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Molecular Identification of *Klebsiella pneumoniae* Using Capsule Genes

A thesis
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رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

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DEDICATION

To

My Parents

For taught me to trust in Allah, all

the unconditional love, guidance, raised me to be the person I am today, supporting and encouraging me to believe in myself

My Husband

For his remarkable patience and unwavering love and support over the many years we have been together

My Brothers and Sister

For have been a great source of motivation, love and inspiration for me

To pearl beads
Broeher's daughter (Aleen and Aya)

I dedicated my thesis work.

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Tamara

Summary

A total of 325 clinical and environmental samples were collected from five hospitals in Baghdad from patients (Male and Female) of age range between (≤ 10-50) years in the period between 1/11/2012 to 7/1/2013. Out of 282 isolates, 56 identified as *Klebsiella* depending on cultural, microscopical and biochemical characteristics. The remaining isolates were identified as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas* and *Proteus*, *Staphylococcus epidermidis*, *Enterobacter* and *Shigella*. Results of biochemical tests that was confirmed by using the API 20E and VITEK 2 system revealed that *Klebsiella* isolates (56 isolates) belong to four species: *K. pneumoniae* (40 isolates), *K. oxytoca* (3 isolates), *K. terrigena* (10 isolates) and *K. ornitholytica* (3 isolates). Thirty-six (90 %) of *K. pneumoniae* were isolated from clinical sources and 4 isolates (10 %) from environmental sources. *K. pneumoniae* isolated more frequently from urine sample and less from wound, ear swab, kitchen and bathrooms samples.

Regarding to the patients gender, it was found that males had a tendency to get infection more than females when 19 (52.8 %) of patients were males and 17 (47.2 %) females. Moreover, the age group ≤ 10 were most subjected to the infection of *K. pneumoniae*.

Antibiotic susceptibility of *K. pneumoniae* isolates against (15) of commonly used antibiotic was determined through disc-diffusion method. Results declared that, generally, the isolates were resistant to the antibiotics used except their sensitivity to imipenem.

DNA was extracted from *K. pneumoniae* isolates. Results showed that the recorded range of DNA concentrations was 47.4-123.8 ng/µl and the DNA purity was (1.6-2.0).

Summary

K. pneumoniae isolates was diagnosed molecularly by using polymerase chain reaction, 16S rRNA, *rmpA* and *kfu* genes were amplified and study the prevalence of the K serotypes, as well as, genes (except *kfu* gene) were amplified by using multiplex PCR. Results showed that all the isolates of *K. pneumoniae* gave a clear band with a molecular size 130 bp when PCR was performed with the primer that target the 16S rRNA. When using the primer specific for the capsule cluster gene *magA* and *k2A*, the result revealed that 23 (57.5 %) isolates belong to K1 serotype which gave a band of 1283 bp in size and 11 (27.5 %) isolates belong to K2 serotype which gave a band of 543 bp in size. These results suggest that *magA* and *k2A* genotype might be a useful marker to identify K1 and K2 serotypes of *K. pneumoniae* and these serotypes have been more prevalent than those that were neither K1 nor K2 (Non-K1/K2) (6 isolates (15 %)).

The *rmpA* and *kfu* genes were amplified, the result stated that *kfu* gene is more frequent than *rmpA* gene, band was appeared of 797 bp in size represented *Kfu* gene in 30 of the isolates, while band was appeared of 536 bp in size represented *rmpA* gene in 11 of the isolates. Moreover, prevalence of *kfu* gene was in 21 isolates of serotype K1 which was more frequent than in serotype K2 (8 isolates) and Non-K1/K2 serotype (1 isolate), while *rmpA* gene was more frequent in the serotype K1 and K2 (5 isolates) than Non-K1/K2 serotype (1 isolates).

Multiplex PCR was performed for *K. pneumoniae* isolates with four primers that target the 16S rRNA, *magA*, *k2A* and *rmpA* genes. Results showed that *K. pneumoniae* serotype K1 with *rmpA* positive isolates gave amplified bands for *magA*, *rmpA* and 16S rRNA genes, the *K. pneumoniae* serotype K2 with *rmpA* negative gave amplified bands for *k2A* and 16S rRNA genes.

Summary

Moreover, *K. pneumoniae* serotype K1 with *rmpA* negative showed positive results with *magA* and 16S rRNA genes. Finally, *K. pneumoniae* Non-K1/K2 with *rmpA* negative showed only positive results with 16S rRNA gene.

Multiplex PCR considered a reliable, relatively rapid, effective, easy application and repeatable and possible to be a powerful and potential tool for the routine clinical identification of *Klebsiella* species.

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List of Abbreviations

Abbreviations	Term
ADH	Arginine dihydrolase
API 20E test	Analytical profile index 20 *Enterobacteriaceae*
$BaCl_2$	Barium chloride
BHI	Brain Heart Infusion broth
bp	Base pair
C3b	Complement protein
C5b, C9	Complement proteins
CIE	Counter-current immunoelectrophoresis
CIT	Citrate
CPS	Capsule polysaccharide
DDH2O	Deionised Distilled water
DNA	Deoxyribonucliec acid
dNTP	Deoxynucleotide tri phosphate
D.W.	Distilled water
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
EMB	Eosin Methylene Blue
ESBL	Extended spectrum beta-lactamases
EtBr	Ethidium bromide
ETC	Extracellular toxic complex
Fur	ferric uptake regulator gene
GAG	Glycosaminoglycan
GEL	Gelatin
HMV	Hyper mucoviscostiy phenotype
IND	Indole
K-Ag	Capsular antigen
k2A	K2 capsule-associated gene A
Kb	Kilo Base
KD	Kilo Dalton
KIA	Kligler iron agar
K. oxytoca	*Klebsiella oxytoca*

K. pneumoniae	*Klebsiella pneumoniae*
K. ornithinolytica	*Klebsiella ornithinolytica*
K. terrigena	*Klebsiella terrigena*
LB	Luria - Bertani
LDC	Lysin decarboxylase
LPS	Lipopolysaccharide
magA	mucoviscosity-associated gene A
$Mgcl_2$	Magnesium chloride
MR	Methy red
NaCl	Sodium chloride
NCCLs	National committee for clinical laboratory standards
Nm	Nanometer
O-Ag	Somatic antigen
ODC	Ornithin decarboxylase
OMP	Outer membrane proteins
orf10	Open reading frame
PCR	Polymerase chain reaction
pH	Power of hydrogen (H^+)
PLA	Pyogenic liver abscess
pmol	Picomole
Primer F	Primer forward
Primer R	Primer reverse
rmpA	Regulator of phenotype gene A
rRNA	Ribosomal Ribonucleic Acid
TBE	Tris-Borate-EDTA
TDA	Tryptophan daaminase
TSI	Triple Sugar Iron
URE	Urease
UTI	Urinary tract infection
UV	Ultra Violet
VP1-VP2	Vogas-Proskauer

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1-1 Introduction

Klebsiella pneumoniae is widely distributed in the gastrointestinal, urinary, and respiratory tracts of healthy people. It cause opportunistic infections mainly nosocomial infections, it is a common hospital-acquired pathogen causing sever respiratory infections such as pneumonia. Other infections caused by this organism include urinary tract infection, wound infection, abscesses, sepsis, inflammation and diarrhea, most *K. pneumoniae* are hospital associated with a high fatality rate if incorrectly treated. Treatment of *Klebsiella* infections is complicated (Chiu *et al*., 2013).

K. pneumoniae have different virulence factors which give the bacteria the ability to invade the host, such as capsular polysaccharide, lipopolysaccharide, serum resistance, siderophore production, fimbriae and other factors such as the production of urea and enterotoxin (Aher *et al*., 2012). However, antibiotic resistance properties are the major factor in its pathogenicity that it resists for wide spectrum of antibiotics and specially β-lactam antibiotics. This is due to the prevalence of infections acquired in hospital which led to the orientation of the research on alternative therapies (Dubey *et al.*, 2013).

K. pneumoniae isolates develop prominent capsular structures composed of complex acidic polysaccharides. Important differences in virulence between the serotypes. Serotype K1 and to a lesser extent K2, are associated with highly invasive disease, it considered among the most pathogenic (Fang *et al.*, 2012). The capsular polysaccharide synthesis (*cps*) gene cluster consists of a serotype specific region with genes that are relatively conserved between serotypes on either side (Doud *et al.*, 2009).

PCR was performed to amplify16S rRNA (Internal transcribed spacer unit) found in all *K. pneumoniae*, *magA* (mucoviscosity associated gene A) to identify the *cps* of *K. pneumoniae* capsule K1 serotype, *k2A* (K2 capsule

associated gene A) to identify the *cps* of *K. pneumoniae* capsule K2 serotype, study the prevalence of *rmpA* (regulator of mucoid phenotype A) and prevalence of *kfu* (an iron uptake system) genes in *K. pneumoniae* by using specific primers. All the target genes are chromosomal except *rmpA* is located on plasmid (Turton *et al.,* 2008).

Due to the common occurrence of *Klebsiella* spp. and high virulance that cause in the absence of accurate and early detection them, severe damage may lead to the death of the patient. Therefore this study was aimed to:

1. Isolation and identification of *K. pneumonia*e from different infections.
2. Isolation of genomic DNA for these isolates.
3. Amplification of specific, virulence factors and capsule biosynthesis genes for *K. pneumoniae* using PCR technique.

1-2 Literature Review:

1-2-1 *Klebsiella* spp.:

It was first discovered by the German microbiologist Edwin Klebs in 1834 and named after his name. Historically, *Klebsiella* called Fried Landers Bacilli by Fried Landers (Brisse *et al.*, 2006).

The genus *Klebsiella* is non-motile (except *Klebsiella mobilis*), Non-sporulating, some of the strains produce Bacteriocin called (klebcin) (Chiu *et al.*, 2013), lactose-fermenting, oxidase negative, urease positive (except *K. terrigena*) (Dubey *et al.*, 2013), catalase positive, not produce H_2S in TSI agar or liquefy gelatin and (G + C) ratio is about 53-59 % (Collee *et al.*, 1996). Temperature range for growth is 12-43 Cº, optimum 37 Cº and Gram-negative with a prominent polysaccharide capsule, this capsule encases the entire cell surface contain a large capsule that is rich in glucouronic acid and pyruvic acid (Talaro, 2002), considerable thickness which gives the colonies their glistening and mucoid appearance on agar plates. Non-Haemolytic on Blood agar (gamma-haemolysis). *Klebsiella* is rod shape 0.3-1 µm in diameter and 0.6-6 µm in length arranged singly, in pairs or in short chains (Wang *et al.*, 2012). *Klebsiella* is facultative anaerobic bacteria and the colonies appear large, mucoid (the degree of mucoidness depends on the amount of carbohydrate in the culture medium as well as varying from strain to strain) (Yu *et al.*, 2007), and pink pigment on MacConkey agar indicating fermentation of lactose and acid production (Todar, 2007).

Klebsiella found in two common habitats: the first habitat is the environment, where they are found in surface water, sewage, soil and on plants and the other habitats being the mucosal surface of mammals such as human, horses or swine, which they colonize. In this respect, the genus *Klebsiella* is like *Enterobacter* and *Citrobacter* but unlike *Escherichia coli* and *Shigella* which are common in human but not in the environment (Goldman and Lorrence, 2009).

Bacteria of the genus *Klebsiella* are opportunistic pathogens that can lead to severe diseases such as septicemia, pneumonia, urinary tract infections, soft tissue infections and infect people who suffer from immunodeficiency system and patients admitted to the hospital (Jazani *et al.*, 2009).

1-2-2 Taxonomy of genus *Klebsiella*:

The genus was originally divided into three main species based on biochemical reactions, they classified to six species depending on DNA homology. These are:

1- *K. pneumoniae* which contain three subspecies (homologous DNAs but different biochemical reactions): *ozaenae*, *rhinoscleromatis* and *pneumoniae*, 2- *K. oxytoca*, 3- *K. planticola*, 4- *K. terrigena*, 5- *K. ornithinolytica*, 6- *K. mobilis* (Holt *et al*., 1994).

Originally, the medical importance of genus *Klebsiella* led to its being subdivided into three species corresponding to the diseases they caused: *K. pneumoniae* (cause pneumoniae), *K. ozaenae* (cause nasal necrosis) and *K. rhinoscleromatis* (cause nasal hardening) (Jawetz *et al*., 2010).

Modern classification systems have been adopted on the basis of similarity in DNA (Tortora *et al*., 2004), classified the *Klebsiella* genus to twelve species: *K. granulomatis*, *K. mobilis*, *K. singaporensis*, *K. oxytoca*, *K. trevisanii*, *K. terrigena*, *K. ozaenae*, *K. ornithinolytica*, *K. planticola*, *K. variicola*, *K. rhinoscleromatis* and *K. pneumoniae.*

1-2-3 *Klebsiella pneumoniae*:

1-2-3-1 General characteristics:

K. pneumoniae is an opportunistic, Gram-negative, rod-shaped bacterium. It is most commonly encountered by physicians world wide as a community-acquired and a hospital-acquired pathogen. Community-acquired *K. pneumoniae* is also called Friedländer's *pneumoniae* named after Carl Friedländer who identified *K. pneumoniae* as the cause of fatal cases of pneumonia (Lau, 2007). *K. pneumoniae* is lactose-fermenting, non-motile, so it

could be distinguished from other motile species of *Enterobacteriaceae* and from non-motile gram negative rods through citrate utilization, oxidase negative, grow on MacConkey agar in large mucoid pink, entirely smooth and round colonies (Benson, 2001). *K. pneumoniae* is urease production, so it could also distinguished from other *Klebsiella* spp.

1-2-3-2 Epidemiology:

Klebsiella is an opportunistic pathogen and is one of enteric bacteria members of the normal intestinal flora. The enteric bacteria generally do not cause disease and in the intestine they may even contribute to normal function and nutrition. The bacteria become pathogenic only when it reach tissues outside of their normal intestinal or other less common normal flora sites (Brooks *et al*., 2007). *K. pneumoniae* is present in the respiratory tract and feces of about 5 % of normal individuals and it is responsible for a small proportion (about 1 %) of bacterial pneumonias. *K. pneumoniae* is the most medically important species of the Family *Enterobacteriaceae* after *E.coli* (Irving *et al*., 2006).

K. pneumoniae is an important hospital-acquired pathogen that is a frequent cause of septicemia, wound and blood infection (Brisse *et al.,* 2009; Turton *et al.,* 2010), urinary tract infection, intra-abdominal infections and pneumonia in immunocompromised individuals, it is also an important pathogen with respect to community-acquired infectious diseases, such as community-acquired pneumonia (Ma *et al*., 2005).

K. pneumoniae strains harbouring extended spectrum beta-lactamases (ESBL) and metallocarbapenemase, conferring resistance to many of antibiotics available that making treatment options limited, have been described in many parts of the world (Keynan and Rubinstein, 2007).

These resistant pathogens considered clinically important because there are cause nosocomial infections that commonly appear in outbreaks and is associated with treatment failure, prolonged hospital stay, increased health expenditure as well as a possible increase in mortality (Leavitt *et al.,* 2009).

The acquired invasive syndrome caused by this pathogen was described in 1981, being characterized by bacteremia, hepatic abscess and metastatic infections, among which endophthalmitis and central nervous system infections (manifested as suppurative meningitis or cerebral abscess) were the most frequently found, although other abscesses in the lungs, pleura, prostate and soft tissues, like osteomyelitis and necrotizing fasciitis (Dora *et al.*, 2013). In Taiwan, *K. pneumoniae*–associated primary pyogenic liver abscess (PLA) has become an important emerging infectious disease. This disease is also a global concern, as is attested by reports from North America (Podschun and Ullmann, 1998), Europe (Yeh *et al.*, 2007) and Asia (Dubey *et al.*, 2013).

1-2-3-3 Virulence factors:

Klebsiella virulence factors differ depending on the sites of infection because the host defense mechanisms differ from site to other (Highsmith and Jarvis, 1985). For example it should be expected that the pattern of virulence factors found in UTI-causing strains of *K. pneumoniae* will differ from that observed in strains isolated from pulmonary sources of patients with pneumonia (Turton *et al.*, 2010).

K. pneumoniae strains possess a variety of virulence factors that facilitate infection and survival in the host. Podschun and Ullmann (1998) documented that the virulence factors of *K. pneumoniae* consist of seven major bacterial factors: capsule (for inhibition of phagocytosis), lipopolysaccharide (for avoidance of host serum complement factors), fimbriae (for adhesion) (Pinsky *et al.*, 2009), siderophores (for iron acquisition), bacteriocin (Yu *et al.*, 2008), serum resistance (Blackburn, 2010) and extended spectrum β-lactamases (ESBLs) (for protection from extended-spectrum cephalosporins) (Lau, 2007) (Figure 1-1).

