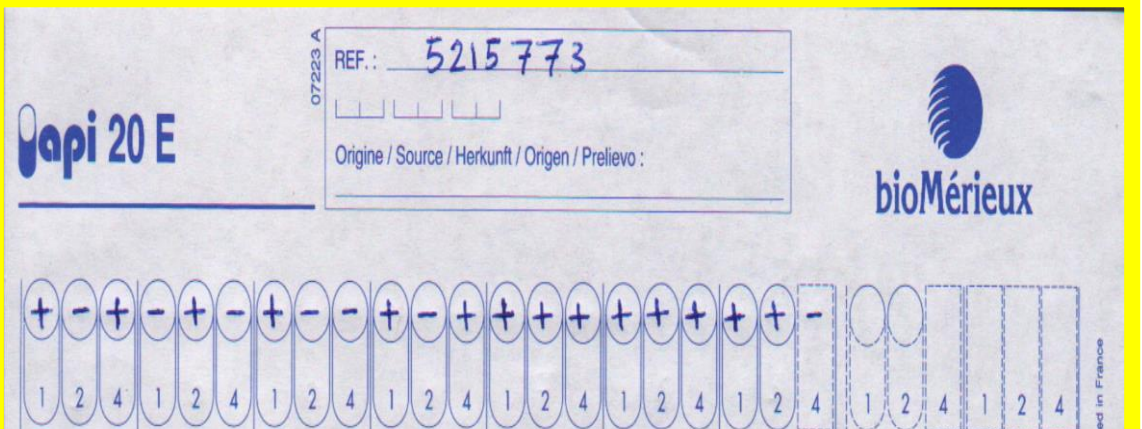

Fig. (٤-١) Re- Identification of *K.pneumoniae* No.١٠ Using api ٢٠E.

٤.٢. Variant Derivatives from K.pneumoniae No.١٠ after Salicylic acid Treatment.

The salicylic acid was used to obtain variants from *K.pneumoniae* sensitive to trimethoprim in order to use them as recipient to Inc P plasmid R٧٥١ which is trimethoprim resistant. Salicylic acid has good role in plasmid curing when use in high concentration ١٠٠µg/ml (Al-Said, ١٩٩٧. السعيد،مجد صبرى١٩٩٧.).

Al-Jlawi (٢٠٠٠) showed that optimum concentration of salicylic acid that lead plasmid cure with out any inhibition effect to the *K. pneumoniae* is ١٥٠µg/ml.

The successive subculture of *K.pneumoniae* No.١٠ on the Supported M٩ medium containing ١٥٠µg/ml of salicylic acid resulted in to obtaining four variant groups that differ in their antibiotic susceptibility pattern and their plasmid contents as shown in Fig (٤-٢).

Group١ of variant groups designated as *K.pneumoniae I* which comprise about ٣١.٦٦٪ of total variant groups characterized by susceptibility toward streptomycin, trimethoprim, and rifampcin in compared with those present in the wild type *K. pneumoniae* No.١٠ as shown in table (٤-١). Electrophoresis study of the plasmids contents in *K.pneumoniae I* group showed there were two bands of plasmid DNA in compare with those found in wild type of the K.*pneumoniae* No.١٠ which contained single band.

Al-Jlawi, (٢٠٠٠) refereed that the presence of salicylic acid in culture medium result different genetic variation ascribed to recombination of genetic material in *K. pneumoniae* No.١٠ and presence of transposable element in this strain. The presence of transposable elements in the *K. pneumoniae* No.١٠ led also to disturb genes responsible for antibiotic resistance .Transposon have ability to make changes in the genetic material of *K.pneumoniae* by various way like deletion , addition, reversion, base pair substitution, and as well as insertional mutagenesis (Simon-Smit *et al*,١٩٨٤; Lewin, ١٩٩٠).

Group ٢ variant which designated as *K.pneumoniae G* comprise about ١٨.٦٦٪ characterized by sensitivity towards tetracycline, streptomycin, nalidixic acid, and rifampcin.

Electrophoresis of plasmid contents of *K.pneumoniae G* revealed that there were three bands of plasmid DNA compare with single band in wild type *K.pneumoniae* No.١٠. Group ٣ designated as *K.pneumoniae K* and group ٤ which designed as K. *pneumoniae* L comprised ٢٧.٦٦٪ and ٢٢٪ respectively of total variant groups. Each of them possesses two bands of plasmid DNA in gel electrophoresis; *K. pneumoniae K* group was sensitive to tetracycline, vancomycin, kanamycin, nalidixic acid, neomycin, trimethoprim, and rifampcin while the *K. pneumoniae L* was sensitive to tetracycline, vancomycin, kanamycin, nalidixic acid, neomycin, and trimethoprim.

McLaughlin (١٩٧٣) and Domenico *et al.*(١٩٨٩) Showed that salicylic acid act as chelating agent that chelating divalent cations especially $Mg^{+٢}$ and $Ca^{+٢}$ ion in the culture medium that play important role in stabilizing of plasma membrane and enzymatic activation that found in per plasma space. The salicylic acid has ability to adsorb the phospholipids causing an increasing in negativity of the plasma membranes of *Klebsiella pneumoniae,* consequently led misdistribution of the plasmids during replication (Al-Jlawi, ٢٠٠٠).Plasma membranes contain specialized site that bind to plasmids and DNA chromosomes during host replications so that salicylic acid may affect on these site thus play role in curing of plasmids (Al-said, ١٩٩٧. السعيد،محمد صبرى١٩٩٧).

Table (٤-١): Successive subcultures of *K.pneumoniae No ١٠. Isolate* on supported M٩ medium containing Salicylic acid (١٥٠µg/ml) at ٣٧ C°.

iant groups														**centage**
٦ *K.pneumoniae* (١٠)														

neumoniae I														٦
neumoniae G														٦
neumoniae K														٦
neumoniae L														

*The calculated percentage for ٣٠٠ single colony transferred by picking and patching in the presence of salicylic acid.

***** The symbol (+) refer to resistance while the (-) is sensitive.

*Ap :ampicillin(١٥٠ µg/ml),Tc :tetracycline(٢٠ µg/ml),Ax :amoxicillin(٣٠ µg/ml),Sm : streptomycin(٥٠ µg/ml),VA : vancomycin(٣٠ µg/ml),Km : kanamycin(٥٠ µg/ml), NV : novobiocin(٣٠ µg/ml),Nal : nalidixic acid (٢٠ µg/ml),N : neomycin (٣٠ µg/ml),Tp : trimethoprim (٥٠ µg/ml),Rif : rifampcin(١٠٠ µg/ml),Cm :chloramphenicol (٥٠ µg/ml),and CTX :cefotaxime (٣٠ µg/ml)

K.p I: represent *K.pneumoniae* variant group ١ after treated with salicylic avid

K.p G: represent *K.pneumoniae* variant group ٢ after treated with salicylic avid