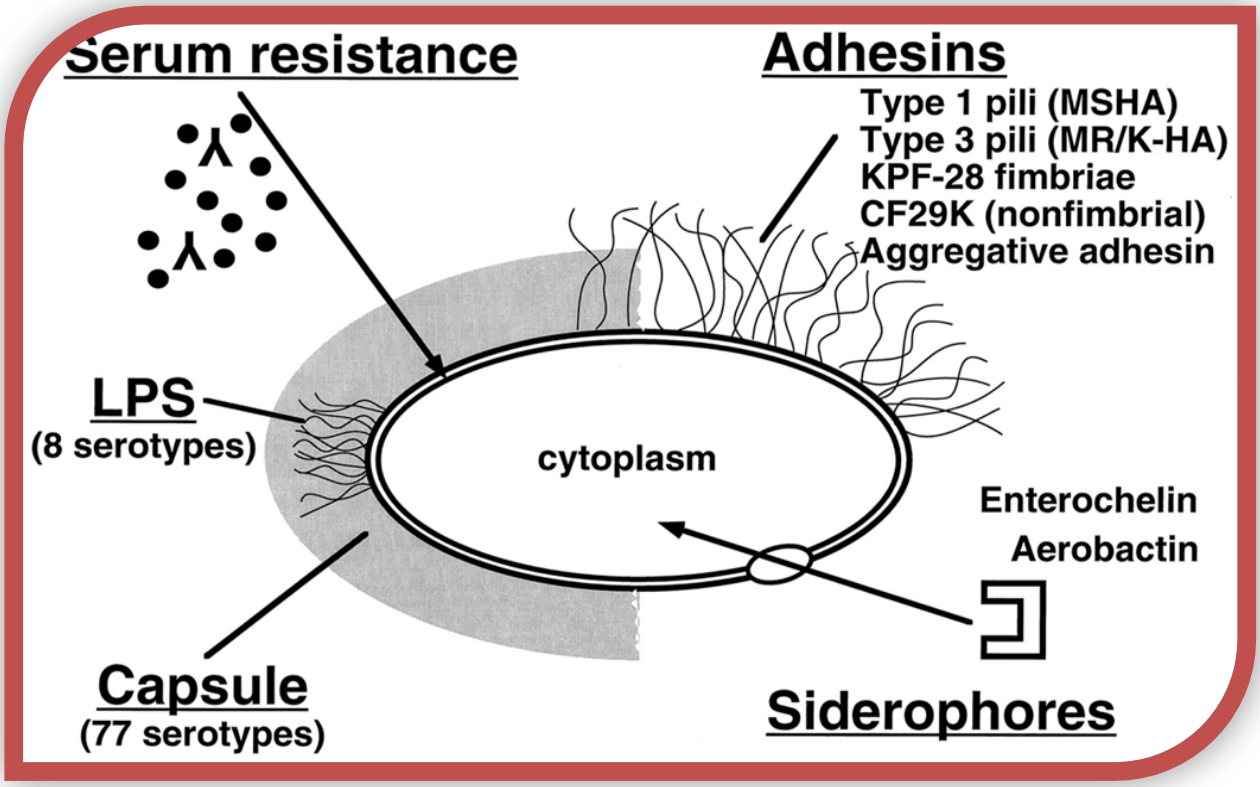
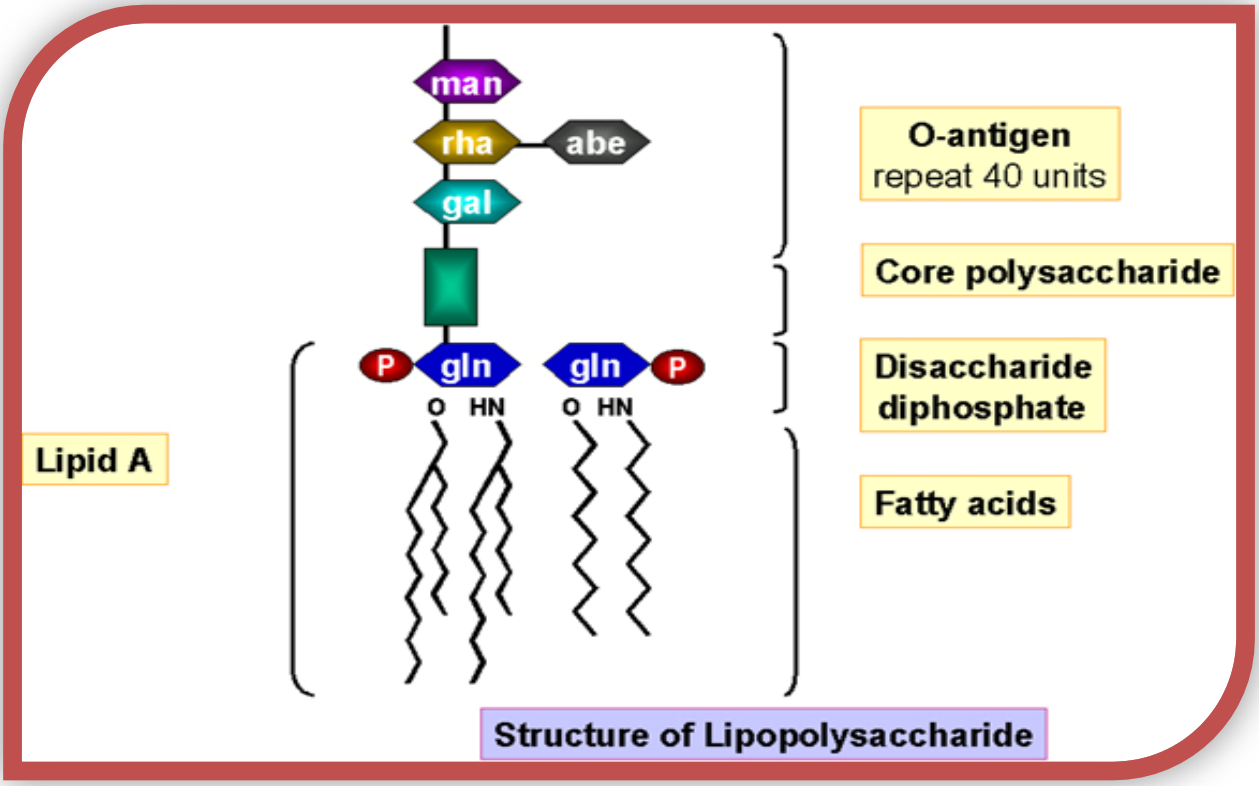


Figure (1-1): Schematic representation of *Klebsiella* pathogenicity factors (Podschun and Ullmann, 1998)

1-2-3-3-1 Lipopolysaccharide and serum resistance:

The lipopolysaccharide (LPS) molecule is one of the outer membrane component of the cell composed of three distinct sections: lipid A (phosphate amine glucose + fatty acids), a core polysaccharide and a side chain called the O-antigen (O-Ag) polysaccharide (Oligosaccharide) (Younes, 2010) as shown in figure (1-2).



man=mannose; rha=rhamnose; abe=arabinose; gal=galactose; gln=glucose; p=phosphate

Figure (1-2): Structure of lipopolysaccharide of *Klebsiella* (Fox, 2011)

Lipid A anchors the LPS molecule into the outer membrane and is also an endotoxin, stimulating the immune system (Alexander and Rietschel, 2001). The core polysaccharide links the O-Ag onto the lipid A molecule and usually is negatively charged due to phosphate substitutions (Ja'far, 2011).

Eight O-antigen types are distinguished in *K. pneumoniae*, O1 being the most frequent. The most important role of the O-antigen is to protect *K. pneumoniae* from complement mediated killing and phagocytosis (Brisse *et al.*, 2006).

The “O” side chains of the LPS may reach through the capsule layer and be exposed to the external environment in certain *Klebsiella* capsule types (Awad, 2012).

The serum bactericidal activity is mediated primarily by complement proteins. After their cascade-like activation, these proteins accumulate as membrane attack complex on the surface of the microorganisms (Blackburn, 2010). This complex consists of the terminal complement proteins (C5 and C9), which produce a transmembranous pore in the plasmic membrane of gram negative bacteria leading to an influx of Na+ and subsequent osmotic lysis of the bacteria (Cheng *et al*., 2010). Lipopolysaccharide contributes to *K. pneumoniae* resistance to serum killing by the host. Merino *et al.* (2000) showed that the O5-antigen LPS is also essential for serum resistance and is an important factor for the adhesion to uroepithelial cells, possibly because of its surface charge and hydrophobicity properties.

Since "LPS" is generally able to activate complement cascade, C3b is subsequently deposited onto the LPS molecules (Clegg and Sebghati, 2001). However, it is fixed preferentially to the longest O-polysaccharide side chains, C3b is far from the bacterial cell membrane. Thus, the formation of the lytic membrane attack complex (C5b–C9) is prevented and subsequent membrane damage and cell death do not take place (Alberti *et al*., 1996).

The "O1" lipopolysaccharide has been linked with the extensive tissue necrosis that complicates *Klebsiella* infections. The production of an extracellular toxic complex (ETC) that has been shown to be responsible in mice for lethality and extensive lung necrosis is composed of 63 % capsular polysaccharide, 30 % lipopolysaccharide and 7 % protein (Sahly *et al*., 2004).

Antibodies produced against the lipopolysaccharide portion (possessing the toxicity) of the ETC have been shown to be protective (Noah *et al*., 2001).

1-2-3-3-2 Siderophores production:

Iron is an essential element for bacterial growth (Griffiths, 1987) and is uptake from the host environment via the bacterial secretion of high affinity, low molecular weight iron chelators called siderophores (Awad, 2012). Iron functioning mainly as a redox catalyst in proteins participating in oxygen and electron transport processes (Ja'far, 2011). Siderophore supply of free

iron available to bacteria in the host environment. It is withdrawal of iron from compounds since this element (Iron) is bound intracellularly to proteins (such as hemoglobin, ferritin, hemosiderin and myoglobin) and extracellularly to high affinity iron-binding proteins such as lactoferrin (in the Milk) and transferrin (in the serum) (Brisse *et al*., 2006). The level of free, bioavailable iron (10^{-18} M) is several thousand fold too low for normal bacterial growth (Al-Shukr, 2005).

Enterobacteriaceae siderophores belong to three major groups: enterobactin (phenolates) (also called enterochelin), aerobactin (hydroxamates) and yersiniabactin (Saharan and Nehra, 2011). Jasim (2012). showed that *K. pneumoniae* yersiniabactin production is increased during pulmonary infection, while during *in vitro* iron-limiting growth conditions, enterobactin is produced at higher levels (All strains were found to produce enterochelin), only a few could produce aerobactin when compared to yersiniabactin. The discrepancies in these results can be attributed to the complexity of these siderophores systems *in vivo* and also the lack of definitive experiments addressing the importance of these iron acquisition systems (Lawlor *et al.*, 2007).

Aerobactin differ from enterobactin in the following points:

1-Enterobactin is much higher affinity for Fe^{+3} than that of ferric aerobactin.

2-Aerobactin is more effective than enterobactin because the greater stability and better solubility.

3-After delivery of iron, aerobactin can be recycled after each turn of iron transport, while enterobactin becomes hydrolyzed by an esterase (Raymond *et al*., 2003).

4-Enterobactin synthesis are expressed by chromosomal gene, which found in most of the enteric bacteria. Most of aerobactin are synthesized by genes carried on plasmid ferric uptake regulator gene (*Fur*) (180 Kilodalton) which was isolated from *Klebsiella* (Grimont and Grimont, 2005), while in (yersiniabactin) *Yersienia* aerobactin genes were carried on chromosome (Markracka *et al*., 2004), it is produced by *Klebsiella* spp. and encoded by the *Yersinia* high-

pathogenicity island, but without known role in prevalence and pathogenesis (Brisse *et al.*, 2006).

1-2-3-3-3 Fimbriae (pili):

Fimbriae are Non-flagellar, filamentous, hair-like long, flexible structures that extend outward from the bacterial surface (Awad, 2012). These structures are up to 10 mm long and have a diameter of 1-11 nm, they consist of homopolymeric structures of fimbriae composed of approximately 1,000 copies of a single structural subunit globular protein called fimbrin or pilin packed in a helical array appendages with a molecular mass of 15-26 KD (Weberhofer, 2008).

The first step in the infectious process is the adherence in which microorganisms must come as close as possible to host mucosal and epithelial cell surfaces and maintain this proximity by attaching to the host cell (Lai *et al*., 2000). The bacterial adhesion must be sufficiently strong to resist drug forces arising from aqueous flushes in the urethra or gaseous flushes in the respiratory tract. Fimbriae exhibiting flexible and stretchable properties similar to that of springs may aid bacteria in remaining attached to host cells by reducing the impact of the flushes (Chen and Wen, 2011).

The adhesive properties in the *Enterobacteriaceae* are generally mediated by different types of fimbriae, there are two predominant types of Pili in *K. pneumoniae* commonly express known as: type 1 (common Pili) and type 3 (Dworkin, 2006). *K. pneumoniae* have two types of adhesion: Non-fimbrial adhesion (there are lipopolysaccharide and capsular antigen) and fimbrial adhesion (consist of Pili type 1 and Pili type 3) (Huang *et al*., 2009).

1-2-3-3-4 Capsular polysaccharide (CPS) (K-antigen):

Capsule polysaccharide (CPS) is recognized as one of the most important virulence factors of *K. pneumoniae,* thickness 160 nm. The capsular is complex acidic polysaccharide (Yasmin, 2012) composed of repeating subunits of 4-6 sugars (thick hydrophilic polysaccharide such as glucose, galactose, mannose, fructose and rhamnose) and very often, acids such as:

uronic acid, glucouronic acid, galacturonic acid, pyruvic acid (as negatively charged components) (Podschun and Ullmann, 1998).

The *K. pneumoniae* K2 capsular polysaccharide has been reported to contain glucose, mannose and N-acetyl-glucuronic acid (Arakawa *et al.*, 1991). Capsule responsible for the glistening, mucoid aspect of colonies on agar plates (Brisse *et al*., 2006).

The capsular material forms thick bundles of fibrillous structures surrounding the bacterial surface in massive layers to protect the bacterium by inhibits phagocytosis by macrophage and neutrophils (Branger *et al*., 2004), and inhibits binding of serum anti-microbial factors such as complement to the bacterial membrane (complement inactivation) (Cortes *et al.*, 2002).

CPS have been classified into 77 serological types, termed K-antigens. The presence of the capsule is critical for the virulence of *K. pneumoniae* (Yoshida *et al*., 2000). *Klebsiella* strains of serotypes K1 to K6 are more associated with severe respiratory infection and septicemia in human than the highered numbered serotypes (Turton *et al.*, 2008). Podschun *et al*. (1992) reported that the degree of virulence conferred by a particular K-antigen might be connected to the mannose content of the CPS, *Klebsiella* serotypes containing mannose repeating sequences killed by macrophages with mannose receptor, such as K7 and K21 which is low virulence because both of them contain these repeating sequences. In contrast, Ofek *et al*. (1993) reported that strains that lack these repeating sequences are not recognized by macrophages and hence phagocytosis dose not take place. This model is consistent with the marked virulence of K2 which completely lacks mannose repeating sequences, while capsular types with low virulence, such as the K7 or K21 antigen, contain repetitive sequences of mannose (Al-Shukr, 2005). Thus, *Klebsiella* strains bearing capsule types devoid of these sequences should be closely associated with infectious disease (Kabha *et al*., 1995).

Studies of the distribution of K-serotypes among *Klebsiella* clinical isolates showed very distinct prevalence patterns of K-types across distinct

geographic regions (Toivanen *et al*., 1999; Fang *et al*., 2004). For example, serotype K1 is frequently isolated in studies from Taiwan, China and Japan but is absent from most seroepidemiology studies in Europe and the United States. K2 and K21 appear to be the only frequently found serotypes in most studies (Brisse *et al*., 2009). Lin *et al*. (2011) pointed that the capsule be linked with sticky polysaccharide network (Mycopolysaccharid web) formation of the so-called (hyper mucoviscostiy phenotype), viscosity is a trait associated with the pathogenesis of *K. pneumoniae*.

The capsular polysaccharide considered virulence factor of *Klebsiella*. It dependent on capsular antigen in the diagnosis of *K. pneumoniae* despite the presence of somatic antigen (O-antigen) because of the small number of serotypes O-antigen (Fang *et al*., 2012). Although there are eight serotypes as well as the difficulty of diagnosis because of disability of O-antigen resulting from the presence of K-antigen thermally fixed, which covers O-antigen. There are 77 capsular types of K-antigen were diagnosed different in the chemical and antigenic type (Podschun and Ulmann, 1998) .

Finally, the isolates often carry heavy CPS that could protect the bacteria from phagocytosis and killing by serum factors (Lee *et al.*, 2006). A part from the antiphagocytic function, *Klebsiella* CPS also helps the bacterial colonization and biofilm formation at the infection sites (Chiu *et al*., 2013).

1-2-3-3-5 Other virulence factors:

There are many other factors contributing in pathogenicity of *Klebsiella* spp. such as: haemolysin, cytotoxins (Endotoxins) and Bacteriocin (Weberhofer, 2008).

Little is known about the hemolysins produced by *Klebsiella* spp. that were considered as non-hemolytic for human red blood cells and found to be hemolytic in rabbit blood agar (Yu *et al*., 2006). Other potential virulence factors produced by *K. pneumoniae* possibly include the production of heat-labile and heat-stable endotoxins (Brisse *et al.*, 2006).

According to Jacob *et al*. (1953) termed bacteriocins to the protein toxins that enter to the bacteria cell by receptors on its surface (Sarika *et al*., 2010). Explain that bacteriocin produced by certain species of bacteria which inhibit or kill the bacteria growth of similar or closely related bacterial strain, that by inducing metabolic block, restriction of DNA, inhibition of protein synthesis by effecting in 16S rRNA, inhibition of peptidoglygan synthesis in cell wall and channel formation that permeability to ions in the cell membrane (Luders *et al*., 2003; Gordon and O'Brien, 2006).

Bacteriocin did not cosider virulence factors directly but it increase the possibility of competition among producing strains (Kayaoglu and Qrsavik, 2004). Riley and Chavan (2007) pointed that bacteriocin produced from *K. pneumoniae* called Klebocin, there are four groups (A, B, C and D).

Bacteriocin production encode by three plasmid genes:

1- Bacteriocin production gene.

2- Immunity gene (to prevent effecting of cell by Bacterioin produced from it).

3- Lysis gene (to release bacteriocin from producing cell).

(Gillor *et al*., 2008).

1-2-4 Outer membrane proteins (OMPs):

The clearance of the *K. pneumoniae* from the infection requires effective host defense mechanisms, to which the bacterial surface takes part. Three components of the outer wall of Gram-negative bacteria shown in figure (1-3) are suspected to be involved in development of immunity there are: LPS, membrane proteoglycans (Al-Sehlawi, 2012), (consists of a "core protein" with one or more covalently attached glycosaminoglycan (GAG) chains) and outer membrane proteins (OMP) (Nucera *et al*., 2006). Outer membrane have medical importance as a selectively permeability barrier protects gram negative bacteria from chemicals that affecting on peptidoglycan layer such as antibiotic and lysozyme (Brooks *et al*., 2004). OMP-A is one of the major proteins of the outer membrane of Gram-negative bacteria. This protein is highly conserved among the *Enterobacteriaceae* and is thought to consist of two domains.

Whereas the effects of LPS and membrane proteoglycans on immune cells have been largely described, only few studies evaluated OMP properties, mainly focused on its immunomodulatory function such as: activates macrophages (Kayaoglu and Qrstavik, 2004).

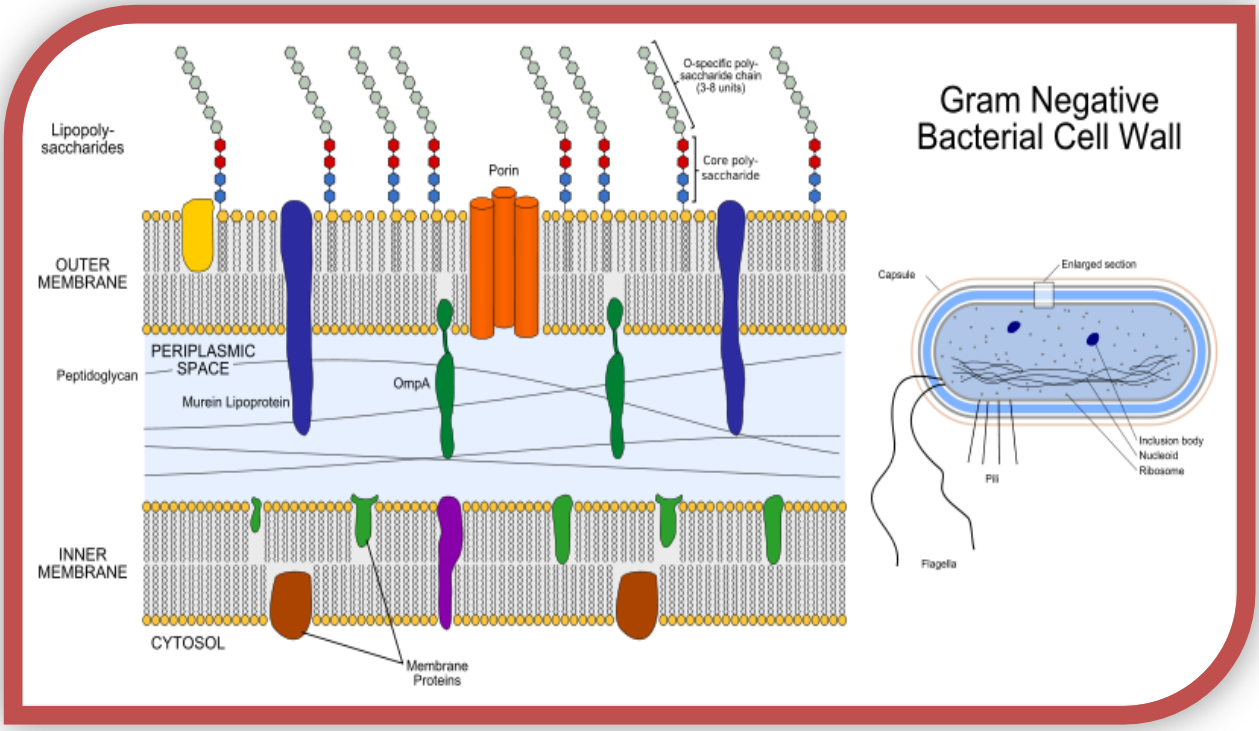


Figure (1-3): Diagram of a gram-negative cell wall
(Brisse *et al.*, 2006)

1-2-5 Typing of *K. pneumoniae*:

Klebsiella spp. is genetically heterogeneous and strains within species and subspecies can be discriminated by a number of methods including: biochemical tests, analysis of antigenic specificities, bacteriophage susceptibility typing, bacteriocin susceptibility or production typing and molecular typing methods (Anbazhagan *et al.*, 2010).

1-2-5-1 Phenotypic typing:

Phenotypic typing is containing four types: biotyping, bacteriocin typing, phage typing and phenotype–HMV (Podschun and Ullmann, 1998). The capsule

binding with sticky polysaccharide network known phenotypic pattern of higher viscosity (Hyper mucoviscostiy phenotype), this viscosity associated with virulence of *K. pneumoniae*, because of K1 and K2 serotyping having this trait made it highly pathogenic (Lin *et al*., 2011).

1-2-5-2 Molecular typing:

Molecular typing methods include two types: Protein based and Nucleic acid based methods, then serotyping discovered by Rebecca Lancefield in 1933 (Hall *et al.*, 2009). Molecular typing methods aimed to discriminate strains based on the differences in their genomic DNA sequence and organization (Van Belkum *et al.*, 2001). Over the last years many molecular typing methods have been applied to investigate outbreaks due to *Klebsiella* strains and to compare strains from different time points and geographic origins (Bortz *et al*., 2008).

The most commonly techniques are the counter-current immunoelectrophoresis (CIE) and the capsular quelling (swelling) reaction used for identification of *K. pneumoniae* serotypes K1 and K2. The costs and availability of the antisera, which can be produced in specialized laboratories, limit the practice of serotyping. Therefore, novel molecular-serotyping tool was developed (Brisse *et al*., 2004). This method is capable to identify all 77 K-types of *K. pneumoniae* using polymerase chain reaction based assays for differentiation of the different strains (Doumith *et al*., 2004).

Serotyping of *Klebsiella* strains based mainly on a division according to the O-antigen (Lipopolysaccharide) and K-antigens (Capsular polysaccharide). O-serotyping classify to 8 different O-antigen types has long been technically difficult to perform because the presence of heat-stable K-antigens classify to 77 types makes determination of O-antigens difficult (Ørskov and Ørskov, 1984) Capsule typing, by contrast, shows good reproducibility and is capable of differentiating most clinical isolates (Chavan *et al*., 2005), So K-serotyping currently is the most widely used technique for typing *Klebsiellae* spp. *Klebsiella* usually have well-developed polysaccharide capsules, which give their colonies their characteristic mucoid appearance (Al-Shukr, 2005).

There are 82 capsule antigens described, 77 different capsular serotypes form the basis for an internationally recognized capsule scheme (Zacharczuk *et al.*, 2011).

The drawback of this method is the large number of serological cross-reactions that occur among the 77 capsule types, Moreover, the typing procedure is cumbersome because of the time needed to perform the test and weak reactions that are not always easy to explain (Turton *et al.*, 2008). In contrast to capsule typing, neither phage typing nor bacteriocin typing alone is sufficiently discriminative and reproducible for epidemiological purposes except under certain conditions (Ørskove and Ørskove, 1984).

The O-serotypes (O1and O2) are the most commonly in virulance strains, while in K-serotype from K1 to K6 are the most dangerous type clinicaly, specifically high virulence on serotypes K1 and K2 because they contain patterns of virulence genes (Yeh *et al*., 2009).

There are releationship between serotype K and O when found O1 that linked with K1 and K2, while K3 releation with O_2. The serotype K1 is the highest virulence of serotype K2 in lesions of the nervous system (Fang *et al.*, 2009), while urinary tract infection, it is responsible from serotypes K8, K9, K10 and K24 (Don *et al*., 2005), a study suggests that serotype K57 has high virulence causing invasive infection and there is other serotypes cause low antigenic response that do not cause infection without very high doses, such as serotype K21 and K7 (Pan *et al*., 2008)

There are several genes location in *cps* gene clusters part of them responsible for K1 and K2 capsule biosynthesis such as K1 genes *magA* (mucoviscosity-associated gene A), *wzc* (encoding tyrosine-protein kinase for K1) and for K2, *k2A* (K2 capsule-associated gene A), *orf10* (open reading frame) encoding putative inner membrane protein for K2 (Zacharczuk *et al*., 2011).

- **Using polymerase chain reaction (PCR) for diagnosis of *K. pneumoniae*:**

PCR is closely patterned after the natural principle of DNA replication, an objective and well-accepted identification method of bacteria and other pathogens (Nucera *et al*., 2006). This testing method is able to amplify a special fragment million times in order to provide objective evidence (Todar, 2007). Beside this, PCR can amplify and detect even smallest amounts of a pathogen, which also improves detection, if the quantity of the tested material is low (Struve *et al.*, 2009).

It is a three-step process, referred to as a cycle, that is repeated a specified number of times. One PCR cycle consists of the following steps: denaturation, annealing and extension (elongation) (Kolmodin and Birch, 2002).

K. pneumoniae is the most clinically important of the *Klebsiella* spp., the ability to identify it rapidly and helpful (Jasim, 2012). The isolates were examined for the presence of various virulence associated genes by PCR technique as well as their biochemical characteristics (Aher *et al*., 2012).

- **Regulatory genes for capsule synthesis of *K. pneumoniae*:**

There are two types of regulatory genes:

A-Chromosomal regulatory genes (*rcsA* and *rcsB*).

B- plasmid regulatory genes (*rmpA* and *rmpB*).

Arakawa *et al.* (1995) is the first who diagnosed chromosomal gene that responsible for the synthesis of capsular polysaccharide serotyping K2, this gene regulated by two promotors for gene transcription. In 2009, Shu *et al*. compared gene sequence for some *K. pneumoniae* isolates, they found that K1, K2, K52 and K57 serotype contain *wacJ* and *wbaP* genes, while *rmlBAC* gene found in K14, K9 and K52, in the other hand K1, K2, K5, K14 and K62 contain *manB and manC*. They suggested that there is a difference in the gene responsible for CPS. All isolate have genes for cps synthesis contain *gnd* gene encoded for gluconate-6-phosphate dehydrogenase, located at 3-end of combine cps genes synthesis. Lin *et al*. (2009) refered to the importance of genes *wza*, *wzc* and overlap between cps synthesis regulation,

phosphorylation enzymes and proteins (protein tyrosine kinase) as select three pr oteins phosphorlated tyrosine for *K. pneumoniae* K1, there are: protein-tyrosine kinase (*wzc*), undecaprenyl-phosphate phosphomannomutase (*manB*) and glycosyltransferase (*wcaJ*). The *wcaJ* gene have importance role in the cps synthesis regulator by transfer the signal responsible for cps synthesis (Lin *et al*., 2009).

The *pgi* gene encode Glucose-6-phosphate isomerase and convert Glucose-6-phosphate isomerase to Fructose-6-phosphate located on gene cluster for cps synthesis, if mutation happened led to problem in gene cluster that result isolate less or without virulence, clearly the genes responsible for hypermucoviscosity production associated with capsule synthesis for decrease defect (Li *et al*., 2012).

Genes encoding membrane proteins located on the gene cluster of cps synthesis in both *E. coli* and *K. pneumoniae* (Dong *et al*., 2006) are: *wzx, wzy, wbaP, wacJ, wzi, wza* and *wzc* (Collins *et al*., 2006). The *wzx and wzy* sequence are more variable, the similarity is very low in serotyping of wzy-C in *magA (K1) and wzy* (K2, K52, K15, K17) (Kong *et al*., 2005).

The other gene associated with hypermucoviscosity carried by virulence strain is (*magA*) mucoviscosity-associated gene A responsible for cps synthesis K1, if mutation happened in *magA* lead to loss capsule (Fang *et al*., 2010), therefore can use this gene for rapid diagnosis correct tracking for source of invasive dangerous disease, the mutation of the *magA* (also called *wzy-k1*) not effect on Lipopolysaccharide unlike *wzy-c* relation with O-Ag synthesis (despite amino acid consist of two genes are similar) (Fang *et al*., 2009).

Transcriptional regulation for cps synthesis done by several genes: *rmpA, rcsAB, rfaH* (Lai *et al*., 2003). Pneumoniae has genes carried on pLVPK big plasmid (180 kb) called *rmpA* (regulator of mucoid phenotype gene A) responsible for hypermucoviscosity and *rmpA2* located beside it (Rahn and Whitfield, 2003). Deletion of *rmpA* result in reduction of cps synthesis that leads to decrease of bacterial virulence, in the other hand the strain contain

mutation in this gene have defect on regular cps biosynthesis this give the importance for hypermucoviscosity (Chuang *et al*., 2006).

Liu *et al*. (2008) refered to the importance of *fur* gene (ferric uptake regulator) because of it is role in regulation of cps synthesis genes (*rcsA*, *rmpA*, *rmpA2*) by iron acquisition, [Fe(II)] this gene effect on regulator cps synthesis genes, since these genes affected with presence of iron.

- **Genes responsible for capsule synthesis of *K. pneumoniae*:**

Despite the discovery of other virulence factors such as fimbriae, siderophores and O-antigen, capsular antigen is considered to be ultimate determinants of *K. pneumoniae* pathogenicity (Zacharczuk *et al*., 2011). Capsular polysaccharide (*cps)* genes consist of cluster of the genes that are responsible for the synthesis of different serotypes of CPS (Kawai, 2006). A number of genes have been identified as markers of virulence, including *magA*, *k2A*, *rmpA*, *kfu* (Doud *et al*., 2009).

***-magA* gene: (**mucoviscosity associated gene A)

The *magA* gene is a chromosomal gene (Yeh *et al*., 2009), it was first described in 2004 by Fang *et al*.. The hypermucoviscosity and *magA* were more dominance in invasive strains of *K. pneumoniae* and that *magA*-negative strains lost their exo-polysaccharide layer and became susceptible to phagocytosis. It is known that *magA* is restricted to the gene cluster of *K. pneumoniae* capsule serotype K1 (Lin *et al*., 2012). Therefore, the variation in the prevalence of *magA* among studies may reflect geographic differences in the prevalence of the K1 serotype. Thus, PCR analysis for *magA* is a rapid and accurate method to detect capsule K1 strains (Yeh *et al*., 2006).

***-k2A* gene:** (K2 capsule associated gene A)

The *k2A* gene of *K. pneumoniae* could be used as a highly specific diagnostic method to identify the *cps* of *K. pneumoniae* capsule K2 serotype, which corresponds to the *magA* region in the *cps* gene clusters of K1 strain (Chuang *et al*., 2006).

-*rmp A* gene: (regulator of mucoid phenotype A) gene

The *rmpA* gene is a plasmid-mediated confer a highly mucoviscous phenotype enhanced and regulator of the capsular polysaccharide synthesis (Rivero *et al*., 2010). It was first described by Nassif *et al.* (1989a). Despite, the relationship between *rmpA* and *K. pneumoniae* clinical syndromes, *rmpA* remained or it unknown for more than a decade. Yu *et al.* (2006) demonstrated that *rmpA*-carrying strains were associated with the hypermucoviscosity phenotype, as well as with the invasive clinical syndrome. Nassif *et al.* (1989b) explained that remove of the *rmpA* gene can decrease virulence in mouse lethality tests by 1000-fold.

- **Other virulence factor genes:**

-*kfu* gene:

The *kfu* chromosomal virulence gene responsible for an iron uptake system (codes for an iron uptake system), is a putative pathogenic gene (Li *et al*., 2012), significantly associated with the virulent hypermucoviscosity phenotype and purulent tissue infections. In addition, *K. pneumoniae* produces several other extracellular virulence factors which affect the host cell metabolism (Aher *et al*., 2012).

- **Other genes used for identification of *K. pneumoniae:***

-16S rRNA gene:

Identification of *K. pneumoniae* subsp. *pneumoniae* based on the 16S-23S internal transcribed spacer (Liu *et al.*, 2008). The rRNA sequences play a central role in the study of microbial evolution, particularly, the 16S rRNA genes have become the standard for the determination of the diversity in the specific populations (Hansen *et al*., 2004). The 16S rRNA genes characterise by following properties:

First, they are universally distributed, allowing the comparison of phylogenetic relationships among all extant organisms.

Second, the rRNAs are generally thought to be part of a core of informational genes, so their relationships provide information for the estimate of evolutionary changes in population.

Third, the rRNAs are functionally highly restricted of sequence stretches ranging from conserved to more variable (Park *et al*., 2011).

Many bacteria have heterogeneous rRNA operons. The three rRNAs, namely 16S, 23S and 5S rRNAs, are typically linked together into an operon, which frequently contains an internal transcribed spacer (Tsuzuki *et al*., 2008).

CHAPTER TWO

MATERIALS AND METHODS

2- Materials and Methods

2-1 Materials:

2-1-1 Equipments and apparatus:

The following equipments and apparatus were used during this study:

NO.	Equipments	Manufacturing company	Origin
1	Autoclave	Tomy	Japan
2	Compound light microscope	Olympus	Japan
3	Cooling centrifuge	Eppendorf	Germany
4	Digital camera	Panasonic	Japan
5	Distillator	GFL	Germany
6	Eppendorff cooling centrifuge	Eppendorf	Germany
7	Horizontal Electrophoresis unit	Apelex	France
8	Incubator	Sanyo	Japan
9	Laminar flow hood	Memmert	Germany
10	Micropipetters	Slamed	Germany
11	Millipore filters	Sartorius	Germany
12	Nanodrop system	Optizen	Korea
13	PCR system (Veriti)	Applied biosystems	Singapore
14	pH-meter	Hanna	Romania
15	Refrigerator	Ishtar	Iraq
16	Sensitive balance	Sartorius	Germany
17	Shaker Incubator	Amerex	U.S.A
18	Turbidity measurement system	Biomerieux	France
19	UV spectrophotometer	Shimadzu	Japan
20	UV transilluminator	Spectroline	U.S.A
21	VITEK2 system	Biomerieux	France
22	Vortex	Vortex-2-genie	U.S.A
23	Water bath	Kottermann	Germany

2-1-2 Chemicals and biological materials:

The following agents were used in the study:

NO.	Materials	Manufacturing company	Origin
1	API 20E test	BioMerieux	France
2	N,N,N,N-tetramethyl-phenylene diamine dihydrochloride (Oxidase reagent)	Sigma	U.S.A
3	Alpha-Nephthol	BDH	England
4	Barium chloride ($BaCl_2$)		
5	Bromophenol blue		
6	Bromothymol blue		
7	D-Mannitol		
8	Ethylene Diamine Tetra Acetic Acid (EDTA)		
9	Glycerol		
10	Hydrogen peroxide		
11	Mannitol		
12	Para-DimethylAmino Benzaldehyde (Kovac's reagent)		
13	Pepton		
14	Sodium chloride (NaCl)		
15	Tryptone		
16	Crystall violet	Fluka	Switzerland
17	Iodine		
18	Ethanol		

19	Methyl red		
20	Potassium Hydroxide (KOH)		
21	Sufranine		
22	Tris-base (Tris-OH)		
23	Methylene blue	CDH	India
24	Nigrosine		
25	Sulphuric acid (H_2SO_4)	GCC	England
26	Urea powder	SD-Fine	India
27	Agar-agar	Himedia	India
28	Kovac's reagent		
29	Yeast extract		
30	Agarose	Promega	U.S.A
31	Ethidium bromide powder		
32	Loading solution		
33	Tris-Boric acid-EDTA Buffer(TBE buffer)		

2-1-3 Antibiotic discs (Bioanalyse / Turkey):

No.	Antibiotics	Disc symbol	Concentration (Mg)
1	Amikacin	AK	30
2	Amoxicillin + Clavulanic acid	AMC	20/10
3	Ampicillin	AM	25
4	Cefotaxime	CTX	30
5	Ceftriaxone	CRO	30
6	Cephalothin	KF	30
7	Cephradine	CE	30
8	Ciprofloxacin	CIP	5
9	Doxycycline	DO	30
10	Gentamicin	CN	30
11	Imipenem	IPM	10
12	Piperacillin	PRL	100
13	Rifampin	RA	30
14	Sulfamethoxazole + Trimethoprime	SXT	1.25/23.75
15	Tetracyclin	TE	30

2-1-4 DNA markers:

No.	DNA Marker	Description	Company	Origin
1	100 bp Ladder	100-1500 base pairs (bp). The ladder consists of 11 double strand DNA fragment ladder with size of (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500 bp). The 500bp present at triple the intensity of other fragments and serve as a reference. All other fragments appear with equal intensity on gel.	Promega	U.S.A
2	1Kb Ladder contain Loading dye	(500-10200) bp. The ladder is composed of 10 double strand purified DNA fragments ranged from (500, 1000, 1600, 2000, 2961, 4000, 5007, 5991, 8000, 10200) bp, the reference bright bands are 500, 1000, 1600, 2000 and 2961.	Bioneer	Korea

2-1-5 Cultural media:

2-1-5-1 Ready to use media:

The following media were prepared and autoclaved after adjusting the pH as mentioned on their containers by the manufacturing companies:

Medium	Company	Origin
Nutrient agar	Difco	U.S.A
Nutrient broth		
Kligler Iron Agar (KIA)		
Simmon citrate agar		
Mueller-Hinton agar		
Brain Heart Infusion broth (BHI)	Oxoid	England
Blood agar Base		
MacConkey agar	LAB	England
Urea agar Base		
MR-VP medium	Himedia	India
Eosin Methylene Blue (EMB)		

2-1-5-2 Laboratory prepared media:

- **Peptone water medium:** (Atlas *et al*., 1995)

This medium composed of the following:

Peptone	20 g
Sodium chloride	5 g
D.W.	Complete to 1L

The pH was adjusted to 7, mixed thoroughly and distributed into tubes and sterilized by autoclaving.

- **Urea agar:** (Collee *et al*., 1996)

It was prepared by:

urea agar base	21.5 g
D. W.	Complete to 1000 ml
adjusting pH of the urea agar base to 7.0 and autoclaved, after cooling to 50 C°	
40 % urea	5 ml

The 40 % urea (previously sterilized by using Millipore filter unit 0.22 µm) was added aseptically and distributed into sterile test tubes (20 ml each) to solidify as slant.

- **Motility semisolid medium:** (Cruickshank *et al*., 1975)

It was prepared by dissolving:

nutrient broth powder	0.8 g
Agar-agar	0.4 g
D. W.	Complete to 100 ml

After preparation, the medium was dispensed in test tubes (5 ml to each one) and sterilized by autoclaving.

- **Blood agar:** (Forbes *et al*., 2002)

It was prepared by dissolving:

Blood agar base	37 g
D. W.	Complete to 1000 ml
Autoclaved and After cooling to 50 C°	
Blood	7 %

Mixed well and distributed into petri-dishes.

- **Mannitol semisolid medium:** (Collee *et al*., 1996)

This medium composed of the following:

Peptone	10 g
D-Mannitol	10 g
Sodium chloride	5 g
Bromothymol blue	0.025 g
Agar	3.5 g
D.W.	Completed to 1 L

pH was adjusted to 7.4, then sterilized by autoclaving.

- **Luria - Bertani broth:** (Cruickshank *et al*., 1975)

This medium composed of the following:

Trypton	10 g
Yeast extract	5 g
NaCl	5 g
D.W.	Completed to 1 L

pH was adjusted to 7.5, then sterilized by autoclaving.

2-1-6 Reagents and stains:

2-1-6-1 Catalase reagent: (Atlas *et al*., 1995)

This solution consists of 3% hydrogen peroxide.

2-1-6-2 Oxidase reagent: (Collee *et al*., 1996)

This reagent was prepared by dissolving 0.1 g of tetramethyl-p-phenylene diamine dihydrochloride powder in 10 ml D.W.

2-1-6-3 Methy red (MR) indicator: (Aneja, 2003)

This indicator was prepared by mixing the following components:

Methyl red	0.2 g
Ethanol (95 % v/v)	300 ml
D. W.	500 ml

2-1-6-4 Vogas - Proskauer (VP) reagent: (Harley and Prescott, 2002)

This reagent was composed of two solutions:

VP1: naphthol solution	
α-naphthol	1 g
Ethanol (95 %)	100 ml
kept in dark bottle and the solution mixed before used	
VP2:40 % Potassium hydroxide solution	
Potassium hydroxide	40 g
D.W.	100 ml
mixed the two solutions before used	

2-1-6-5 Gram stain:

Ready to use stains and reagents:crystal violet, gram iodine, ethanol 70 % and safranin stain, which were used for staining bacterial isolates for microscopic examination.