K.p K: represent *K.pneumoniae* variant group ٣after treated with salicylic avid

K.p L: represent *K.pneumoniae* variant group ٤after treated by salicylic avid

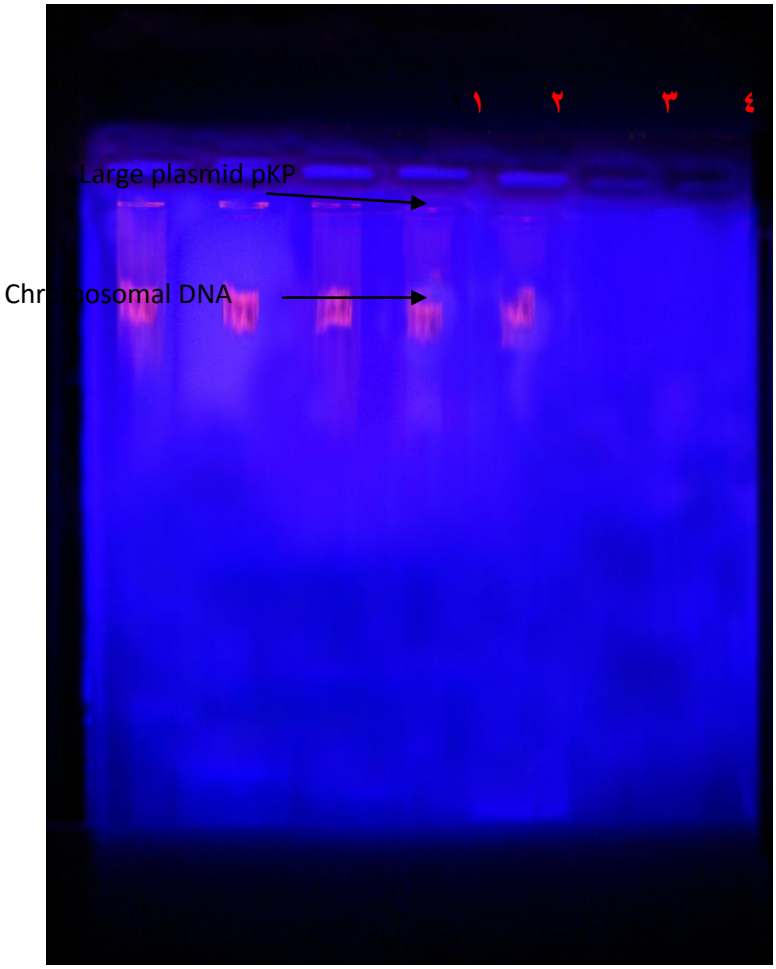

Fig. (٤-٢) plasmid profile of variant groups of *Klebsiella pneumoniae No.* ١٠ after treating with ١٥٠ µg/ml of Salicylic Acid.

The DNA plasmid extracted by alkaline lysis (Kado and Liu, ١٩٨١) and migrated on Agorose gel ٠.٧٪, ٥٠ volt, and ٦hr.

Lane ١: *Klebsiella pneumoniae* No.١٠ with out treating with salicylic acid.

Lane ٢: *K.p I* (Variant group ١ of *K.pneumoniae* No.١٠)

Lane ٣: *K.p G* (Variant group ٢ of *K.pneumoniae* No.١٠)

Lane ٤: *K.p K*(Variant group ٣ of *K.pneumoniae* No.١٠)

Lane ٥: *K.pL*(Variant group ٤ of *K.pneumoniae* No.١٠)

٤.٣ Detection of transposon in *K. Pneumoniae* I Variant

٤.٣.١ Transposons are responsible for genetic instability.

The relocation of transposons can lead to genetic instability .this may result in the simple loss of a phenotype if transposition results in the insertion of a transposable element into a structural gene (Hefforn *et al.*١٩٧٥).

The successive subcultures of *K.pneumoniae I* (that derived from *Klebsiella pneumoniae* No.١٠ after treating with salicylic acid) on medium containing ١٥٠µg/ml ampicillin led to excision of transposon from positions that they located in *pKPI* to other location of *pKPI* it self (Intramolecular transposition) or to other plasmids or chromosomes (Intermolecular transposition), with company of block of structural genes, including the antibiotic resistance genes.

The excision of transposon is due to the concentration of ampicillin that makes stress on the host and therefore induced the SOS response in bacteria which consequently stimulate the excision of transposon (Rusina et al., ١٩٩٢) and (Miller et al., ٢٠٠٤).

The successive subculturing (٥٤٠ generation) of *K. pneumoniae I* in Mullar-Hinton Agar medium containing ١٥٠ µg/ml at ٣٧ Cº the last subculture (١٠th) was revealed two groups of variant as shown in table (٤-٢). The first group designed as *K. pneumoniae I*١ which comprised about ٩٢.٥ % of total variant groups and characterized by losing the neomycin resistance while the second group members which designed as *K. pneumoniae I*٢ constitute about ٧.٥ % of total variant groups and characterized by losing neomycin and tetracycline resistance in comparison with *K. pneumoniae I* variant.

Goryshin *an*d Rezinkoff, (١٩٩٨) showed the Tn٥ can randomly insert in to genome of an organism, and in doing so, disrupt function of genes that they may incorporated in to. Hefforn, (١٩٧٥) reported that the gene that specified streptomycin phospho transferase inactivate when TnA had been inserted in to this gene. DE lacruz and Grinsted, (١٩٨٢) showed that the transposition of Tn٢١ some times inactivated tetracycline gene that responsible for tetracycline resistance in *pACYC ١٨٤* by insertion.

When the same *K. pneumoniae I* variant subjected to the same treatment but incubated at ٣٠ Cº, the colonies of last subculture (١٠th) revealed four groups of variants as shown in table (٤-٣).The first group designed as *K. pneumoniae I*٢ constitute about ٦٠ % of total variant groups characterized by losing Neomycin resistance in comparison of *K. pneumoniae I* variant .The second group which designed as *K. pneumoniae I*٤ comprised about ٢٠ % of total variant groups characterized by losing neomycin and vancomycin resistance in comparison of K.pneumoniae I variant .The third group which designed as *K. pneumoniae I*٥ constitute about ١١.٥ % of total

variant groups characterized by losing Neomycin , Vancomycin and Kanamycin resistance in comparison of *K. pneumoniae I* variant .The fourth group which designed as *K. pneumoniae* $I_{٦}$ comprised about ٨.٥ % of total variant groups characterized by losing neomycin ,vancomycin, kanamycin and novobiocin resistance in comparison of *K. pneumoniae I* variant.

These results indicated that transposition of transposon in *K. pneumoniae I* variant at ٣٠ C° is more than these occurred at ٣٧ C° and therefore the antibiotic change pattern in ٣٠ C° is more than those at ٣٧ C°.

This result was similar to both Cornelis (١٩٧٩) that stated that the rate of transposition of prokaryotic transposon is temperature dependent and Kretschmer and Cohen (١٩٧٩**)** that showed that the transposon $Tn_{٣}$ in *E.coli* has an optimum temperature for transposition falls between ٢٦ - ٣٠ C°; but the rate of transposition falls directly above ٣٠ C°. The falls in this transposition demonstrated by Cornels, ١٩٧٩ , by changes in the physiological condition in the cell that lead to cause a conformational change in either the transposase or resolvase, making them less efficient.

Table (٤-٢): different phenotypic classes obtained from successive subcultures of *K.pneumoniae I* variant on Mullar-Hinton containing ampicillin (١٥٠µg/ml) at ٣٧ C°.