2-1-6-6 Capsule stain:

Ready to use stains, it is contain methylene blue (Aqueous staining) 0.25 % and nigrosine (Negative staining) 10 % w/v.

2-1-7 Solutions and buffers:

2-1-7-1 Standard solutions and buffers:

- **Physiological normal saline (Normal saline solution):** (Collee *et al*., 1996)

It was prepared by dissolving 0.85 g of NaCl in 100 ml D.W. and sterilized by autoclaving and stored at 4 C°.

- **Macfarland standard solution:** (Benson, 2001)

Solution A	
Barium chloride ($BaCl_2$)	1 g
D.W.	100 ml
Solution B	
Concentrated sulphuric acid (H_2SO_4)	1 ml
D.W.	completed to 100 ml
Macfarland No. 0.5 (1×10^8 cfu/ml):	
Solution A	0.05 ml
Solution B	9.95 ml

Mixed by vortex for 2 min. and stored in dark bottle until used.

- **Tris-EDTA buffer (TE buffer):** (Sambrook and Russell, 2001)

It was prepared by mixing the following materials:

Tris-OH	0.05 M
EDTA	0.001 M
D.W.	Completed to 1 L

pH was adjusted to 8, then sterilized by autoclaving and stored at 4 C° until used.

2-1-7-2 DNA extraction kit buffers (Bioneer, Korea):

- **DNA genomic extraction kit:**

Consisted of the following:

- GT buffer
- GB buffer
- W1 buffer
- Wash buffer
- Elution buffer

2-1-7-3 Electrophoresis solutions and buffers:

- **Tris-Borate-EDTA buffer 10X (TBE buffer 10X):** (Sambrook and Russell, 2001)

Tris-OH	0.089 M
Boric acid	0.089 M
EDTA	0.002 M

The pH was adjusted to 8, autoclaved at 121 C° for 15 min.

- **Ethidium bromide solution 10mg/ml:** (Sambrook and Russell, 2001)

It was prepared by dissolving 0.2 g of ethidium bromide in 20 ml distilled water and stirred on magnetic stirrer for four hours to ensure that the ethidium bromide was dissolved, then it was filtrated, and stored in a dark bottle at 4 Cº, until used.

- **Loading buffer (Blue-Orange loading dye (6X)):** (Promega, USA)

It was provided in a premixed containing:

Orange G	0.4 %
Bromophenol blue	0.03 %
Xylene cyanol FF	0.03 %
Ficoll 400	15 %
Tris-HCl (pH=7.5)	10 mM
EDTA (pH=8.0)	50 mM

The loading buffer is used for loading DNA samples into gel wells and tracking migration during electrophoresis, stored at -20 C°.

2-1-7-4 Polymerase chain reaction (PCR) solutions:

- **PCR premix (1X):** (Bioneer, Korea)

The master mix contained optimum concentrations of reaction requirements ($Mgcl_2$ 1.5 mM, Taq polymerase 1 U, each dNTPs 200 µM) that

efficient amplification of DNA template by PCR, it also contained two dyes (blue and yellow) that allow monitoring of progress during gel electrophoresis.

2-1-8 Primers (Bioneer / Korea):

The following primers were used in this study to identify the target genes in *Klebsiella* spp. isolates:

No	Target gene	Primer	Oligo sequence (5'-3')	Product size (bp)	Ref.
1	*magA*	magA-F	GGT GCT CTT TAC ATC ATT GC	1283	(Turton *et al.*, 2008)
		magA-R	GCA ATG GCC ATT TGC GTT TGC GTT AG		
2	*k2A*	k2A-F	CAACCATGGTGGTCGA TTAG	543	(Rivero *et al.*, 2010) (Doud *et al.*, 2009)
		k2A-R	TGGTAGCCATATCCCTT TGG		
3	16S rRNA	16S rRNA-F	ATT TGA AGA GGT TGC AAA CGA T	130	(Turton *et al.*, 2010)
		16S rRNA-R	TTC ACT CTG AAG TTT TCT TGT GTT C		
4	*rmpA*	rmpA-F	ACT GGG CTA CCT CTG CTT CA	536	(Nadasy *et al.*, 2007) (Turton *et al.*, 2010)
		rmpA-R	CTT GCA TGA GCC ATC TTT CA		
5	*kfu*	kfu-F	GAAGTGACGCTGTTTCT GGC	797	(Yu *et al.*, 2008)
		kfu-R	TTTCGTGTGGCCAGTGA CTC		

2-2 Methods:

2-2-1 Sterilization methods: (Baily *et al.*, 1990)

Three methods of sterilization were used:

- **Wet-heat sterilization (autoclaving):**

Microbial culture media, buffers, reagents and solutions were sterilized by the autoclave at 121 C° (15 Ib/inch^2) for 15 min. unless otherwise stated.

- **Dry-heat sterilization (oven):**

Electric oven was used to sterilize glassware at 180 C° for 3 hrs.

- **Membrane filtration:**

Urea solutions and antibiotic solutions were sterilized throughout 0.22 and 0.45 µm Millipore filters and Whatman No.1 filter papers.

2-2-2 Samples collection:

A total of three hundred and twenty five samples were aseptically collected using sterile containers and transport swabs damped with normal saline from different Hospital's Environment and patients visiting some Hospitals (Ibn Al-baladi, Central Children, Al-kindy, Al-Wasity and Medical city / Educational lab.) in Baghdad. Samples were collected from different age groups and gender in the period between 1/11/2012 to 7/1/2013.

Types and numbers of clinical and hospital environmental samples are listed in Table (2-1).

Table (2-1): Types and numbers of clinical and hospital environmental samples

No.	Samples source	No. of sample (%)	Sample type
1	Urine	50 (15.3 %)	**Clinical**
2	Wound	68 (21 %)	
3	Burn	80 (24.6 %)	
4	Sputum	75 (23.1 %)	
5	Ear swab	10 (3.1 %)	
6	Blood	28 (8.6 %)	
Total		**311 (95.7%)**	
7	Operation hall	2 (0.62 %)	**Hospital Environment**
8	Wards	4 (1.23 %)	
9	Kitchen	3 (0.92 %)	
10	Bathrooms	5 (1.53 %)	
Total		**14 (4.3 %)**	

2-2-3 *Klebsiella* isolation:

All isolates were recovered from clinical and hospital environmental samples after cultured on MacConkey, eosin methylene blue agar and blood agar plates. These plates were incubated at 37 Cº aerobically and after overnight incubation, they were checked for bacterial growth. The colonies with pink color and mucous texture were sub cultured onto MacConkey agar to confirm lactose-fermenting (pink) from non-lactose fermenting bacteria (colorless), on EMB agar *Klebsiella* colonies appear in pink color, while onto Blood agar appears pale and gave gamma-Heamolysis result (Holt *et al*., 1994).

2-2-4 Identification of *K. pneumoniae:*

Identification of suspected isolates was done according to the colony morphology, staining reaction and biochemical tests (Atlas *et al*., 1995; Macfaddin, 2000; Garrity, 2005).

2-2-4-1 Colony morphology:

All isolates were identified primary according to general cultural characteristic (color, shape, texture and size) of colony onto MacConky agar, Blood agar and EMB agar after incubated overnight at 37 C°. Other characteristics were observed like lactose fermentation and blood lysis.

2-2-4-2 Staining reaction:

- Gram stain: (Atlas *et al*., 1995)

All the bacterial isolates were examined after stained with Gram stain to examine cells shape, grouping, gram reaction microscopically.

- Capsule stain:

The capsule stain test was conducted for detecting capsule production as following:

- Grown colony incubated at 37 C^{o} for 24 hrs. on MacConky agar.
- Preparing a typical slide from colony.
- The slide was allowed to air dry do not heat fixed.
- The slide was flood with methylene blue for 3-5 min.
- The slide was rinsed with nigrosine for 1 min.
- Finally Examined with oil immersion.

2-2-4-3 Biochemical tests:

Following tests were used to identify *K. pneumoniae*:

- **IMVC test:** (Macfaddin, 2000).
- **A- Indole production test:**

Peptone broth was inoculated with fresh culture of bacteria and incubated at 37 C^{o} for 24 hrs, then 10 drops of Kovac's reagent was added for each test tube. Appearance of red ring at the top of the broth within 10 min. indicates a positive result. This test used to detect the bacterial capacity to produce treptophanase enzyme which hydrolyze treptophane to indole, pyruvic acid and ammonia.

B- Methyl red test:

Methyl red-Voges Proskauer broth was inoculated with the bacterial culture and incubated at 37 C˚ for 24 hrs. Five drops of methyl red solution was added to each tube and the result was read immediately. Red color indicates a positive result this test used to detect the bacterial ability to ferment glucose and produce acid as a final product.

C- Voges-Proskauer test

MR-VP medium prepared was inoculated with the bacterial culture, incubated at 37 C^{o} for 24 hrs., then 0.6 ml of VP1and 0.2 ml of VP2 were added; red color after 15 min. indicated a positive result, while the negative result confirmed when the color of the reagent remain unchanged. This test used to detect bacterial ability to ferment glucose and produce acetone.

D- Citrate utilization test:

Simmon citrate agar slants were stabbings with fresh bacterial isolates and incubated at 37 C^{o} for 24-48 hrs. Changing the color from green to blue is indicating a positive result. This test used to detect the bacterial ability to utilize sodium citrate as the carbon source.

- **Hypermucoviscosity test:** (Macfaddin, 2000)

The bacterial culture was inoculated onto MacConky agar or nutrient agar, then incubated at 37C^{o} for 24 hrs. The colony touched by a loop then lifted vertically from the surface of agar plate, mucous phenotype was defined as being present when a string-like growth was observed. *Klebsiella* spp. formed a string < 5 mm in length except *K. pneumoniae* colonies showed ≥ 5 mm demonstrating the hypermucoviscosity phenotype.

- **Sugars fermentation and gas production test (Kligler iron agar test (KIA)):** (Macfaddin, 2000; Garrity, 2005)

KIA slants were inoculated with an inoculating needle by stabbing the botton and streaking the surface of the slant in a zigzag pattern, then tubes were incubated at 37 C^{o} for 24 hrs. Results were recorded as follows (table 2-2).

Table (2-2): Result obtained from KIA agar test

Slant / Butt.	Color or Bubble	Reaction
acid / Alkaline	Yellow / Red (Butt. / top)	Glucose only fermented
Acid / acid	Yellow / Yellow (Butt. / top)	Glucose (anaerobic conditions) and Lactose (aerobic conditions) fermented
Alkaline / alkaline	Red / Red (Butt. / top)	No fermentation of glucose and lactose
H_2S production (In the Butt.)	Black precipitation	reaction between H_2S and ferrous sulphate in the medium
Gas production (in the Butt.)	Bubbles formation	pushing the agar to the top indicates CO_2 formation

The positive result was noticed by changing the color of the phenol red indicator from red to yellow as a result for acid production from sugar fermentation, while the negative result observed by keeping the phenol red indicator its red color, bubbles and black residue doesn't formed. This media was used for initial identification members of *Enterobacteriaceae*.

- **Urease test:** (Atlas *et al*., 1995)

Urease activity in *K. pneumoniae* was detected by inoculating the surface of urea agar slants with the bacterial growth and incubated at 37 C˚ for 24 hrs. Changing the color of medium to purple-pink indicates a positive result, while keeping the media its yellow-orange color indicates a negative result. This test

used to detect bacterial capacity to produce urease enzyme which hydrolyze urea to ammonia and carbon dioxide.

- **Motility test:** (Garrity, 2005)

Semi solid nutrient medium was inoculated by single vertically stabbing into the center of the agar with a needle (inoculated with the suspected colony), then incubated at 37 C° for 24 hrs. Motile organism recognized by movement away from the stab line or a cloudy appearance through the medium.

- **Oxidase test:** (Atlas *et al*., 1995)

This test was done by using filter paper moistening with few drops of a freshly prepared solution of Oxidase reagent (tetramethyl-p-phenylene diamine dihydrochloride). Aseptically a clump of cells cultured on brain heart infusion agar was picked up from the growth with a sterile toothpick and smeared on the filter paper. The development of a violet or purple color within 2-10 sec. indicates a positive results.

- **Catalase test:** (Atlas *et al*., 1995)

Single colony was placed onto a clean glass microscope slide with a sterile toothpick, then a drop of hydrogen peroxide (3 %) was placed onto the colony. The production of gaseous bubbles indicates the presence of catalase that hydrolyzes hydrogen peroxide to water and oxygen.

- **Mannitol fermentation:** (Collee *et al*., 1996)

Mannitol semisolid agar medium was inoculated and incubated at 37 C˚ for 24 hrs. The changing of medium color to yellow indicates positive results for mannitol fermentation.

2-2-5 Maintenance and preservation of *Klebsiella* isolates

Maintenance of bacterial isolates was performed according to Sambrook and Russell **(**2001) as follow:

2-2-5-1 Short term storage:

Single pure colonies of bacterial isolates were maintained for a period of few weeks for routine laboratory works by subcultured on nutrient agar plates, wrapped tightly with parafilm and stored at 4 Cº.

2-2-5-2 Medium term storage:

Tubes that contained 8 ml of sterile nutrient agar in slants position were inoculated with the bacterial isolate, and then incubated at 37 Cº overnight, before stored at 4 Cº.

2-2-5-3 Long term storage:

A test tube contained 10 ml of sterile brain heart infusion broth inoculated with a single colony of *Klebsiella* and incubated at 37 Cº for 24 hrs., then 8.5 ml of the cell suspension was mixed with 1.5 ml of glycerol (15 %), and stored for long time at -20 C°.

2-2-6 API 20E identification of *Klebsiella* isolates:

The reagents and indicators IND, TDA and VP1-VP2, which were used in API 20E system had been prepared according to the Manufacturer's instruction kit of (BioMrrieux) for *Enterobacteriaceae*, *Klebsiella* spp. isolates were examined by this kit as following:

2-2-6-1 Preparation of the strips:

The API 20E strip as it listed in appendix (1) consists of 20 microtubes containing dehydrated substrates. Five milliliters of tap water was distributed into wells of the tray to create humid atmosphere, then API test strip was withdrawn from the sealed package and placed into the incubation tray.

2-2-6-2 Preparation of the inoculums:

Single colony was picked with a sterilized loop from MaCconky agar and suspended into sterile 5 ml of NaCl (0.85 %) and mixed well, this inoculated suspension must be used immediately after preparation.

2-2-6-3 Inoculation of the strip:

- Bacterial suspension was transferred to the tilted API test strip by a sterile pasture pipette, the microtube was filled by placing the pipette tip against the side of the cupules.
- The tube and cupule sections of the **CIT**, **VP** and **GEL** microtubes were filled.
- The cupule sections of **ADH**, **LDH**, **ODC**, **H2S** and **URE** microtubes were filled completely with sterilize mineral oil to prepare anaerobic conditions.
- The Incubation box (Tray and lid) was closed and incubated at 37 Cº for 24 hrs.
- After incubation, the reagents were added to **TDA**, **IND** and **VP** microtubes.

2-2-6-4 Reading the strip:

The reagents were added as the following:

- One drop of Indol reagent (kavoc's reagent) was added to IND tube and was read immediately.
- One drop of TDA reagent (3.4 % ferric chloride) was added to TDA tube.
- One drop of each VP1 (40 % potassium hydroxide) and VP2 (6 % alpha naphthol) reagents was added to VP tube and read after 10 min.

The results were read according to appendix (1) and identification is obtained with the numerical profile, by dividing the strip into 7 groups, each group contains 3 tests with numbers 1-2-4. Each positive test was given the digit number that is special according to it sequence in the strip, the negative test was given the digit number 0. Consequently, the sum of the 3 digit number for each group was taken if it is between 0-7, A seven-digit profiler number is obtained for the 20 tests of the API 20 E strip and balanced with the digital number that exist in Analytical Profile Index that provided from manufacturer company.

2-2-7 VITEK 2 system for identification of *Klebsiella* isolates:

VITEK 2 system (figure 2-1) is used for diagnosis of bacterial isolates, it is consists of 64 biochemical tests and 20 antibiotic tests.

The VITEK 2 system was used in this study to confirm the identification of *Klebsiella* spp. as follow:

- A single pure colony of bacterial isolate was suspended in 3 ml of physiological normal saline in a sterile manufacture's tube.
- Bacterial suspension was compared with standard turbid static solution (Turbidity measurement system) to measure the turbidity supplier of the company, where the final concentration inside the tube must range between 0.5-0.63.
- The tubes were puted in its own rack after it was added to each tube examination VITEK 2 Cassette depending on the diagnostic gram stain, where there is a special strip for gram negative bacteria.
- The rack containing tubes and cassete was transferred to the system, puted in the first in the field of fillers (filler) automatically populates the cassete with bacterial suspension and after the finishing of the process gave the end signal from the device.
- The second field reader (reader) was transferred to it, its first cut the tapes and gives order (burden) is a digital signal since kept. The rack containing tubes, moving from the device to give the data for each sample on a computer attached to the VITEK system.
- The taps were left for 24 hrs. at 37 Cº then read the results for diagnosis of bacteria (appendix 2)

Figure (2-1): VITEK 2 system

2-2-8 Antibiotic susceptibility Test (Disc-diffusion method): (Barry, 1976)

Antimicrobial sensitivity test of *K. pneumoniae* isolates was carried out against the antibiotics shown in (2-1-3) using the standard disc diffusion method as follows:

Five milliliters of sterile 85 % sodium chloride were inoculated with fresh culture of each *Klebsiella* isolate and compared with 0.5 Macfrland. A sterile cotton swab is dipped into the inoculom and swabbed evenly across the surface of Muller-Hinton agar plate.

The inoculated plates were placed at room temperature for 10 min. to allow absorption of excess moisture, then the antibiotic discs were placed firmly on the inoculated plates with a forceps to ensure contact with the agar then incubated at 37 C^{o} for 24 hrs. After incubation, diameters of the inhibition zones

were measured in mm. and compared with that of standards of the National committee for clinical laboratory standards (NCCLs).

2-2-9 Extraction of DNA from the *Klebsiella* isolates:

Genomic DNA was extracted according to the manufacturer's instructions as follows:

2-2-9-1 Genomic DNA mini-kit bacterial protocol:

- Bacterial culture was inoculated in 5 ml Luria - Bertani broth and incubated at 37 Cº for 24 hrs.
- A 1.5 ml was distributed in 1.5 ml eppendorff tube, centrifuged at 14000-16000 xg for 1 min., and then the supernatant was discarded and pellet was taken.
- A 200 µl of GT buffer was added, the cell pellet was resuspended by vortex or pipetting, and incubated at room temperature for 5 min.
- A 200 µl of GB buffer was added and mixed by shaking vigorously for 5 sec.
- The sample lysate was incubated at 60 Cº for 10 min. until is clear, during incubation the tube was inverted every 3 min.
- A 200 µl of absolute ethanol was added to the sample lysate and immediately mixed by vortex.
- A GD column was placed in a 2 ml collection tube then all mixture (including precipitate) was transferred to the GD column, centrifuged at 14000-16000 xg for 2 min. The 2 ml collection tube containing the flow through was discarded and the GD column was placed in a new 2 ml collection tube.
- A 200 µl of W1 buffer was added to the GD column, centrifuged at 14000-16000 xg for 30 sec. The flow through was discarded and the GD column was placed back in the 2 ml collection tube.
- A 600 µl of wash buffer was added to the GD column, centrifuged at 14000-16000 xg for 30 sec. The flow through was discarded and the GD column was

placed back in the 2 ml collection tube, centrifuged again at 14000-16000 xg for 3 min. to dry column matrix.