*The calculated percentage for ٢٠٠ single colony transferred by picking and patching in the presence of ampicillin (١٥٠µg/ml) incubated at ٣٧ C°.

Variant Groups	Ap	Tc	Ax	Sm	VA	Km	NV	Nal	N	Tp	Rif	Cm	Ctx	**Percentage %**
Group ١ ***(K.p I١)***	+	+	+	-	+	+	+	+	-	-	-	+	+	٩٢.٥
Group ٢ ***(K.p I٢)***	+	-	+	-	+	+	+	+	-	-	-	+	+	٧.٥
Original ***(K.p I)***	+	+	+	-	+	+	+	+	+	-	-	+	+	١٠٠

*The symbol (+) refer to resistance while the (-) is sensitive.

*Ap :ampicillin(١٥٠ µg/ml),Tc :tetracycline(٢٠ µg/ml),Ax :amoxicillin(٣٠ µg/ml),Sm : streptomycin(٥٠ µg/ml),VA : vancomycin(٣٠ µg/ml),Km : kanamycin(٥٠ µg/ml), NV : novobiocin(٣٠ µg/ml),Nal : nalidixic acid (٢٠ µg/ml),N : neomycin (٣٠ µg/ml),Tp : trimethoprim (٥٠ µg/ml),Rif : rifampcin(١٠٠ µg/ml),Cm :chloramphenicol (٥٠ µg/ml),and CTX :cefotaxime (٣٠ µg/ml)

***K.p I١* and *K.p I٢*:** represent variant strains derived from *K. pneumoniae I* after successive subculture on ampicillin medium incubated at ٣٧ C°.

Table (٤-٣): different phenotypic classes obtained from sequential subculturing of *K. pneumoniae I variant* on ampicillin containing medium (١٥٠µg/ml) at ٣٠ C°.

*The calculated percentage for ٢٠٠ single colony transferred by picking and patching in the presence of ampicillin (١٥٠µg/ml) incubated at ٣٠ C°.

Variant Groups	Ap	Tc	Ax	Sm	VA	Km	NV	Nal	N	Tp	Rif	Cm	Ctx	**Percentage %**
Group ١ ***(K.p I٣)***	+	+	+	-	+	+	+	+	-	-	-	+	+	٦٠
Group ٢ ***(K.p I٤)***	+	+	+	-	-	+	+	+	-	-	-	+	+	٢٠
Group ٣ ***(K.p I٥)***	+	+	+	-	-	-	+	+	-	-	-	+	+	١١.٥
Group ٤ ***(K.p I٦)***	+	+	+	-	-	-	-	+	-	-	-	-	+	٨.٥
Original ***(K.p I)***	+	+	+	-	+	+	+	+	+	-	-	+	+	١٠٠

*The symbol (+) refer to resistance while the (-) is sensitive.

*Ap :ampicillin(١٥٠ µg/ml),Tc :tetracycline(٢٠ µg/ml),Ax :amoxicillin(٣٠ µg/ml),Sm : streptomycin(٥٠ µg/ml),VA : vancomycin(٣٠ µg/ml),Km : kanamycin(٥٠ µg/ml), NV : novobiocin(٣٠ µg/ml),Nal : nalidixic acid (٢٠ µg/ml),N : neomycin (٣٠ µg/ml),Tp : trimethoprim (٥٠ µg/ml),Rif : rifampcin(١٠٠ µg/ml),Cm :chloramphenicol (٥٠ µg/ml),and CTX :cefotaxime (٣٠ µg/ml)

***K.p I٣, K.p I٤, K.p I٥ and K.p I٦**: represent variant strains derived from K. pneumoniae I after successive subculture on ampicillin medium incubated at ٣٠ Cº.

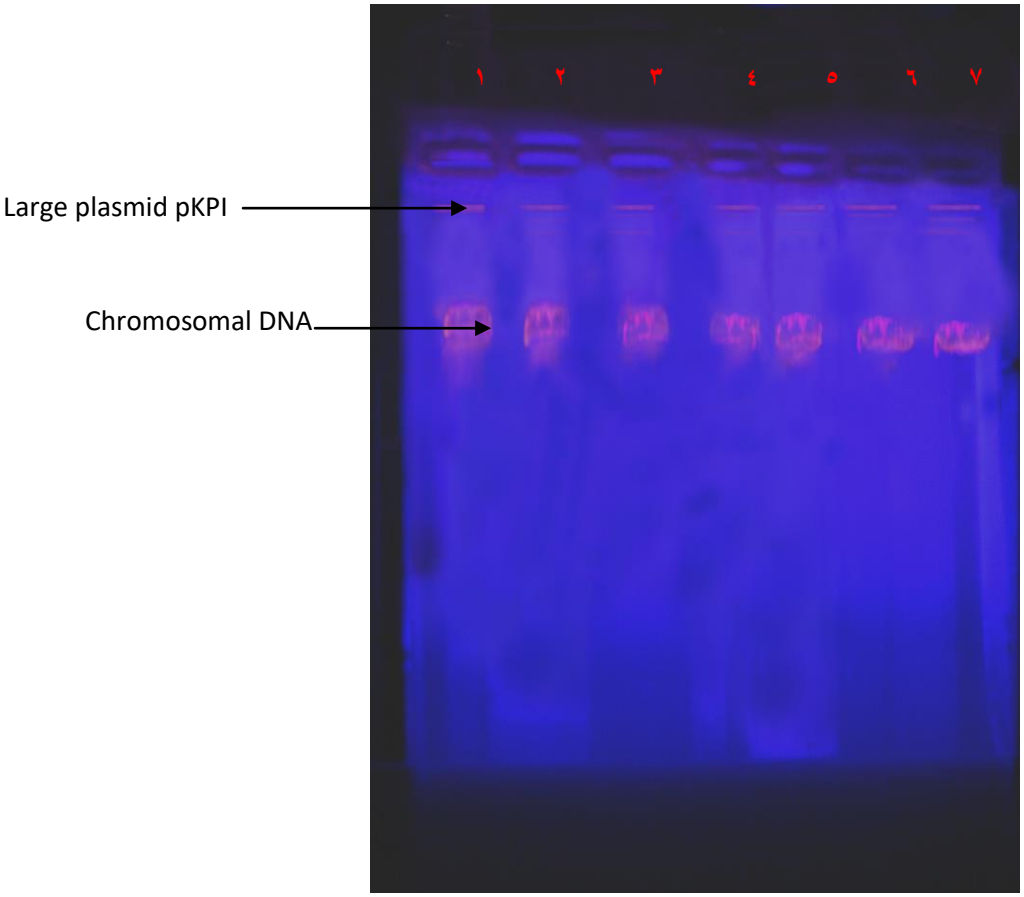

Fig. (٤-٣) Electrophoresis of Plasmids DNA contents of several groups of *K. pneumoniae I* variant at ٣٧C°and ٣٠ C°.

The DNA plasmid extracted by alkaline lysis (Kado and Liu, ١٩٨١) and migrated on Agorose gel ٠.٧٪, ٥٠ volt, and ٦ hr.

Lane ١*: K.p I* variant *(K.pneumoniae* variant group ١ after treated by salicylic avid).

Lane٢: *K.p I*١ derived from *K. pneumoniae I* after successive subculture at ٣٧ Cº.

Lane ٣*: K.p I*٢ derived from *K. pneumoniae I* after successive subculture at ٣٠ Cº.

Lane ٤: *K.p I*٣ derived from *K. pneumoniae I* after successive subculture at ٣٠ Cº.

Lane٥: *K.p I*٤ derived from *K.pneumoniae I* after successive subculture at ٣٠ Cº.

Lane٦: *K.p I*٥ derived from *K.pneumoniae I* after successive subculture at ٣٠ Cº.

Lane٧*: K.p I*٦ derived from *K.pneumoniae I* after successive subculture at ٣٠ Cº.

٤.٣.٢ Type of antibiotic encoded by transposon

Successive subculture of *K.pneumoniae I* variant in increased concentration of ampicillin, novobiocin, chloramphinecol, tetracycline, and amoxillin in solid culture revealed that *K.pneumoniae I* harboring transposon conferring ampicillin resistance.

The results in table (٤-٤) showed that *K.pneumoniae I* variant was sensitive to antibiotic like chloramphinecol, novobiocin, tetracycline, and amoxicillin at concentration ≥ than ٢٠٠µg/ml, ٥٠ µg/ml, ٥٠µg/ml, and ١٠٠µg/ml respectively except for ampicillin which remain resistant even at concentration about ١٠٠٠ µg/ml.

The survive of *K.pneumoniae I* variant in medium containing more than ١٠٠٠µg/ml in compare with *K.pneumoniae*(control) which was unable to survive in the same concentration of ampicillin, led us to suggest that the transposon conferring ampicillin resistance, but no other antibiotic like amoxicillin, tetracycline, novobiocin and chloramphenicol.

Hefforn *et al. (*١٩٧٥) Reported that if transposition occurs into a regular gene, then a normally repressed gene may become expressed constitutively, and at much higher levels than is usual. this has consequence for the phenotype of the insertional mutant it is quite possible that ampicillin meditated transposon(TnA) insertion within structural gene has inactivated or modified plasmid gene that control the rate of initiation of plasmid replication or some aspects of replication control. Rubens *et al. (*١٩٧٦) referred that some transposon carry strong promoter sequence and these may affect the expression of genes downstream of the site of insertion, causing polar mutation.

Table (٤-٤): Determination of type of antibiotic resistance carried by transposable genetic element in *K.pneumoniae I variant.*

Isolates	Antibiotic Type	Concentrations (µg /ml)											
		٢٥	٥٠	١٠٠	٢٠٠	٣٠٠	٤٠٠	٥٠٠	٦٠٠	٧٠٠	٨٠٠	٩٠٠	١٠٠٠
K. pneumoniae I variant	Cm	+	+	+	-	-	-	-	-	-	-	-	-
	NV	+	+	-	-	-	-	-	-	-	-	-	-
	Ap	+	+	+	+	+	+	+	+	+	+	+	+
	Tc	+	+	-	-	-	-	-	-	-	-	-	-
	Ax	+	+	+	-	-	-	-	-	-	-	-	-
K.pneumoniae (control)	Ap	+	+	+	-	-	-	-	-	-	-	-	-

The symbol (+) refer to resistance while the (-) is sensitive.

AP: ampicillin, Tc: tetracycline, Ax: amoxicillin, NV: novobiocin,

Nal: nalidixic acid, and Cm: chloramphenicol.

٤.٤ An attempt to isolate transposon on Inc p conjugative *pR٧٥١*

Upon mating of *E.coli* GMP٤١٧٦ (*pR٧٥١*) with *K.pneumoniae I* variant ampicillin, trimethoprim–resistant Tranconjugant (TR_1) were selected at frequency of ٧.٢٥×١٠$^{-٤}$ per recipient. The ٥٣ kb conjugative plasmid, *pR٧٥١* which confer trimethoprim resistance, was successfully transferred to *K.p I variant* (Tp^S) by conjugation.

Peterson *et al. (١٩٨٢)* referred that when two or more plasmids reside in the same cell, they may coexist stably without affecting one another, or may interact in some way. One possible interaction is physical exchange of segments of DNA. This can occur by transposition or recombination. Transposition of one segment of DNA to another is widely observed and since it can occur in the absence of *recA* function in a host cell, it is therefore not by homologous recombination (Calos and Millar, ١٩٨٠).

Successive subcultures of TR_1 on Mueller-Hinton agar containing ١٥٠µg/ml of ampicillin incubated at both ٣٧ C° and ٣٠ C° to induce transposon to transpose from *pKpI* to Inc p plasmid *R٧٥١* as shown in table (٤-٥) and (٤-٧) respectively .

٢٠٠ colonies of TR_1 from the last ١٠th subculture of those grown at ٣٧C° transferred by picking and patching to media containing ampicillin, trimethoprim, chloramphenicol, kanamycine and tetracycline, two phenotypic classes of TR_1 *K.pneumoniae* obtained table (٤-٦).Group I of TR_1 designated as TR_1A which constitute about ٩٢.٥% and characterized by susceptibility to only Streptomycin. Group II of TR_1 designated as TR_1B comprised ٧.٥٪ of total TR_1 phenotype classes and characterized by susceptibility toward Streptomycin and Chloramphenicol.

Three phenotypic classes of TR_1 yielded, when ٢٠٠ colonies of last subculture (١٠th) of TR_1 at ٣٠C° transfer to the media containing ampicillin, trimethoprim, chloramphenicol, kanamycine and tetracycline. Group ١ of TR_1 which designed as TR_1C comprise ٦١.٥% of total TR_1 phenotypic classes and characterized by susceptibility to only streptomycin table (٤-٨). Group ٢ of TR_1 which designated as TR_1D constitute ٣٦.٥٪ of total TR_1 change in antibiotic resistance pattern distinguish from original TR_1 by sensitivity toward tetracycline and Chloramphenicol in addition to streptomycin., while group ٣ which designated as TR_1E comprise only ٢٪ of TR_1 phenotypic classes appeared to be sensitive to trimethoprim, chloramphenicol, streptomycin, and tetracycline.

The DNA of first six subcultures of those grew at ٣٧C° had chosen to be extracted and subjected to gel electrophoresis, Fig (٤-٥) revealed the presence of two plasmid bands one of them resemble *pKPI* and other smaller in size in molecular weigh than *pKPI* but it is higher than *R*١٧٥ plasmid.

The DNA from Subcultured No. ٢, ٣, ٤, ٥, ٩, and ١٠ of *K.pneumoniae* TR١ that grown at ٣٠C° table (٤-٧) were subjected to gel electrophoresis, the results revealed presence of two bands in each ٣, ٤, ٥, ٩ and ١٠ subculture, one of them resemble that of *pKpI* and the other smaller in molecular weigh than *pKPI* but higher than conjugative *inc P* plasmid as shown in Fig (٤-٦).The results revealed also presence of bright high molecular band may be formed as result of cointegration between *pKPI* and *R*١٧٥ plasmid in subculture No.٢.as shown in Fig (٤-٦ lane ٢).

From the contegrate formation; we suggest that the transposable element found in *K.pneumoniae I* isolates which confer resistance to ampicillin is belonging to Tn٣ family.