- The dried GD column was transferred to clean 1.5 ml eppendorff tube.
- A 100 µl of preheated elution buffer was added or TE to the center of the column matrix, let stand for 3-5 min., centrifuged at 14000-16000xg for 30 sec. to elute the purified DNA.

2-2-9-2 Measurement of DNA concentration and purity:

The concentration of DNA was measured by Nanodrop system according to the Nanodrop Optizen manual, 1 µl of each DNA samples was used. DNA purity was measured depending on the ratio of sample absorbance at wave lengths 260 and 280 nm.

2-2-9-3 Agarose gel preparation: (Green and Sambrook, 2012)

Agarose gel of 1.5 % concentration was utilized to confirm the size of genomic DNA bands and to confirm the size of the PCR products.

The agarose gel consist of 1.8 g dissolved in 120 ml of 1X TBE buffer using a microwave. After the agarose solution cools down to 55-60 Cº, a 1 µl of 0.5 µg/ml final concentration ethidium bromide (EtBr) was added. Then, the solution was poured into the gel tank with the combs in place and let to cool for 30 min. The combs were removed carefully and tank was placed in the electrophoresis system containing running buffer consisting of 1X TBE, the buffer is poured until it covers the gel for about 1-2 mm. Ten microliter of each PCR product along with the negative control and DNA ladder (100 bp, 1 kb or both) were loaded into the wells, the system cover was then placed and the system was turned on. Electrophoresis was performed for 2hrs. with a 70 volt/35 mAmp current. The DNA bands were visualized with a UV transilluminator was followed and photographed by using digital camera.

2-2-10 Polymerase chain reaction (PCR) diagnosis

In order to diagnose the target genes, PCR was used as diagnostic techniques. The extracted DNA was subjected to amplification as follow:

2-2-10-1 Preparation of primers solution:

The lyophilized primer was dissolved using deionizer distal water DDH_2O to obtain 100 pmol/µl in the master tube, then 10 pmol/µl was prepared as a working solution by taking 10 µl from master tube and completed the volume to 100 µl by adding DDH2O.

2-2-10-2 Monoplex PCR mixture and PCR program conditions:

PCR reactions were performed in 20 µl volumes in PCR tubes under sterile conditions, all the volume of the reaction mixture was completed to 20 µl using sterile DDH_2O. All amplification experiments included a negative control blank which contained all PCR material with the exception of target DNA. Mixture and program condition of primers were listed as following:

- Diagnosis of *K. pneumoniae* serotype K1 by *magA* gene

Serotype K1 was diagnosed with PCR by using the primer specific for *magA* gene. The master mix (lypholyzed) contained optimum concentrations of reaction requirements ($Mgcl_2$ 1.5 mM, Taq polymerase 1 U, each dNTPs 200 µM). The reaction mixture was illustrated in table (2-3):

Table (2-3): PCR mixture

PCR reaction components	Volume (µl)
Primer F (10 picomols/µl)	1
Primer R (10 picomols/µl)	1
DNA template	2.5
DDH_2O	15.5
Total volume	**20**

The optimal condition for detection of *magA* gene was adjusted as in the table (2-4).

Table (2-4): Program of PCR thermocycling conditions

No.	Steps	Temp. (C°)	Time	No. of cycle
1	Initial denaturation	94	5 min.	1 cycle
2	Denaturation	94	30 sec.	35 cycle
3	Annealing	55	1 min.	
4	Extension	72	1 min.	
5	Final extension	72	10 min.	1 cycle

- Diagnosis of *K. pneumoniae* serotype K2 by *k2A* gene

The second set of PCR reaction was performed for diagnosing serotype K2 by using the primer specific for *K2A* gene. The reaction mixture was of the same composition as described in table (2-3), but conditions of this set of primer were different as follow: the denaturation step was taken 45 sec. and the final extension of 3 min. at 72 C° was applied.

- Diagnosis of *K. pneumoniae* by 16S rRNA gene

This gene was amplified by using 16S rRNA specific primer. The reaction mixture was of the same composition as described in table (2-3) with reducing the amount of DNA to 2 µl. The optimal condition for detection 16S rRNA gene was adjusted as in the table (2-4) with reducing the the number of cycles to 30 and final extension time to 5 min.

- Diagnosis of *K. pneumoniae* by *rmpA* gene

The fourth set of PCR reaction was performed for amplifing *rmpA* gene. The reaction mixture was of the same composition as described in table (2-3). The conditions of this set of primer were different as following: the denaturation step was taken 45 sec. and final extension of 3 min. at 72 C° was applied.

- Diagnosis of *K. pneumoniae* by *kfu* gene

Kfu gene was amplified with PCR by using the specific primer. The reaction mixture contained the entire component at the following concentration:

0.6 µl of each primer F and R (10 picomols/µl), 1.5 µl of DNA and completed the reaction volume up to 20 µl by DDH_2O. The reaction conditions involved initial denaturation at 94 C˚ for 5 minute; then 30 cycles of 94 C˚ for 20 sec., 60 C˚ for 1 min., 72 C˚ for 1 min. and 72 C˚ for 3 min. as a final extension.

2-2-10-3 Multiplex PCR reaction and program conditions:

Four primers were used in the multiplex PCR: *magA*, *K2A*, 16S rRNA and *rmpA*. The mixture of multiplex PCR reaction consists of (table 2-5):

Table (2-5): Mutiplex PCR mixture

Primers	PCR reaction components	Volume (µl)
magA	10 picomols/ µl of each primer F and R	0.7
k2A	10 picomols/ µl each primer F and R	0.7
16S rRNA	10 picomols/ µl each primer F and R	0.7
rmpA	10 picomols/ µl each primer F and R	0.7
DNA		3
DDH_2O		11.4
Total volume		**20**

Amplification was carried out according to the following thermal and cycling condition as shown in table (2-6):

Table (2-6): Program of multiplex PCR thermocycling conditions

No.	Steps	Temp. (C˚)	Time	No. of cycle
1	Initial denaturation	94	5 min.	1 cycle
2	Denaturation	94	30 sec.	35 cycle
3	Annealing	60	1.5 min.	
4	Extension	72	1.5 min.	
5	Final extension	72	10 min.	1 cycle

CHAPTER THREE

RESULTS AND DISCUSSION

3- Results and Discussion:

3-1 Isolation of *Klebsiella* isolates:

A total of 325 clinical and environmental samples were collected from different Hospital's Environment and patients in Ibn Al-baladi, Central Children, Al-kindy, Al-Wasity and Medical city / Educational lab. in Baghdad /Iraq. Samples were collected from different age groups and gender during the period from 1/11/2012 to 7/1/2013. Three hundred and eleven (95.7 %) were clinical samples, while the rest (14) were environmental samples (4.3 %).

3-2 Identification of *Klebsiella* isolates:

Several morphological, physiological and biochemical tests were made to identify bacterial isolates. Two hundred and eighty two isolates were obtained from three hundred and twenty five samples. Results showed that *Klebsiella* spp. constitute 19.9 % (56 isolates) of these isolates, and identified as *K. pneumoniae* (40 isolates) 14.2 %, *K. oxytoca* (3 isolates) 1.1 %, *K. ornitholytica* (3 isolates) 1.1 % and *K. terrigena* (10 isolates) 3.5 %.

The other bacterial isolates were *E. coli* (94 isolates, 33.3 %), *Staphylococcus aureus* (34 isolates, 12.1 %), *Pseudomonas* and *Proteus* (32 isolates, 11.3 % for each one). While *Staphylococcus epidermidis*, *Enterobacter* and *Shigella* were isolated in percentages 8.5 % (24 isolates), 2.5 % (7 isolates) and 1.1 % (3 isolates), respectively.

Bacterial isolates were identified according to their cultural, microscopical and biochemical characteristics that were in agreement with Holt *et al.* (1994), Atlas *et al*. (1995) and Collee *et al*. (1996).

Moreover, identification of the bacterial isolates was confirmed by using API system (API 20E) and VITEK 2 system.

3-2-1 Colony morphology:

Primary identification of bacterial isolates were done after incubated aerobically on MacConkey agar, blood agar and EMB agar plates at 37 C^{o} for 24-48 hrs. On MacConkey agar, *Klebsiella* colonies were lactose fermenting

colonies and gave pink color, regular edge, round, mucoid texture with large size, (figure 3-1 A) and *K. oxytoca* were (3-4 mm) in diameter while *K. terrigena* was (1.5-2.5 mm) with a weakly mucoid aspect. In the identification of *Klebsiella*, a selective enrichment technique including culturing of samples on MacConkey agar was used. This medium contains bile salts and crystal violet which promotes growth of *Enterobacteriaceae* and related enteric Gram negative rods, in addition to suppresses growth of Gram positive bacteria and some fastidious Gram negative bacteria. Lactose in this medium is the sole carbon source that differentiates between lactose fermenting bacteria and Non-lactose fermenting bacteria. The first is characterized by producing pink colonies due to the conversion of neutral red indicator dye when it is below pH 6.8. Adversely, the Non-lactose bacterial growth appears colorless or transparent (Holt *et al*., 1994).

On the enrichment Blood agar medium, bacterial isolates were large, mucoid, white to grey and Non-heamolytic colonies, this used for differentiate *Klebsiella* from other bacterial species that similar with growth on MacConkey agar but heamolytic blood like *Serratia* spp. (Don *et al*., 2005).

On the EMB agar, this medium is usually used to differentiate between *Klebsiella* and *E. coli* because the aniline dyes (eosin and methylene blue) in this medium combines to form a precipitate at acidic pH and appearing as a metallic green sheen, thus serving as indicators for acid production. Therefore, *Klebsiella* colonies appear in pink color but *E. coli* colonies be dark surrounded by a green metallic sheen due to the highly amount of acid produced by fermentation (Atlas *et al*., 1995). Moreover, all *K. pneumoniae* isolates had a positive string test result, indicating hypermucoviscosity phenotype as determined by the formation of a string of, approximately, 7cm in length (figure 3-1 B). Nadasy *et al.* (2007) stated that this character was not observed with the noninvasive *K. pneumoniae* strains isolated from patients with noninvasive infections.

(A) **(B)**

Figure (3-1): Mucoid colonies of *K. pneumoniae* on MacConkey agar at 37 C° for 24 hrs. (A) single colony (B) the Hypermucoviscosity phenotype (string test)

3-2-2 Staining reaction:

The suspected *Klebsiella* isolates, were found to be Gram negative, Non-motile, small straight rods and arranged singly or in pairs under the compound light microscope (figure 3-2 A) as described by Garrity (2005). All isolates showed a distinct capsule as clear zone surrounding bacteria when they examined under oil immersion after capsule staining as shown in figure (3-2 B).

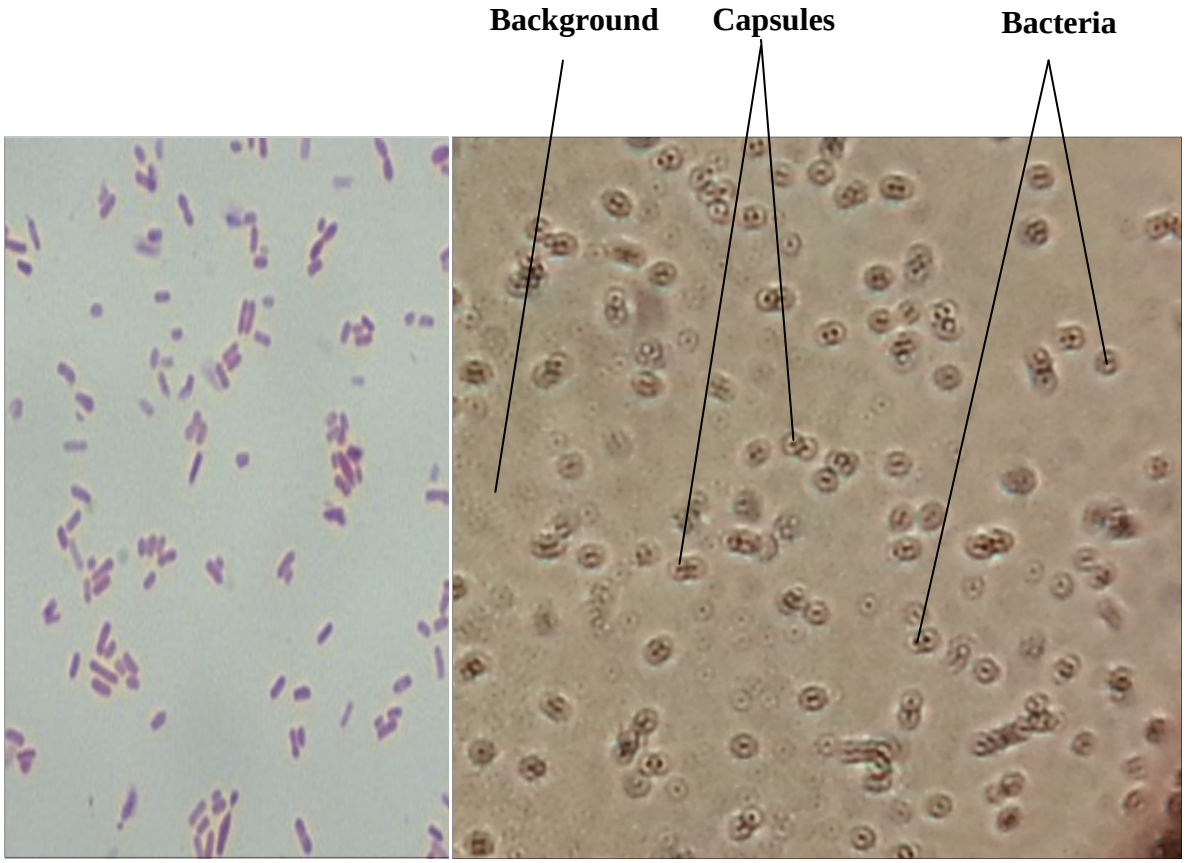


Figure (3-2): *Klebsiella pneumoniae* (A) gram stain (B) capsule stain

3-2-3 Biochemical tests:

The biochemical tests were used for further identification of bacterial isolates. Table (3-1) showed that all isolates of *Klebsiella* were positive or negative for methyl red and positive for voges-proskauer (VP), meaning that acetoin and 2,3-butanediol were produced from glucose partial fermentation and that neutral end products predominate over the acidic end products (Brisse *et al.*, 2006). The results of IMViC differentiate them from other lactose fermenter genera; *E. coli, Citrobacter* and *Serratia*. *Klebsiella* showed negative result for indole (except *K. oxytoca*). In the indole test, ability to hydrolyze tryptophan to indole is a characteristic of certain enteric bacteria possessing the enzyme tryptophanase, an enzyme that decomposes amino acid

tryptophan to indole, pyruvic acid and water. Indole negative bacteria such as *Klebsiella* was not produced tryptophanase, so that when Kovac's reagent was added to a broth free of indole, a red ring will not be formed at the top of the broth (Collee *et al*.,1996).

Utilization of citrate is one of several important physiological test used to diagnose members of *Enterobacteriaceae*, *Klebsiella* showed positive reactions for citrate, so that the citrate in simmon citrate medium is important to detect weather the bacteria isolates able to grow on it as a unique carbon and energy source. In addition, Simmon's medium also contains bromothymol blue as a pH indicator. *Klebsiella* is produced CO_2, it reacts with components of the medium to produce an alkaline compound, the alkaline pH turns the pH indicator (bromthymol blue) from green to blue, reflecting it as positive citrate test (Macfaddin, 2000).

In Kligler Iron Agar (KIA) test, it differentiates the genera of *Enterobacteriaceae* from each other based on their carbohydrate fermentation patterns and H_2S production. KIA slants contain 1 % lactose and 1 % glucose. The pH indicator (phenol red) changed the medium color from orange-red to yellow in the presence of acids. KIA also contains sodium thiosulfate, a substrate for H_2S production, and ferrous sulfate that produces black precipitate to differentiate H_2S producing bacteria from others.

Results (table 3-1) showed that *Klebsiella* isolates turned the color of both the slant and butt, which produced acidic slant (yellow) and acid butt (yellow) accompanied by gas production (bubbles formation), but without black precipitate formation, which indicates that lactose and glucose fermentation had occurred and no H_2S was produced. These results agreed with those declared by Garrity (2005).

Table (3-1): Cultural, Microscopical and Physiological characteristics of different bacterial isolates

No.	Isolates / Test		*K. pneumoniae*	*K. oxytoca*	*K. terrigena*	*K. ornitholytica*	*E. coli*	*S. aureus*	*S. epidermidis*	*Proteus*	*Pseudomonas*	*Enterobacter*	*Shigella*
1	Cell shape		Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Cocci	Cocci	Bacilli	Bacilli	Bacilli	Bacilli
2	MacConkey agar		LF	LF	LF	LF	LF	LNF	LNF	LNF	LNF	LNF	LNF
3	Gram stain		–	–	–	–	–	+	+	–	–	–	–
4	Capsule stain		+	+	+	+	–	–	–	–	–	–	–
5	Motility		–	–	–	–	V	–	–	V	V	V	–
6	Urease		+	+	+	+	–	ND	ND	+	+	–	–
7	Indole		–	+	–	–	+	–	ND	–	–	–	V
8	MR		V	V	V	V	V	ND	ND	V	–	–	+
9	VP		+	+	+	+	–	V	ND	–	–	V	–
10	Citrate utilization		+	+	+	+	–	ND	ND	V	+	V	–
11	Kliglar iron agar (KIA)	H_2S	–	–	–	–	–	ND	ND	+	–	–	–
		CO_2	+	+	+	+	+	ND	ND	ND	ND	–	–
		Acid	A/A	A/A	A/A	A/A	A/A	ND	ND	A/K	K/K	A/A	A/K
12	Oxidase		–	–	–	–	–	–	–	–	V	–	–
13	Catalase		V	V	V	V	V	+	+	V	V	V	V
14	Mannitol fermenter		V	V	V	V	V	V	–	–	V	V	V
15	live at 10 C°		–	+	+	+	ND	ND	ND	ND	ND	ND	ND
16	produce gas from lactose at 44.5 C°		+	–	–	–	ND	ND	ND	ND	ND	ND	ND

(+) positive result (–) negative result (ND) Not determined (K) alkaline (A) acid

V= variable result (LF) Lactose ferment (LNF) Lactose Non-ferment

Whereas urease test differentiate *Klebsiella* isolates from *Enterobacter* isolates as it was positive for *Klebsiella* and negative for *Enterobacter* (Forbes *et al.*, 2002). Urease enzyme catalyzes the breakdown of urea, and the bacteria that can produce this enzyme is able to detoxify the waste products and to drive metabolic energy from its utilization which change the medium color from yellow to purple-pink, indicating urease positive test. *Klebsiella* can produce urease enzyme and gives urease positive test (Atlas *et al.*,1995).

In the motility test, *Klebsiella* isolates were non-motile. The movement of the growth away from the stab line or a hazy appearance through the semisolid medium indicates that the bacteria are motile. But the linear growth means negative result a property which *Klebsiella* is characterized by Gwendolyn (1988).

Klebsiella isolates were oxidase negative and catalase positive or negative result (Bernere and Farmer, 2005).

At the species level, the results of indole test differentiate *K. pneumoniae* from *K. oxytoca* it was positive for *K. oxytoca* and negative for other (Atlas *et al.*, 1995). *K. pneumoniae* were unable to live at 10 C°, which distinguish them from *K.oxytoca* and *K. terrigena* since they can live at this temperature (Grimont and Grimont, 2005). All isolates of *K. pneumoniae* found to be Non-motile which separated them from the motile species *K. mobilis* (Brisse *et al.*, 2006). All the isolates of *K. pneumoniae* were able to produce gas from lactose at 44.5 C^{o} which distinguish them from *K. ozaenae, K. rhinoscleromati, K.oxytoca* and *K. terrigena* since they are unable to produce gas from lactose at 44.5 C^{o}.