Reed (١٩٨١) showed that Tn٣ transpose from one replicon to another in a two- step process in the first , element mediated fusion of two replicons produces a co integrate structure, this fusion which requires the TnpA protein necessarily for replicative process ;cointegrate contain an additional copy of the element and duplication of a ٥ base pair target sequence. The second step in transposition is the resolutions of co integrate by site-specific recombination. .

Hefforn *et al*. (١٩٨٣) documented that Tn٣ is an ampicillin –resistance transposons, which is ٤٩٥٧ bp in length and contain a ٣٨-bp terminal inverted repeat at the left of the terminus (IRL) and at the right inverted repeat (IRR).

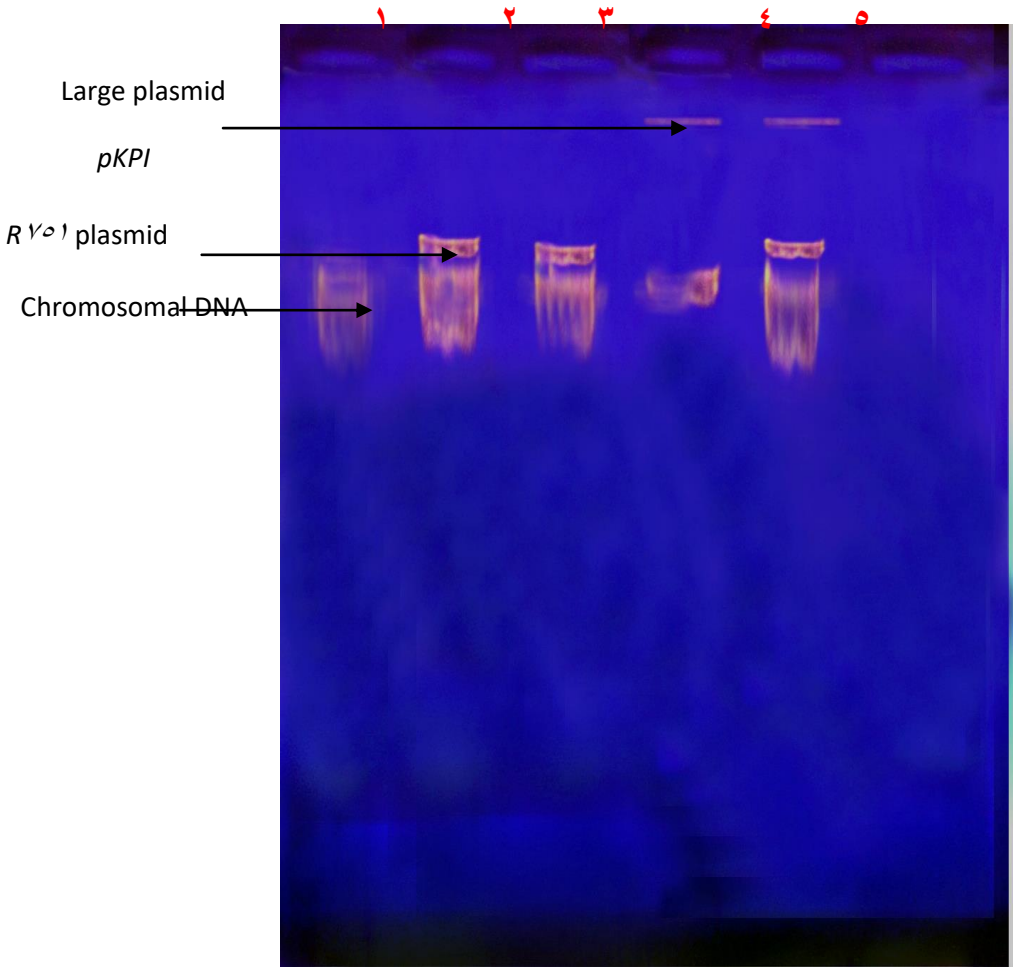

Fig. (٤-٤) **Electrophoresis of tranconjugant obtained from mating *K.pneumoniae I variant and E. coli* JMP٤١٧٦ (*pR٧٥١*) Sm^r,Tp^r at ٣٧C°.**

The DNA plasmid extracted by alkaline lysis (Kado and Liu, ١٩٨١) and migrated on Agorose gel ٠.٧٪, ٥٠ volt, and ٦hr.

Lane ١: *E.coli* MM٢٩٤ Rif^r

Lane ٢ and ٣*: E.coli* JMP٤١٧٦ (pR٧٥١) Sm^r,Tp^r

Lane ٤: *K.pneumoniae I* variant

Lane ٥: TR_1(Tp^r, Ap^r).

Table (٤-٥): Subsequent subcultures of Tranconjugant TR١ on medium contain ١٥٠µg/ ml ampicillin incubated at ٣٧C°.

Number of Subcultures	Muller –Hinton Agar contain ١٥٠µg/ ml of Ampicillin					
	Amp	Tp	Cm	Km	Sm	Tc
١	+	+	+	+	-	+
٢	+	+	+	+	-	+
٣	+	+	+	+	-	+
٤	+	+	+	+	-	+
٥	+	+	-	+	-	+
٦	+	+	-	+	-	+
٧	+	+	-	+	-	+
٨	+	+	-	+	-	+
٩	+	+	-	+	-	+
١٠	+	+	-	+	-	+

***** The symbol (+) refer to resistance while the (-) is sensitive

***** Ap: ampicillin (١٥٠ µg/ml), Tp: trimethoprim (٥٠ µg/ml) Km: kanamycin (٥٠ µg/ml), Cm: chloramphenicol (٥٠ µg/ml), and Tc: tetracycline (٢٠ µg/ml)

Table (٤-٦): Variant groups of TR١ obtained by transferring ٢٠٠colonies of last ١٠th subculture of TR١ from these grown at ٣٧ C° by picking and patching.

R١ phenotypic class	Ap	Tp	Cm	Km	Sm	Tc	Percentage %
Group ١ TR١A	+	+	+	+	-	+	٩٢.٥
Group ٢ TR١B	+	+	-	+	-	+	٧.٥
TR١ before treatment	+	+	+	+	-	+	١٠٠

*The calculated percentage for ٢٠٠ single colony transferred by picking and patching in the presence of ampicillin (١٥٠µg/ml) incubated at ٣٧ C°.

*The symbol (+) refer to resistance while the (-) is sensitive.

*The symbol (+) refer to resistance while the (-) is sensitive.

Ap : ampicillin(١٥٠ µg/ml),Tc : tetracycline(٢٠ µg/ml),Sm : streptomycin(٥٠ µg/ml),Km : kanamycin(٥٠ µg/ml), Tp : trimethoprim (٥٠ µg/ml), and Cm :chloramphenicol (٥٠ µg/ml).

*TR١A : represent group ١ of total phenotypic class after successive subculture of TR١ on ampicillin medium and incubated at ٣٧ Cº.