3-2-4 API 20E system identification of *Klebsiella* isolates:

Results of biochemical tests for *Klebsiella* spp. (56 isolates) were confirmed by using API 20E system as shown in appendix (3) and figure (3-3). Results of all bacterial isolates were compatible with the above results.

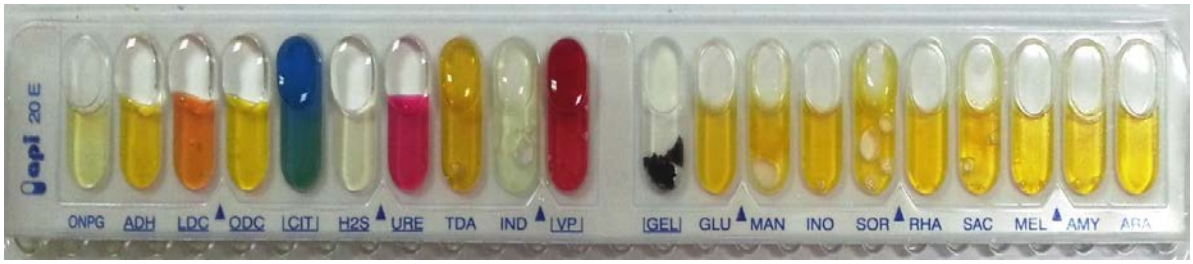


Figure (3-3) API 20E system for characterization of *K. pneumoniae*

The API 20E diagnostic system may it gave excellent results and differentiate *Klebsiella* spp. Moreover, the diagnosis of this system is high accuracy (Turton *et al*., 2008). However, the diagnosis by conventional methods included biochemical tests and API 20E system are expensive and time consuming.

3-2-5 VITEK 2 system for identification of *Klebsiella* isolates:

This system was used to confirm a final diagnosis of *K. pneumoniae.* This system was detected bacteria faster, efficient and away from the contamination that may prevent detection of the pathogen.

Results of the tests used in this system (appendix 4) confirmed the results obtained from morphological, biochemical and API 20E system. So all isolates (56) that previously identified as *Klebsiella* spp. are proved to be *Klebsiella*.

The VITEK 2 system provides biochemical as well as antibiotic susceptibility tests. Appendix (5) showed positive results of the *K. pneumoniae*.

3-3 Distribution of bacterial isolates according to source of samples:

Results presented in table (3-2) show that out of 268 clinical samples, 36 (13.4 %) *K. pneumoniae* isolates were recovered, while from the 14 hospital environmental samples, 4 (28.6 %) isolates were found to be *K. pneumoniae*. Ten (3.7 %) of all *Klebsiella* isolates were belong to *K. terrigena*, all were isolated from clinical samples only. However, 3 (1.1 %) of *K. oxytoca*

and *K. ornitholytica*, 2 isolates (0.7 %) and 1 isolate (7.1 %) were recovered from clinical and hospital environment samples, respectively.

Abdul Razzaq *et al.* (2013) reported that 29.1 % (46/158) isolates were identified as *K. pneumoniae*. Omar-Zahid, (2009) found that *Klebsiella* spp. formed 54.16 % of total isolates from clinical specimens and 79.12 % was identified as *K. pneumoniae*.

Table (3-2): Types and numbers of bacterial isolates obtained from samples of five hospitals

Source of sample	No. (%)	No. (%) of *Klebsiella* spp.	No. (%) of *K. pneumoniae*	No. (%) of *K. oxytoca*	No.(%) of *K. ornitholytica*	No.(%) of *K. terrigena*	No. (%) of other Gram (-) isolates	No. (%) of other Gram (+) isolates
Clinical sample	**268 (82.5)**	50 (18.7)	36 (13.4)	2 (0.7)	2 (0.7)	10 (3.7)	162 (60.4)	56 (20.9)
Hospital environment sample	**14 (4.3)**	6 (42.9)	4 (28.6)	1 (7.1)	1 (7.1)	0 (0)	6 (42.9)	2 (14.3)
Total	**282 (100)**	56 (19.9)	40 (14.2)	3 (1.1)	3 (1.1)	10 (3.5)	168 (59.6)	58 (20.6)

Results revealed that *K. pneumoniae* (40 isolates) was the dominant (71.4 %) among all (56 isolates) other species of *Klebsiella*. This result was in agreement with the report documented by Hansen (1997) who found that this species was the most frequently occurring among other species, when its account for 86 % of *Klebsiella* species isolated clinically. Also agreed with results reported by Paterson *et al*. (2004), Kusum *et al.* (2004) and Kucukates (2005). Moreover, Najmadeen (2006) indicated that *K. pneumoniae* accounted

81.24 % of *Klebsiella* isolated clinically in seven hospitals in Sulaimaniyah \Iraq. Horan *et al*. (1988) referred that nosocomial *Klebsiella* infections are caused mainly by *K. pneumoniae*, the medically most important species of the genus.

Podschun *et al*. (2001) reported that the percentage of *K. oxytoca* was increased in Europe and reached to 26 % of the isolates of this genus, This bacterium was isolated from the central care unit and causing various diseases, as a result of the development of resistance to antibiotics and possession of B-lactamase enzymes type TEM-59.

Results illustrated in table (3-3) show that *K. pneumoniae* was isolated more frequently from urine (21 isolates, 52.5 %) and less from wound, ear swab, Kitchen and bathrooms (1 isolate, 2.5 %). While one isolate of *K. oxytoca* was isolated from urine, sputum and ward (1 isolate, 33.3 %). *K. ornitholytica* was isolated more frequently from urine (2 isolates, 66.7 %) and ward (1 isolate, 33.3 %). In the case of *K terrigena* was isolated more frequent in urine (3 isolates, 30 %) then in wound, burn and blood (2 isolates, 20 %) and less in sputum (1 isolate, 10 %).

Podschun and Ullmann (1994) in an epidemiological study reported that *K. pneumoniae* was dominant among all other *Klebsiella* spp. They isolated this bacterium from urine, blood, stool and environmental sources.

Results revealed also that *K. terrigena* considered the second highest percentage of the *Klebsiella* spp. in the urine, although, this species documented for the first time as environmental isolate which was isolated from water and soil. However, Goegele *et al*. (2007) declared the first case of *K. terrigena* infection dangerous in human that cause an inflammation of heart. Moreover, this table elucidated that *K. pneumoniae* were isolated in high percentage (52.5 %) from urine samples. These results were consistent with those of Petite *et al*. (1991) Lafta (2010), who found that the percentage of *K. pneumoniae* that isolated from urinary tract infection reached to 50 % of

Klebsiella spp., this confirms the fact that *K. pneumoniae* play important role in causing UTI.

Table (3-3): Distribution o f *Klebsiella* spp. among different clinical and environmental isolates.

Sample type	Isolates / Sample source	No. (%) of Samples	No. (%) Of *K. pneumoniae*	No. (%) of *K. oxytoca*	No. (%) of *K. ornitholytica*	No. (%) of *K. terrigena*
Clinical	Urine	**50 (17.7)**	21 (52.5)	1 (33.3)	2 (66.7)	3 (30)
	Wound	**68 (24)**	1 (2.5)	0 (0)	0 (0)	2 (20)
	Burn	**80 (28.4)**	2 (5)	0 (0)	0 (0)	2 (20)
	Sputum	**75 (26.6)**	2 (5)	1 (33.3)	0 (0)	1 (10)
	Ear swab	**10 (3.5)**	1 (2.5)	0 (0)	0 (0)	0 (0)
	Blood	**28 (9.9)**	9 (22.5)	0 (0)	0 (0)	2 (20)
Hospital Environment	Operation hall	**2 (0.71)**	2 (5)	0 (0)	0 (0)	0 (0)
	Wards	**4 (1.4)**	0 (0 %)	1 (33.3)	1 (33.3)	0 (0)
	Kitchen	**3 (1.1)**	1 (2.5)	0 (0)	0 (0)	0 (0)
	Bathrooms	**5 (1.8)**	1 (2.5)	0 (0)	0 (0)	0 (0)
Total		**282 (100)**	**40 (14.2)**	**3 (1.1)**	**3 (1.1)**	**10 (3.5)**

In the study of Al-Dhahri (2002) revealed that the percentage of *Klebsiella* spp. isolated from UTI was 17.2 %, while in a study conducted in Syria, the proportion of *Klebsiella* spp. isolated from the hospital in people

with inflammation of the UTI was 53.09 %. As Al-Hilfi (2009) explained that, the percentage of *Klebsiella* spp. was 2.1 % when isolated from the UTI. Mahesh *et al*. (2010) reported that highest cases were recorded by urine source and the lowest by wound source.

Results revealed that *K. pneumoniae* is the second of the most important causes of blood poisoning, the prevalence of *K. pneumoniae* in blood was 22.5 %, followed by *K. terrigena* 20 %, and no growth of *K. oxytoca* and *K. ornitholytica* was noticed. It was found that the death rate of *K. pneumoniae* infection was much higher than other causes and exceeded the rate of deaths caused by *Streptococcus pneumoniae.* Almost patients with *K. pneumoniae* infection suffering one of infection as pneumonia infections and intestine inflammatory and still is one of the most infections related with evolution of the bloodstream infections (Lin *et al.*, 2010). In another study conducted by Osman (2008) in Sudan, he found that infection rate of this species causes blood poisoning reached 16.2 % and considered as third causes of blood infection. These results pointed that blood infection with *K. pneumoniae* was more than infection with other *Klebsiella* spp., which may be due to the high virulence of *K. pneumoniae*, it has large capsule and resistant to antibiotics (Ghorashi *et al*., 2011) and play an important role in the resistance to the immune system's defenses (Evrard *et al.*, 2010).

3-4 Distribution of *K. pneumoniae* according to gender and ages of patients:

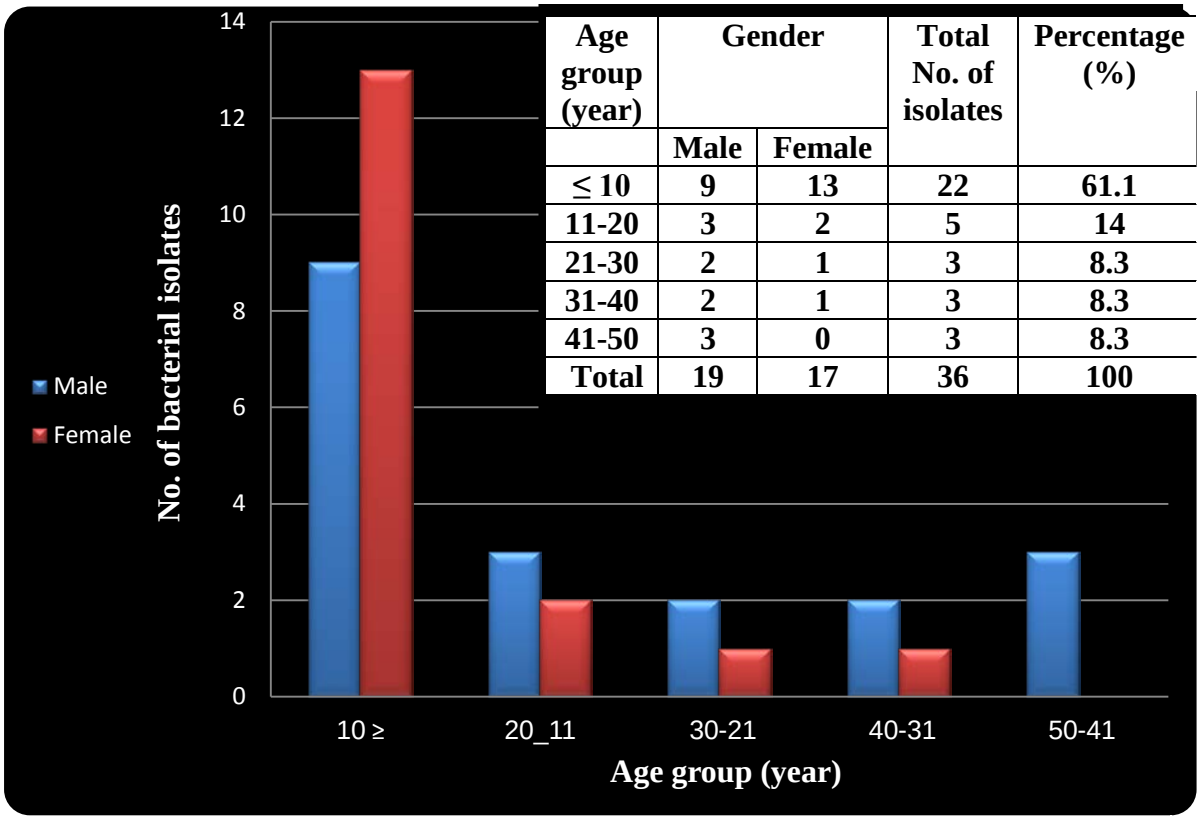
Out of 40 isolates of *K. pneumonia*, 36 were isolated from clinical samples (patients) as mentioned above (table 3-2).

Results (figure 3-4) showed that regarding gender of patients, males had a tendency to get infection more than females when 19 (52.8 %) of patients were males and 17 (47.2 %) females. These results referred that males have more chance of *K. pneumoniae* infection than female. Similarly, Fang *et al.* (2012) revealed that out of 33 patients, male was 19 cases while female 14

cases. In the study of Okada *et al.* (2010) they found that patients included in their study, were 54 males and 26 females. However, Khan *et al*. (2010) stated that female infection was higher (54.5 %) than male (45.6 %). In addition, Magliano *et al.* (2012) viewed that 209 cases were males and 995 cases females, patients were mainly of the group ≥ 60 years. They referred that male patients above the age of 60 years were subjected to greater frequency of *K. pneumoniae* infection.

Results in figure (3-4) illustrated that 22 (61.1 %) and 5 (14 %) of the patients in Iraq were of the age ≤ 10 and 11-20 years, respectively. While 3 (8.3 %) patients were belong to the 21-30, 31-40 and 41-50 year age groups. The high percentage of infection recorded in the age group ≤ 10, which may be due to certain cause such as week immunity and/or collected a large number of samples in age group ≤ 10 comparative with the samples of other age groups. The prevalence of infection with *K. pneumoniae* in this study showed similar results as in developing countries which peaks at most in the middle age (less than 20 years old) group (Struve *et al*., 2005). Such results are similar to those of Khan *et al.* (2010) who found that patients were mainly of the group less than 10 years.

In addition, results showed that highest cases were recorded with age group ≤ 10 year by urine source (21 case) and the lowest (1 case) was recorded by ear and wound swabs.



Age group (year)	Gender		Total No. of isolates	Percentage (%)
	Male	Female		
≤ 10	9	13	22	61.1
11-20	3	2	5	14
21-30	2	1	3	8.3
31-40	2	1	3	8.3
41-50	3	0	3	8.3
Total	19	17	36	100

Figure (3-4): Distribution of *K. pneumoniae* isolates according to gender and age groups of patients

3-5 Antibiotic resistance of *K. pneumoniae* isolates:

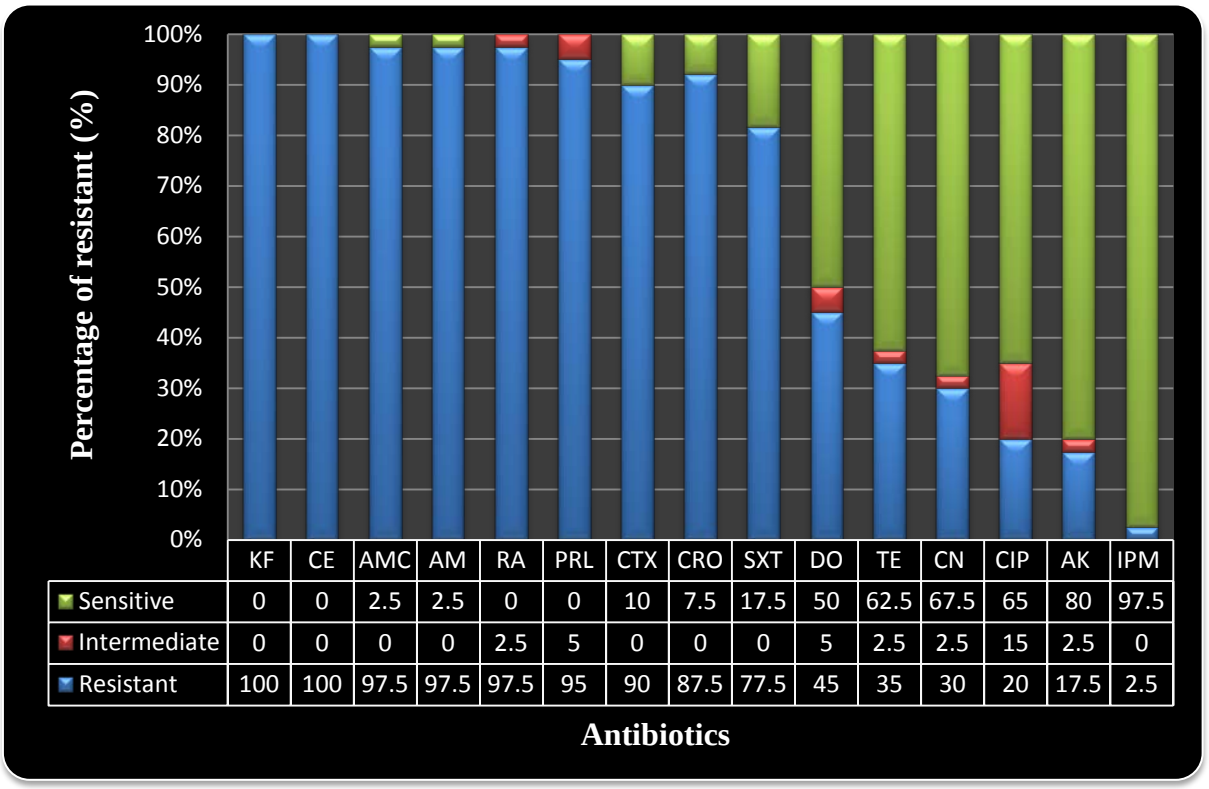
Susceptibility of the 40 isolates of *K. pneumoniae* was examined towards 15 different antibiotics using disc diffusion method recommended by the National Committee for Clinical Laboratory Standards (NCCLs) guideline.

Results presented in figure (3-5) show that *K. pneumoniae* isolates were resistant to the antibiotics of cephalosporin group included cephradine, cephalothin, cefotaxime and ceftriaxone. All isolates (100 %) were resistant to cephradine and cephalothin. While 90.5 % and 87.5 % of isolates were resistant to cefotaxime and ceftriaxone, respectively. This result was in agreement with Amin *et al.* (2009) in Pakistan who noticed that the percentage of resistance to cefotaxime and ceftriaxone were 82.5 % and 85 %, respectively. However, Nasehi *et al.* (2010) reported that *K. pneumoniae* isolates were 27 % resistant to ceftriaxone and this may be related to

possessing of β- lactamase enzymes (cephalosporinase) which are able to the inactivate cephalosprins through cleavage β-lactam ring of the drug.

While resistance to the penicillin group included ampicillin, amoxicillin-clvulanic acid and piperacillin which were 97.5 % for ampicillin and amoxicillin + clavulanic acid and 95 % for piperacillin. Resistance of *K. pneumoniae* to the cephalosporin group and penicillin group may be related to possessing of β-lactamase enzymes (cephalosporinase and penicillinase) which are able to the inactivate pencillins and cephalosprins through cleavage β-lactam ring of the drug (Stock and Wiedmenn, 2001; Pagani *et al.*, 2006). β-lactamase are chromosomally or plasmid encoded and most of these plasmids are self transmissible plasmids (Prescott *et al.*, 1999). Lim *et al.* (2009) reported that 10 % of *K. pneumoniae* was resistant to the Amoxicillin + clavulanic acid. However, Amin *et al*. (2009) found that 10 % of isolates were resistant to the Amoxicillin + clavulanic acid and 12 % were resistant to the ciprofloxacin. These results were consistent with those of Hostackai and Klokocnkovai (2001) and Al-Mulla (2003) who found that all isolates (100 %) were resistant to ampicillin. The ampicillin is one of the common antibiotics used for the treatment of UTI (Rice *et al.*, 1996), so the wide spread of resistance in Iraqi isolates represent a major problem in treatment of the infection. Thomson and Amyes (1993) showed that 88.2 % of *Klebsiella* isolated from infections in Scotland were resistant to ampicillin. Also resistance to penicillins and this is due to the lack of penicillin binding proteins (PBPs) or the microorganisms could change their permeability to the drug (Bermudes *et al.*, 1999).

The results agreed with results of Ahmad (2000) who mentioned that most of *K. pneumoniae* isolates were resistant to piperacillin.



	KF	CE	AMC	AM	RA	PRL	CTX	CRO	SXT	DO	TE	CN	CIP	AK	IPM
Sensitive	0	0	2.5	2.5	0	0	10	7.5	17.5	50	62.5	67.5	65	80	97.5
Intermediate	0	0	0	0	2.5	5	0	0	0	5	2.5	2.5	15	2.5	0
Resistant	100	100	97.5	97.5	97.5	95	90	87.5	77.5	45	35	30	20	17.5	2.5

Figure (3-5) Antibiotic resistant of *K. pneumoniae* isolates

Cephalothin (KF) (30 µg), Cephradine (CE) (30 µg), Amoxicillin + Clavulanic acid (AMC) (20/10 µg), Ampicillin (AM) (25 µg), Rifampin (RA) (30 µg), Piperacillin (PRL) (100 µg), Cefotaxime (CTX) (30 µg), Ceftriaxone (CRO) (30 µg), Sulfamethoxazole + Trimethoprime (SXT) (1.25/23.75 µg), Doxycycline (DO) (30 µg), Tetracyclin (TE) (30 µg), Gentamycin (CN) (30 µg), Ciprofloxacin (CIP) (5 µg), Amikacin (AK) (30 µg) and Imipenem (IPM) (10 µg).

Results (figure 3-5) showed also that the resistance of *K. pneumoniae* isolates to the aminoglycosids group included amikacin and gentamicin were 17.5 % and 30 %, respectively. Similarly, Nasehi *et al.* (2010) found that resistance to amikacin was 17.5 %. While results for Akindele and Rotilu (2000) was rather different from the data. They reported that about 79 % of *Klebsiella* isolates were resistance to gentamicin. In addition, Reish *et al.* (1993) and Roilides *et al.* (2000) found that all the *Klebsiella* strains in their studies were resistant to gentamicin. In this regard, Feizabadi *et al.* (2007)

found that *Klebsiella* isolates producing extended spectrum β-lactamase enzymes were resistant to aminoglycosids.

There are three mechanisms for resistance of *K. pneumoniae* to amikacin: first: modification of antibiotic by modification enzymes, second: chromosomal mutation in target protein encoded genes, third: decreases permeability of bacteria to the antibiotic (Levinson and Jawetz, 2000).

While resistance to tetracycline group, which included doxycyclin and tetracycline were 45 % and 35 %, respectively. Marranzano *et al.* (1996) declared that 90 % of the *K. pneumoniae* isolates were sensitive to the tetracycline.

Resistance of *Klebsiella* isolates to ciprofloxacin which belongs to the quinolones group was 20 %. Fluit *et al*. (2001) reported that the resistance to quinolones is releated to change in antibiotic-enzyme (GyrA) binding site.

In the other hand, resistance of this bacterium isolates to rifampin, which classified from rifamycin group was recorded 97.5 %.

While resistance to Sulfonamide group included Trimethoprim + Sulphamethoxazole) was 77.5 %. This combination blocks two consecutive steps in bacterial biosynthesis of essential nucleic acids and proteins and is usually bactericidal (Fluit *et al*., 2001) .

Finally, it was found that *K. pneumoniae* susceptibile to imipenem which belongs to the group of Carbapenems, 97.5 % of this bacterium isolates were sensitive to this antibiotic (figure 3-5). Livemore and Brown (2001) reported that carbapenem (imipenem) antibiotics have strong activity against ESBLs from *Klebsiella* spp. Feizabadi *et al*. (2007) observed that *K. pneumoniae* isolated from respiratory tract was sensitive against imipenem. In another study *K. pneumoniae* isolated from different clinical specimens was susceptible to imipenem (Feizabadi *et al*., 2008). *K. pneumoniae* possess Carbapenemase enzymes that responsible for the Carbapenem resistant (Deshpande *et al*., 2010). The result of the susceptibility of *K. pneumoniae* to the imipenem

agreed with results of Lim *et al.* (2009) and Nasehi *et al*. (2010) who revealed that only one isolate was resistant to the imipenem. According to this result it can considered that imipenem as best treatment against *K. pneumoniae.*

Results showed that clinical isolates were multidrug resistance and recorded higher resistance than environmental isolates. This multidrug resistance led to antibiotic ineffectiveness against bacteria responsible for life threatening diseases. The multidrug resistance of bacteria to several antibiotics could be due to certain mutations that occur as a result of overuse and misuse of antibiotics in hospitals, especially with persons receiving broad spectrum or multiple antibiotics (Tullus *et al*., 1988). So that the antibiotic use must be regulated in the hospitals to prevent these problems. In addition to mutation, plasmids that carrying resistance genes play important role in spreading the multidrug resistance between bacteria (Stock and Wiedemann, 2001).

3-6 Molecular detection of *K. pneumoniae* using PCR amplification:

3-6-1 Extraction of DNA from *Klebsiella* isolates:

Genomic DNA was extracted from all *K. pneumoniae* isolates (40) using genomic DNA minikit (2-2-9-1). Extraction was done according to the manufacturer's instructions of geneaid company after cultured on the Luria - Bertani broth. DNA was extracted to provide a PCR template for amplification. Results showed that the recorded range of DNA concentration was 47.4-123.8 ng/µl and the DNA purity was 1.6-2.0. The obtained quantities and purity of DNA are fair enough for amplification by PCR. Higher amounts of DNA template increase the risk of generating of Non-specific PCR products. Lower amounts of template reduce the accuracy of the amplification. A pure DNA preparations has expected of 1.8 which are based on the extinction coefficients of nucleic acids at 260 nm and 280 nm (Green and Sambrook, 2012).

Such results were also observed when the DNA samples analyzed by gel electrophoresis, in which DNA bands were detected indicating purified DNA samples as shown in figure (3-6).

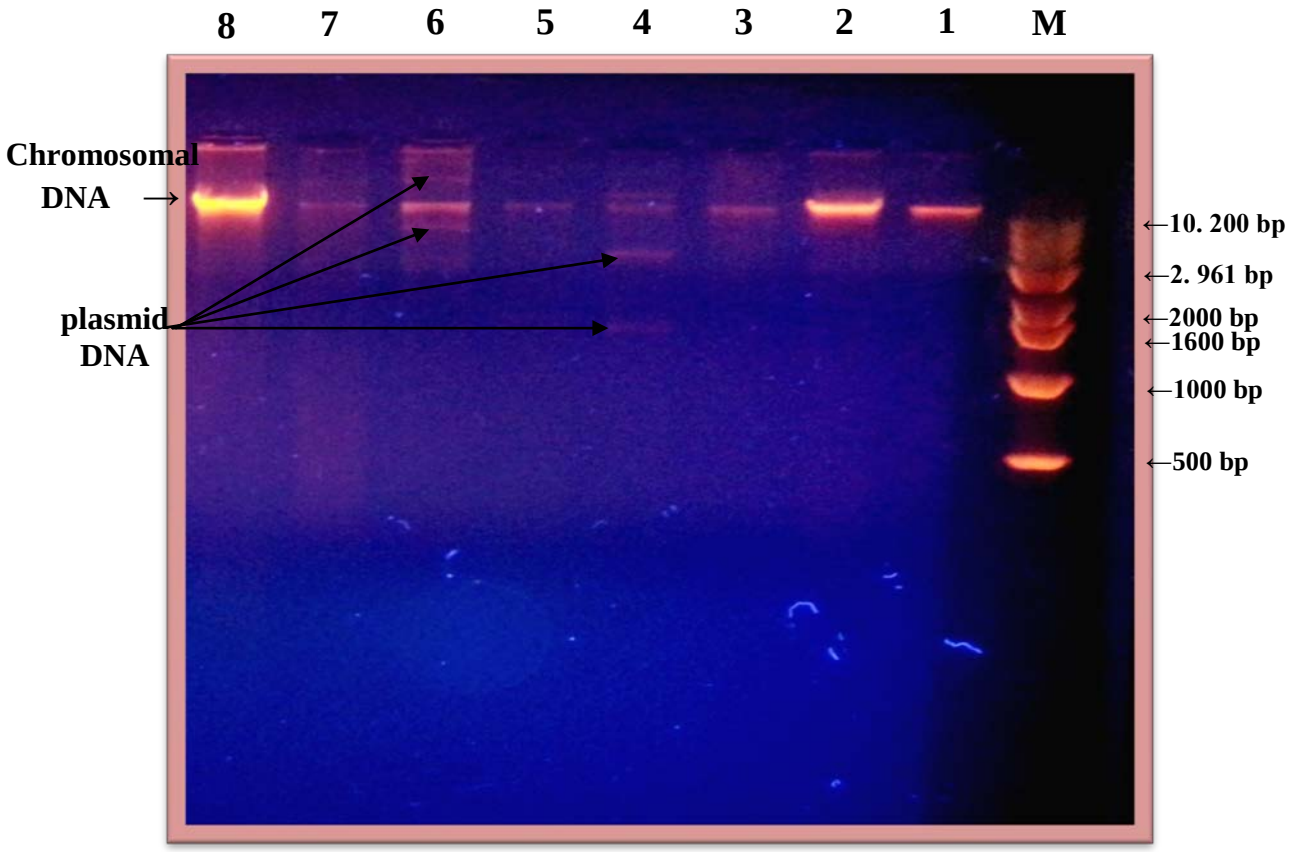


Figure (3-6) Gel electrophoresis for genomic DNA of *K. pneumoniae* isolates. Electrophoresis was performed on 1.5 % agarose gel and run with a 70 volt/35 mAmp current for 3 hrs. visualized under UV after staining with ethidium bromide. Lane **M** is a (1 Kb) ladder, Line: **1** –T121, **2** – T123, **3** – T120, **4** – T18, **5** – T73, **6** – T40, **7** – T88, **8** – T63.

3-6-2 Amplification of 16S rRNA gene:

All isolates (40) were subjected to molecular identification through PCR amplification of 16S rRNA using K 16S-F and K 16S-R primers which represents specific primers for the PCR amplification of *K. pneumonia* 16S rRNA. Results showed that the amplified fragments were about 130 bp in size as shown in figure (3-7), which is the same size obtained by Turton *et al.* (2010) when they used the same primer. All (40) isolates gave positive results (130 bp bands), and identified as *K pneumoniae*. Resuts of PCR amplification proved that all isolates were *K. pneumoniae,* and confirmed the previous results.

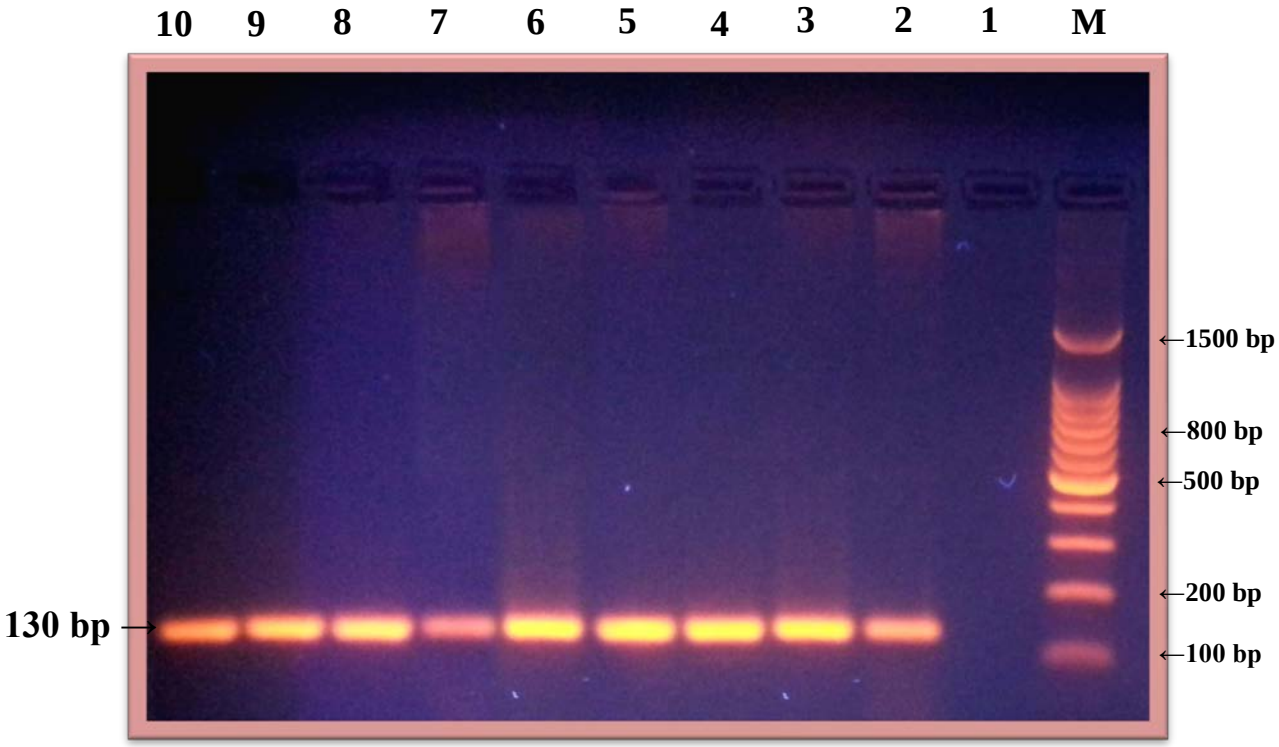


Figure (3-7): Gel electrophoresis for amplification of 16S rRNA gene of *K. pneumoniae*. Electrophoresis was performed on 1.5 % agarose gel and run with a 70 volt/35 mAmp current for 2 hrs. Lane **M** is a (100 bp) ladder, Line: **1** – DNA-free negative control, **2** – T1 (clinical isolate), **3** – T11 (clinical isolate), **4** – T70 (clinical isolate), **5** – T78 (environmental isolate), **6** – T26 (environmental isolate), **7** – T91 (environmental isolate), **8** – T5 (clinical isolate), **9** – T120 (clinical isolate), **10** – T52 (clinical isolate).

3-6-3 Amplification of *magA* and *k2A* genes:

K. pneumoniae serotype K1 was diagnosed with PCR by amplified *magA* gene using a specific primer pair (magA-F and magA-R). Forty *K. pneumoniae* isolates were subjected to amplification using this primer, 23 isolate (57.5%) were positive for *mag*A gene. These results demonstrated that these pathogenic isolates (23) have a K1 serotype. Figure (3-8) showed that PCR product was roughly 1283 bp in size, which is the same size obtained by Turton *et al.* (2008) when they used the same primer.

Chuang *et al.* (2006) demonstrated that *magA* is located within an operon that is specific to serotype K1 *cps* gene clusters regardless of their sources. Similarly, Struve *et al.* (2005) investigated 495 worldwide isolates and

Yeh *et al.* (2006) screened 134 *K. pneumoniae* isolates, they found that *magA* is restricted to the gene cluster of *K. pneumoniae* capsule serotype K1 and that all the Non-K1 strains were *magA* negative. Thus, PCR analysis for *magA* is a rapid and accurate method to molecular diagnosis of *K. pneumoniae* serotype K1 isolates.

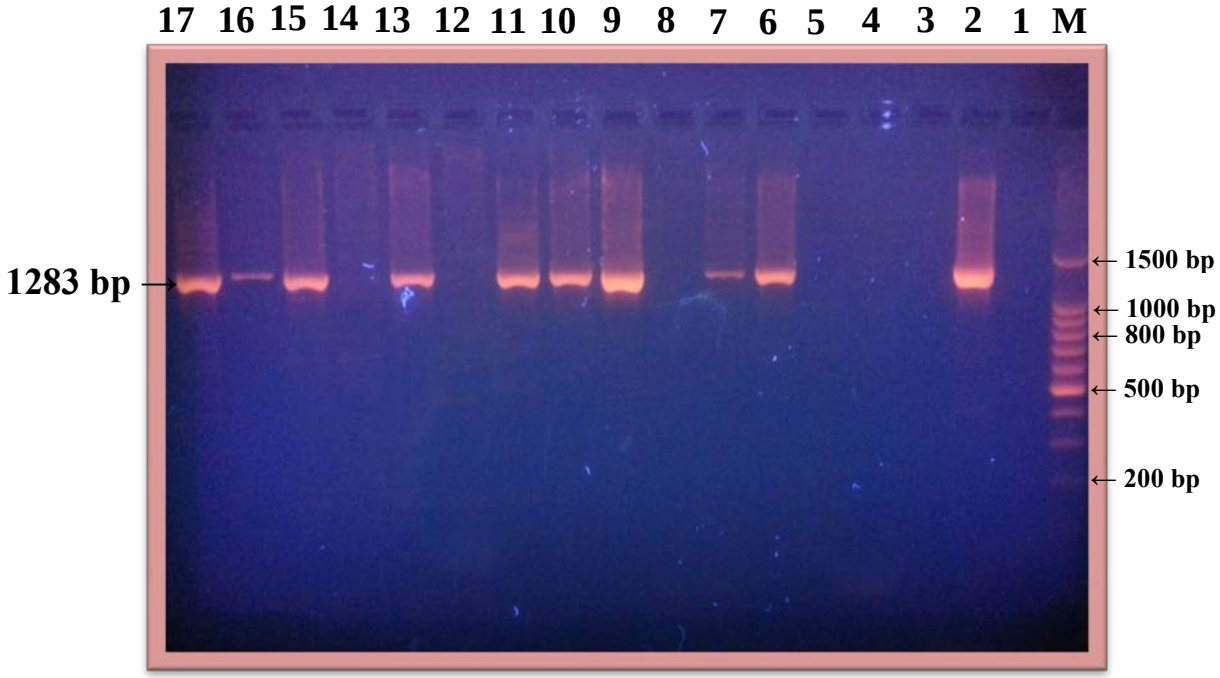


Figure (3-8): Gel electrophoresis for amplification of *magA* gene using specific primers of *K. pneumoniae* serotype K1. Electrophoresis was performed on 1.5 % agarose gel and run with a 70 volt/35 mAmp current for 2 hrs. Lane **M** is a (100 bp) ladder, Line: **1** – DNA-free negative control, **2** – T1 (clinical isolate), **3** – T11 (clinical isolate), **4** – T70 (clinical isolate), **5** – T26 (environmental isolate), **6** – T5 (clinical isolate), **7** – T78 (environmental isolate), **8** – T91 (environmental isolate), **9** – T93 (clinical isolate), **10** – T92 (clinical isolate), **11** – T105 (clinical isolate), **12** – T98 (clinical isolate), **13** – T28 (clinical isolate), **14** – T38 (clinical isolate), **15** – T122 (clinical isolate), **16** – T108 (environmental isolate), 17 – T123 (clinical isolate).