*TR١B : represent group ٢ of total phenotypic class after successive subculture of TR١ on ampicillin medium and incubated at ٣٧ Cº

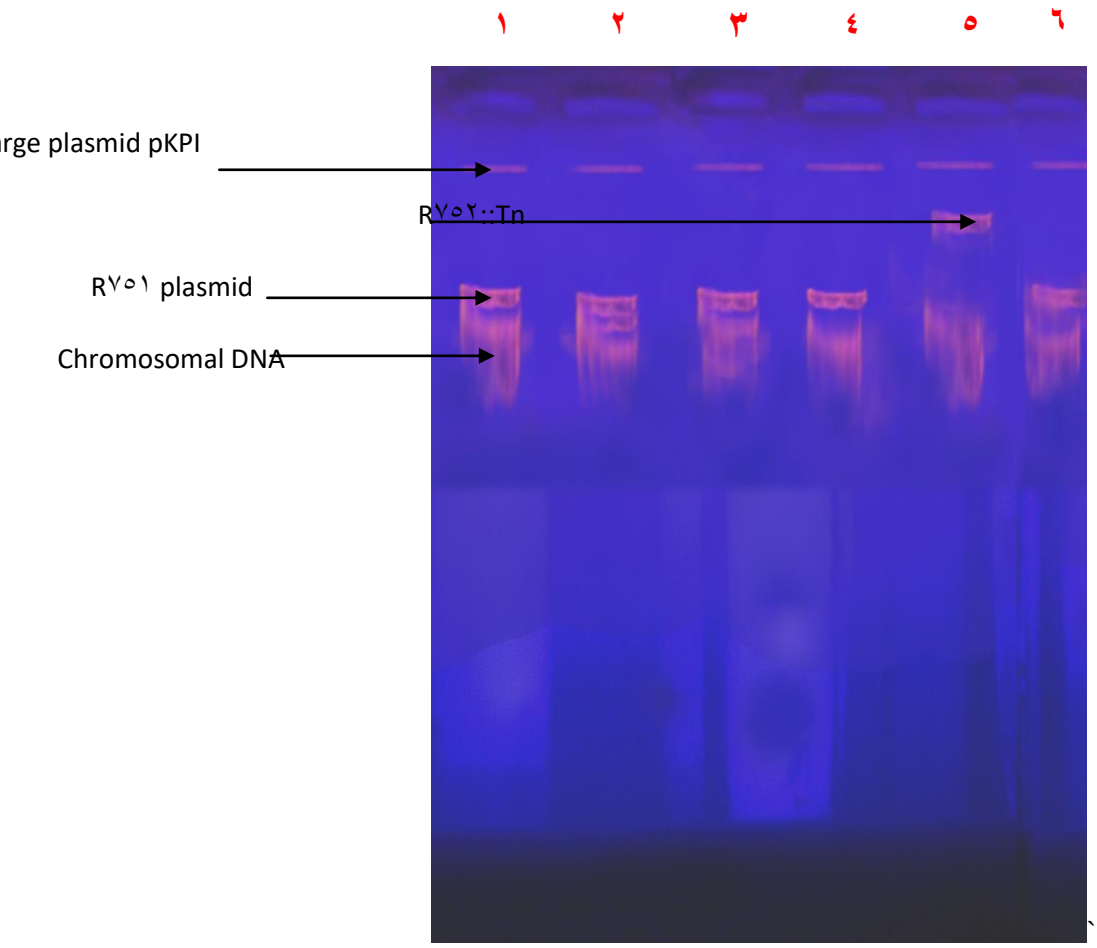

Fig.(٤-٥) Electrophoresis for tranconjugant TR١ (Tp^r, Ap^r) Plasmids DNA contents of several subcultures at ٣٧C°.

The DNA plasmid extracted by alkaline lysis (Kado and Liu, ١٩٨١) and migrated on Agorose gel ٠.٧٪, ٥٠ volt, for ٦hr.

Lane ١: TR١ from first subculture

Lane ٢: TR١ from second subculture

Lane ٣: TR١ from third subculture

Lane ٤: TR١ from fourth subculture

Lane ٥: TR١ from fifth subculture

Lane ٦: TR١ from sixth subculture

Table (٤-٧): Successive subcultures of tranconjugant TR١ on medium contain ١٥٠µg/ ml ampicillin incubated at ٣٠C°

Number of Subcultures	Muller –Hinton Agar contain ١٥٠µg/ ml Ampicillin					
	Amp	Tp	Cm	Km	Sm	Tc
١	+	+	+	+	-	+
٢	+	+	+	+	-	+
٣	+	+	+	+	-	+
٤	+	+	+	+	-	-
٥	+	+	+	+	-	-
٦	+	+	-	+	-	-
٧	+	+	-	+	-	-
٨	+	+	-	+	-	-
٩	+	-	-	+	-	-
١٠	+	-	-	+	-	-

*The symbol (+) refer to resistance while the (-) is sensitive

*****The symbol (+) refer to resistance while the (-) is sensitive.

*****The symbol (+) refer to resistance while the (-) is sensitive.

Ap : ampicillin(١٥٠ µg/ml),Tc : tetracycline(٢٠ µg/ml),Sm : streptomycin(٥٠ µg/ml),Km : kanamycin(٥٠ µg/ml), Tp : trimethoprim (٥٠ µg/ml), and Cm :chloramphenicol (٥٠ µg/ml).

ɔsequent subcultures	**Ap**	**Tp**	**Cm**	**Km**	**Sm**	**Tc**	**Percentage %**
Group ١ TR١C	+	+	+	+	-	+	٦١.٥
Group ٢ TR١D	+	+	-	+	-	-	٣٦.٥
Group ٣ TR١E	+	-	-	+	-	-	٢

Table (٤-٨): Variant groups of TR١ obtained by transferring ٢٠٠colonies of last ١٠th subculture of TR١ from these grown at ٣٠ C° by picking and patching

TR١ before treatment	+	+	+	+	-	+	١٠٠

*The calculated percentage for ٢٠٠ single colony transferred by picking and patching in the presence of ampicillin (١٥٠µg/ml) incubated at ٣٠ C°.

*The symbol (+) refer to resistance while the (-) is sensitive.

Ap : ampicillin(١٥٠ µg/ml),Tc : tetracycline(٢٠ µg/ml),Sm : streptomycin(٥٠ µg/ml),Km : kanamycin(٥٠ µg/ml), Tp : trimethoprim (٥٠ µg/ml), and Cm :chloramphenicol (٥٠ µg/ml).

*TR١C, TR١D and TR١E : represent groups١,٢ and ٣ of total phenotypic class after successive subculture of TR١ on ampicillin medium and incubated at ٣٠ Cº.

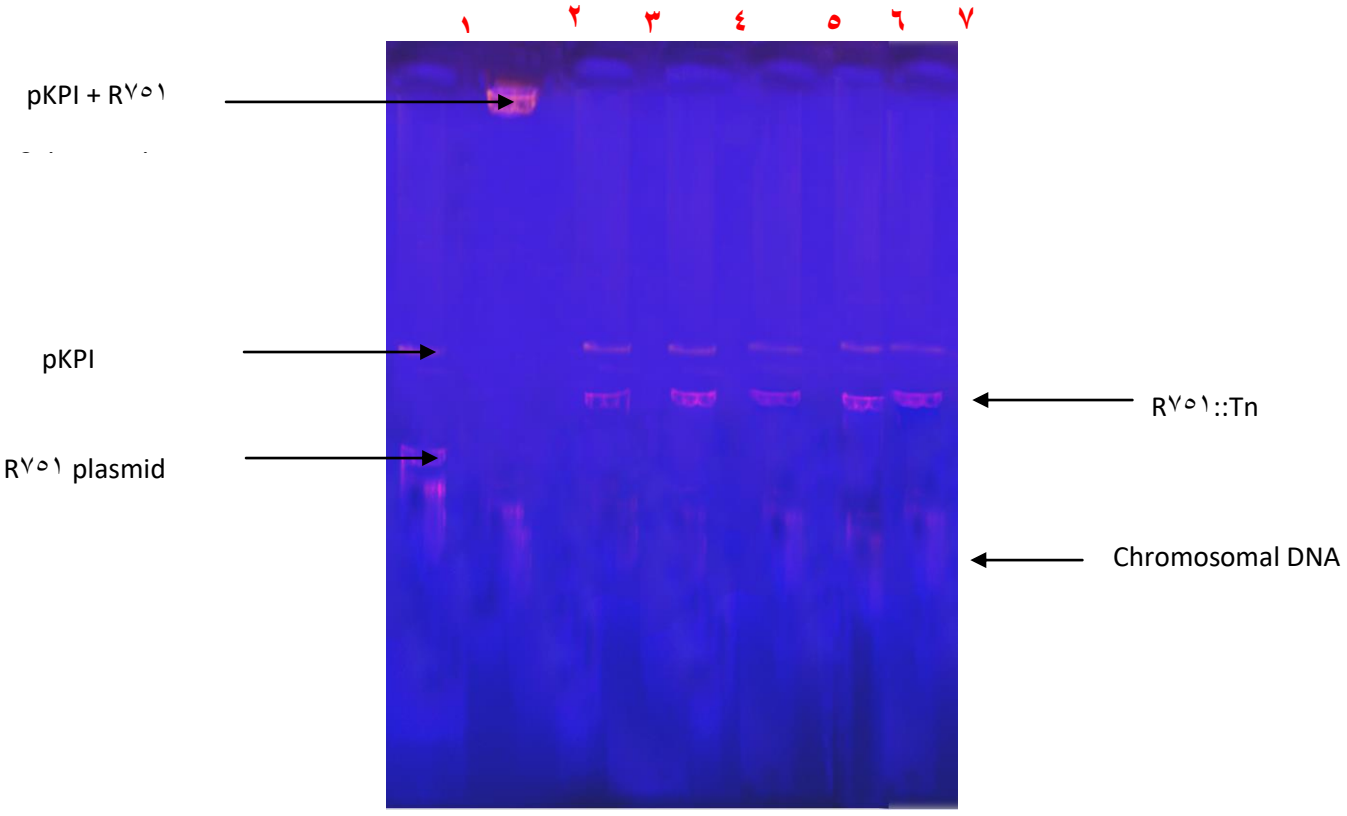

Fig. (٤-٦) Electrophoresis of Tranconjugant TR١ (Tp^r, Ap^r) Plasmids DNA contents of several subcultures at ٣٠C°.

The DNA plasmid extracted by alkaline lysis (Kado and Liu, ١٩٨١) and migrated on Agorose gel ٠.٧٪, ٥٠ volt, and ١٦hr.

Lane ١: *K. pneumoniae* transconjugant $TR_١$

Lane ٢: subculture No.٢ from these that grow at ٣٠ C°.

Lane ٣: subculture No.٣ from these that grow at ٣٠ C°.

Lane ٤: subculture No.٤ from these that grow at ٣٠ C°.

Lane ٥: subculture No.٥ from these that grow at ٣٠ C°.

Lane ٦: subculture No.٩ from these that grow at ٣٠ C°.

Lane ٧: subculture No.١٠ from these that grow at ٣٠ C°

Transposition of $Tn_٣$ occurred via cointegrate intermediate which produced by action of *tnpA* gene that expressed transposase and *tnpR* which expressed resolvase that divide the co integrate into it's two original molecules by site-specific recombination between two copies of the duplicate transposon; the result is a copy of the element at it's original locus and new copy at the target site (Reed, ١٩٨١).

In order to show that conjugative *R٧٥١* plasmid acquired the ampicillin transposon from large *pKPI*, conjugation between $TR_١$B which harbored both mega plasmid *pKPI* and conjugative plasmid *R٧٥١* plasmid with *E. coli* MM ٢٩٤, ampicillin, trimethoprim –resistant tranconjugant ($TR_٢$) were selected at frequency of ٦٢×١٠^{-٤} per recipient. $TR_٢$ harbored single plasmid $pTR_٢$ that appeared to carry determinants for resistance to trimethoprim and ampicillin and the size of $pTR_٢$ appeared larger than parent *R٧٥١* plasmid which confer resistance only to trimethoprim (Fig ٤-٧ lane ٢)

Upon mating of TR١D which harbored mega plasmid *pKPI* and conjugative plasmid *R٧٥١* plasmid with *E.coli* MM ٢٩٤, ampicillin, trimethoprim –resistant tranconjugant ($TR_٣$) were selected at frequency of ١٢×١٠^{-٧} per recipient. The plasmid contents study revealed of $TR_٣$ presence of plasmid $pTR_٣$ that higher in molecular weigh than *R٧٥١* plasmid and may represent R٧٥١::Tn (Fig ٤-٧ lane ٣).

Upon mating of TR١E which harbored mega plasmid *pKPI* and conjugative plasmid *R٧٥١* plasmid with *E.coli* MM٢٩٤, ampicillin, Rifampcin –resistant tranconjugant ($TR_٤$) were selected at frequency of ٦×١٠^{-٣} per recipient. The plasmid profile of $TR_٤$ (Ap^{r}, Tp^{S}) in Fig (٤-٧ lane ٤) showed presence of a band represent $pTR_٤$ plasmid which differed in their molecular weigh from that of parent *R٧٥١* plasmid (٤-٧ lane ٥).

The explanation for the presence of conjugative plasmid IncP *R٧٥١* in $TR_٤$ and Lacking of ability to survive in the presence of trimethoprim in medium is that the transposon may inserted to position where the trimethoprim resistance genes is located.

As in our study, Rubens *et al.(*١٩٨١)have shown that a transposable DNA sequence encoding resistance to beta –lactamase , located on ١٥٠ Kbp plasmid isolated from *Klebsiella* strains, is capable of transposing to other plasmid that exist within the same cell during a prolonged epidemic of nosocomial infection.

The transposon that carry ampicillin determinant in our *K.pneumoniae I* variant strain successfully transferred from *pKPI* to the co resident ٥٣ kb conjugative *R٧٥١* plasmid which consequently transferred to *E. coli* MM٢٩٤.

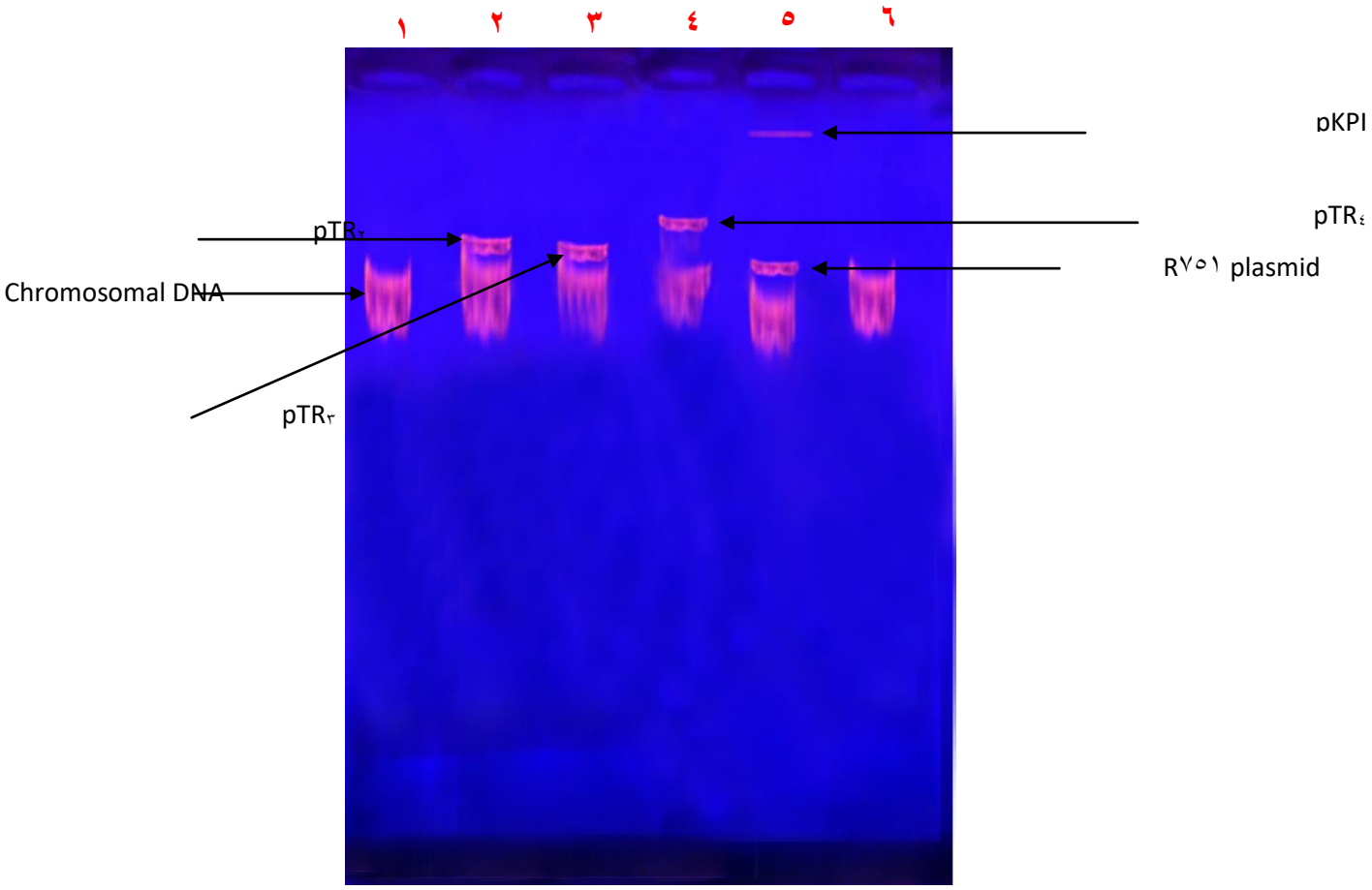

Fig. (٤-٧) Electrophoresis for Plasmids DNA contents for Tranconjugant TR٢, TR٣, and TR٤.

The DNA plasmid extracted by alkaline lysis (Kado and Liu, ١٩٨١) and migrated on Agorose gel ٠.٧٪, ٥٠ volt, and ٦hr.

Lane ١: E.*coli* MM٢٩٤.

Lane ٢: *E. coli* MM٢٩٤ transconjugant TR٢.

Lane ٣: *E. coli* MM٢٩٤ transconjugant TR٣

Lane ٤: *E. coli* MM٢٩٤ transconjugant TR٤

Lane ٥: *K.pneumoniae I* transconjugant TR١

Recommendation

This study advises to determine nucleotide sequence and G-C ratio of this transposon which confers ampcillin resistance to *K. pneumoniae* in order to confirm whether our transposon is similar to Tn۳ family or not. We also recommend further analyzing of this transposon by using suitable DNA probes of the Tn۳ family transposon family and hybridized them with our transposon for further identification. Finally we advise for further Identification of the type of beta-lactamase that expressed by our transposon by isoelectric focusing and determine the type of beta-lactamase.

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We, the examining committee, certify that we have read this Thesis and examined the student in its contents and that in our opinion; it is adequate for awarding Degree of Master of Science in Biotechnology.

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Summary

The isolate *Klebsiella pneumoniae* No١٠ which was previously isolated by (Al-Jlawi, ٢٠٠٠), is genetically instable and harbor a single non-conjugative large plasmid *pKP* (١٢٠Kbp) encoding resistance to ampicillin, tetracycline, streptomycin, kanamycine, trimethoprim, chloramphenicol, and nalidixic acid in addition to mucoid phenotype of bacteria.

Four variant groups (*K.pneumoniae I*, *K.pneumoniae G, K.pneumoniae K and K.pneumoniae L*) were derived from the isolate by successive subcultring in M_9 medium supplemented with ١٥٠µg/ml salicylic acid. The derived groups differ from the wild *K.pneumoniae* No.١٠ in their plasmid profile and antibiotic resistance pattern. *K.pneumoniae I* was sensitive to trimethoprim, streptomycin and rifampicin and thus, was used to serve as a recipient for the conjugative *Inc P* plasmid *R٧٥١* (Tp^r).

Ampicillin at concentration ١٥٠ µg/ml induces genetic instability of *K.pneumoniae I* when subcultured about ٥٤٠ generation in ampicillin medium also this instability was greater at ٣٠ Cº than at ٣٧ Cº.

This isolate was resistance to high ampicillin concentration more than ١٠٠٠ µg/ml in comparison with the other antibiotics including chloramphinicol, tetracycline, novobiocin, and amoxillin.This result suggested that possibility presence of transposon that encoding to ampicillin resistance in the isolate.

The ampicillin transposon (Tn::Ap) was induced to jump from the *pKPI* plasmid of *K.pneumoniae* transconjugant TR_1 that harbor *pKPI* and *R٧٥١* plasmid into conjugative *R٧٥١* plasmid by ampicillin ١٥٠µg/ml at both ٣٠ Cº and ٣٧ Cº. Agarose gel electrophoresis of the DNA contents of these cells types revealed presence of plasmid band higher in molecular weigh than p*R٧٥١* but less than *pKPI* .these results suggest possibility of jumping Ap-Tn from *pKPI* to *pR٧٥١.*

Three type of transconjugant of *E.coli* MM٢٩٤ (rif^r) TR_2, TR_3 and TR_4 obtained from mating of *E. coli* MM٢٩٤ (rif^r) and *K.pneumoniae I* cells in which transposition of Ap-Tn occurred. The gel electrophoresis of DNA contents from TR_2, TR_3 and TR_4 revealed presence of plasmid band higher in molecular weigh than the original *pR٧٥١* due to presence of an extra DNA fragment (transposon) that inserted to R٧٥١ plasmid.

المـصادر العربيه

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