K. pneumoniae serotype K2 was diagnosed with PCR by using a primer pair (k2A-F and k2A-R) specific for amplification *k2A* gene. Forty *K. pneumoniae* isolates were subjected to amplification using this primer. Results in figure (3-9) illustrated that PCR product was about 543 bp in size, which is the same size obtained by Rivero *et al.* (2010). The k_2A fragment of 543 bp

was detected in 11 (27.5 %) of *K. pneumoniae* isolates. These results referred that these (pathogenic) isolates have a K2 serotype. PCR analysis for the open reading frame (ORF)–9 region *k2A* of *K. pneumoniae* serotype K2, which corresponds to the *magA* region in the *cps* gene clusters of K1 isolates, could be used as a highly specific molecular diagnostic method to identify the *K. pneumoniae* capsule K2 serotype (Chuang, 2007).

According to the results of molecular serotypes of K1 (23 isolates) and K2 (11 isolates) the rest isolates (6 isolates that were negative to amplification of *magA* and *k2A*) considered as Non-K1/K2. Non-K1/K2 strain is a less virulent and cross-react with K1 and K2 in serotyping but did not yield *magA* and *k2A* specific amplicon. The lack of such cross-reactions may be an advantage of developed assay when compared with a classical serotyping (Cheng *et al.*, 2013).

The results were in agreement with Fung *et al*. (2002) who reported that the prevalence of serotype K1 and serotype K2 was 52.3 % and 22.7 % respectively, and consistent with Victor *et al*. (2007) who reported that *K. pneumoniae* serotype K1 is dominant on the other serotypes in the different infections. Moreover, These results agreed with results of Doud *et al*. (2009) who referred that K1 and K2 serotype of *K. pneumoniae* is the most common type of isolates. However, Chuang *et al*. (2006) reported that prevalence of K1 and K2 was 83.3% and 2.4% respectively.

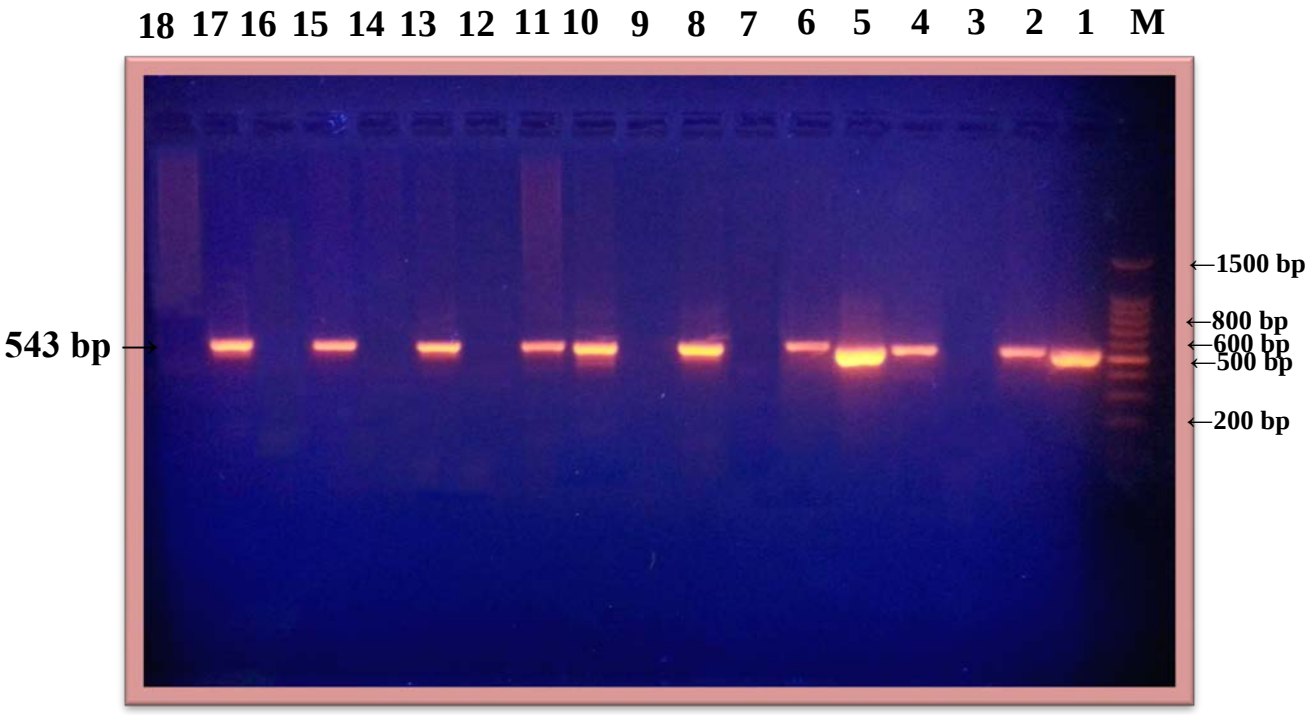


Figure (3-9): Gel electrophoresis for amplification of *k2A* gene using specific primers of *K. pneumoniae* serotype K2. Electrophoresis was performed on 1.5 % agarose gel and run with a 70 volt/35 mAmp current for 2 hrs. Lane **M** is a (100 bp) ladder, **Line**: **1** – T13 (clinical isolate K2), **2** – T11 (clinical isolate K2), **3** – T1 (clinical isolate K1), **4** – T40 (clinical isolate K2), **5** – T26 (environmental isolate K2), **6** – T120 (clinical isolate K2), **7** – T70 (Non- K1/K2 clinical isolate), **8** – T121 (clinical isolate K2), **9** – DNA-free negative control, **10** – T33 (clinical isolate K2), **11** – T57 (clinical isolate K2), **12** – T37 (Non-K1/K2 clinical isolate), **13** – T73 (clinical isolate K2), **14** – T81 (Non K1/K2 clinical isolate K1), **15** – T80 (clinical isolate K2), **16** – T108 (environmental isolate K1), **17** – T98 (clinical isolate K2), **18** – T38 (Non-K1/K2 clinical isolate).

In addition the results were disagreement with results elucidated by Lin *et al.* (2010) who noticed that serotypes K1, K2 and Non-K1/K2 accounted 14.3 % (7/49), 38.8 % (19/49) and 46.9 % (23/49) of all *K. pneumoniae* isolates, respectively. Yeh *et al.* (2007) reported that the most common and predominant serotype was K1 46.6% (34/73), followed by Non-K1/K2 32.9 % (24/73), and K2 20.5% (15/73), respectively. They referred also that the

serotype K1 and K2 isolates were significantly more prevalent than those that were neither K1 nor K2 (Non-K1/K2) (49/73 versus 24/73).

3-7 Amplification of *rmpA* and *kfu* genes:

3-7-1 prevalence of the virulence genes *rmpA* and *kfu* in *K. pneumoniae* isolates:

Two other virulence factors were studied which include the extracapsular polysaccharide synthesis regulator gene (*rmpA*) related to the hypermucoviscosity phenotype (Yu *et al.*, 2007) and the ferric iron uptake system gene (*kfu*) required for the metabolism of iron to sustained growth in the host (Ma *et al.*, 2005; Dora *et al.*, 2013).

In the present study *rmpA* and *kfu* genes were amplified with PCR by using a primer pair (rmpA-F and rmpA-R) and (kfu-F and kfu-R) specific for amplification *rmpA* and *kfu* gene, respectively. The amplified DNA with the rmpA and kfu primers resulting in a PCR product with a band of a molecular size of about 536 and 797 bp, respectively as shown in figure (3-10) and (3-11).

Forty *K. pneumoniae* isolates were subjected to amplification using these primers, 11 isolate (27.5 %) and 30 isolates (75 %) were positive for *rmp*A and *kfu* genes, respectively. Detection of these genes may indicate the virulence potential of the isolates (Aher *et al.*, 2012).

Suescun *et al.* (2006) documented that the *rmpA* was located on a 180-Kb virulence plasmid. This plasmid is a multi-copy plasmid and responsible for expressing the mucoid phenotype of *K. pneumoniae*. It was found that *rmpA* carrying plasmid of the *K. pneumoniae* isolates, the plasmid contained also many virulence-associated genes (Yeh *et al.*, 2007).

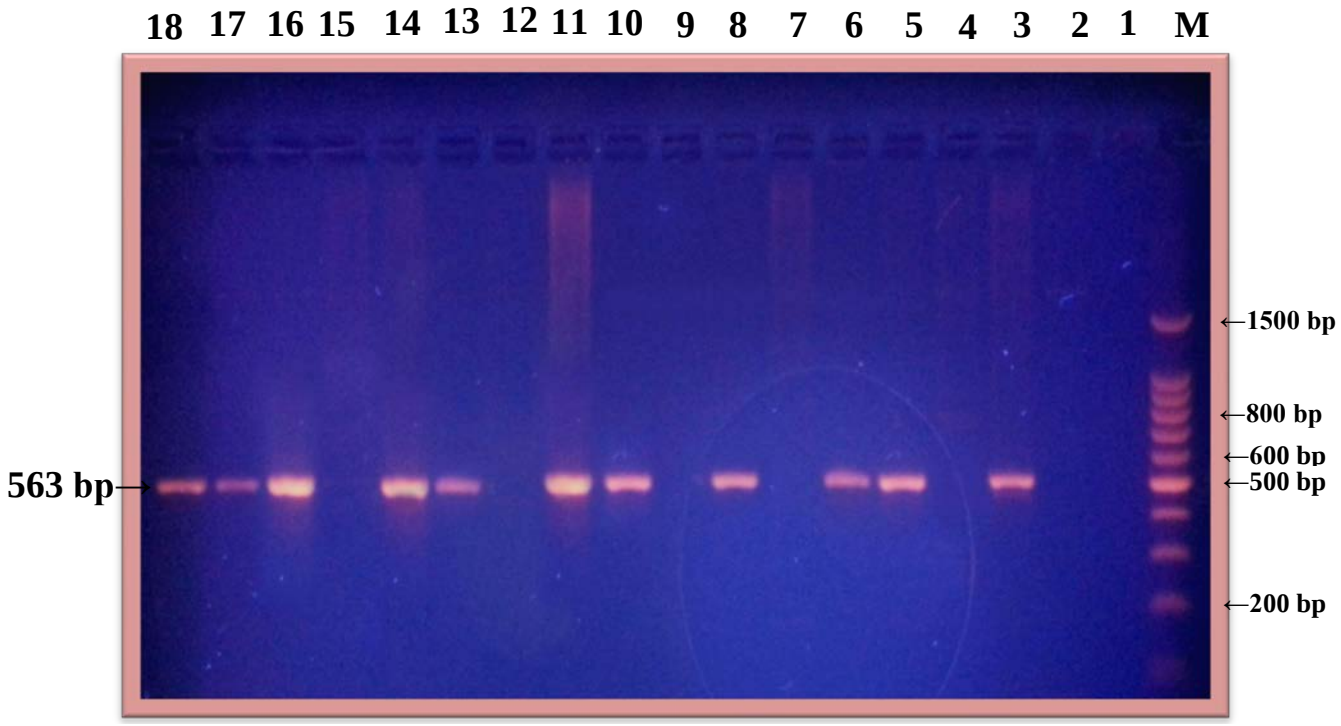


Figure (3-10) Gel electrophoresis for amplification of *rmpA* gene using specific primers for *K. pneumoniae*. Electrophoresis was performed on 1.5 % agarose gel and run with a 70 volt/35 mAmp current for 2 hrs. Lane **M** is a (100 bp) ladder, **Line**: **1** – DNA-free negative control, **2** – T11 (clinical isolate), **3** – T1 (clinical isolate), **4** – T21 (clinical isolate), **5** – T5 (clinical isolate), **6** – T13 (clinical isolate), **7** – T78 (environmental isolate), **8** – T18 (clinical isolate), **9** – T91 (environmental isolate), **10** – T26 (environmental isolate), **11** – T33 (clinical isolate), **12** – T108 (environmental isolate), **13** – T40 (clinical isolate), **14** – T63 (clinical isolate), **15** – T123 (clinical isolate), **16** – T73 (clinical isolate), 17 – T98 (clinical isolate), **18** – T105 (clinical isolate).

Yu *et al.* (2006) revealed that prevelance of *rmpA* gene was in 72 from 151 isolates (48 %). Aher *et al.* (2012) reported that out of total 8 isolates, 2 isolates (25 %) were positive for *kfu* gene. Yu *et al.* (2008) studied the prevalence of various virulence attributed in the causative isolates, they detect *rmpA* gene in 96 % of the isolates and *kfu* gene in 66 % of the isolates. They found also that among 45 liver abscess isolates with positive hypermucoviscosity phenotype, the prevalence of *rmpA* gene was 97.8 %.

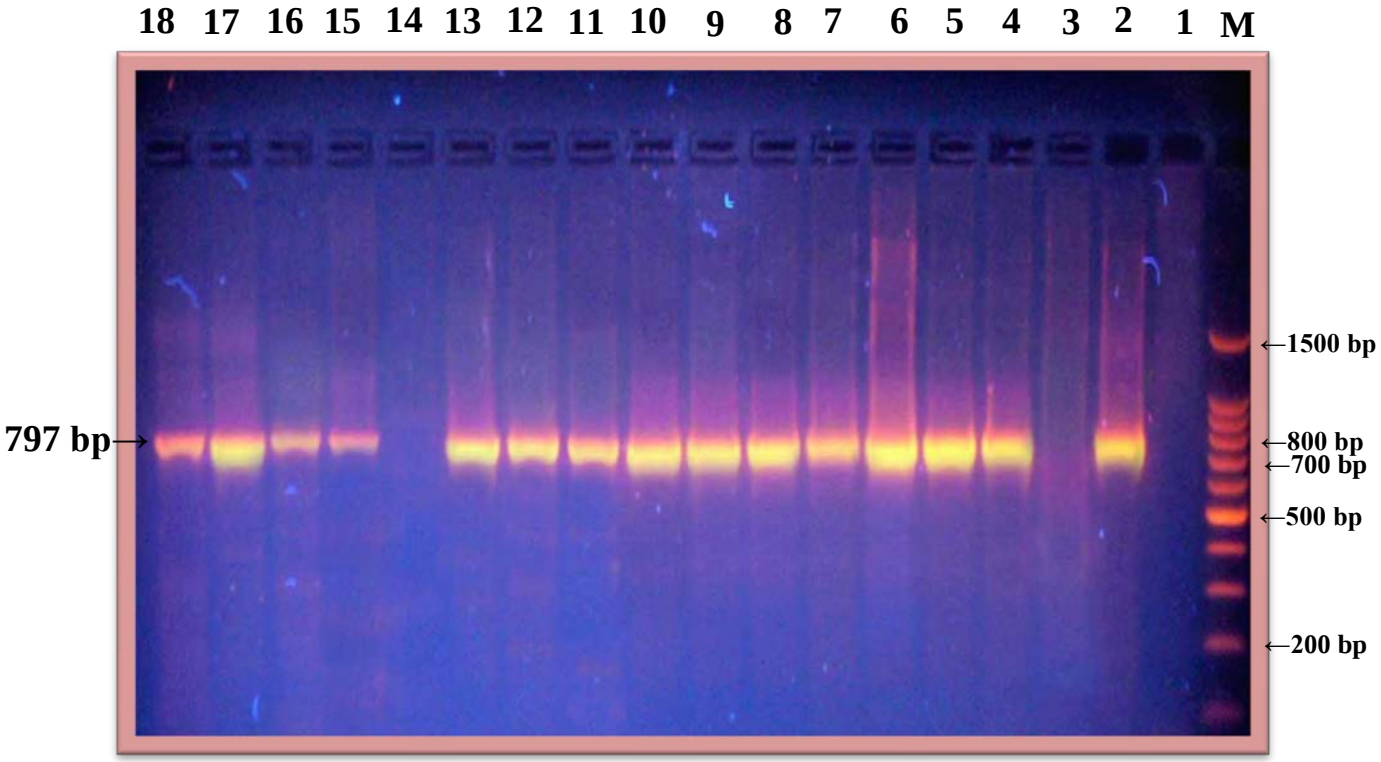


Figure (3-11) Gel electrophoresis for amplification of *kfu* gene using specific primers for *K. pneumoniae*. Electrophoresis was performed on 1.5 % agarose gel and run with a 70 volt/35 mAmp current for 2 hrs. Lane **M** is a (100 bp) ladder, **Line**: **1** – DNA-free negative control, **2** – T1 (clinical isolate), **3** – T13 (clinical isolate), **4** – T5 (clinical isolate), **5** – T8 (clinical isolate), **6** – T11 (clinical isolate), **7** – T18 (clinical isolate), **8** – T26 (environmental isolate), **9** – T40 (clinical isolate), **10** – T33 (clinical isolate), **11** – T63 (clinical isolate), **12** – T78 (environmental isolate), **13** – T80 (clinical isolate), **14** – T93 (clinical isolate), **15** – T105 (clinical isolate), **16** – T108 (environmental isolate), 17 – T120 (clinical isolate), **18** – T122 (clinical isolate).

The results of the present study were in agreement with reports of Fang *et al.* (2004), Ma *et al.* (2005) and Yu *et al.* (2006) who designed these oligos complementary to each end specifically amplified the product size of 797 bp of the sequence of the *kfu* gene. However, Yu *et al.* (2006) detected the prevalence of virulence associated gene (*kfu* gene) at the rate of 35 %.

The *rmpA* and *magA* are the most frequently occurring ones, the latter being associated with *K. pneumoniae* hypermucoviscosity and high virulence

(Kawai, 2006; Chang *et al*., 2013). The molecular detection of these genes provides an avenue for early diagnosis of the infection from susceptible hosts. In view of all the findings, further investigation is needed to define the role of other host and pathogen factors, which may assist in the progression of disease at the physiological and molecular levels (Aher *et al*., 2012).

3-7-2 Prevalence of *rmpA* and *kfu* within different serotypes of *K. pneumoniae*:

Table (3-4) illustrated the prevalence of *rmpA* and *kfu* genes and their association with capsular serotypes (K1, K2, and Non-K1/K2) of *K. pneumoniae* isolates. In each serotype group, there was a difference in the prevalence of *rmpA* and *kfu* genes between isolates. Results (table 3-4) showed that distribution of *rmpA* gene in the serotype K1, serotype K2 and Non-K1/K2 was 5 (21.7 %), 5 (45.5 %) and 1 (16.7 %), respectively while the distribution of *kfu* gene was 21 (91 %), 8 (72.7 %) and 1 (16.7 %), respectively.

Table (3-4): Prevalence of *rmpA* and *kfu* genes within different serotypes of *K. pneumoniae* isolates

No.	Virulane factors	K1 Serotypes	K2 Serotypes	Non-K1/K2 Serotypes	Total
		23 isolates	11 isolates	6 isolates	
1	*rmpA*	5 (21.7 %)	5 (45.5 %)	1 (16.7 %)	11
2	*kfu*	21 (91 %)	8 (72.7 %)	1 (16.7 %)	30

Yu *et al*. (2008) demonstrated that distribution of *rmpA* gene within serotype K1, serotype K2 and Non-K1/K2 were 100 %, 100 % and 86 %, respectively while *kfu* gene were 100 %, 0 % and 50 %, respectively. Yeh *et al*. (2007) reported that *rmpA* plays a minor role in virulence with Non-K1/K2 isolates while plays a major factor with the serotype K1 or K2 isolates.

Moreover, serotype K1 or K2, rather than *magA* and *rmpA*, correlated best with the virulence of *K. pneumoniae* isolates.

Abdul Razzaq *et al.* (2013) referred that although, previous studies on *rmpA* gene were restricted with serotype K2 strains, *rmpA* gene also exists in serotype other than K2. They found that 21 isolates were positive for this gene where 16 positive found among K2 serotypes results, 4 in Non-K1/K2 isolates and only one in K1 serotypes isolates. They suggested that *rmpA* gene was more prevalent in K2 than K1 and in Non-K1/K2 isolates; this will enhance the severity of *K. pneumoniae* isolates. Also Fung *et al*. (2011) mentioned that the *rmpA* gene is present in serotype K1 and serotype K2. Yeh *et al.* (2007) revealed that all isolates (34) of serotype K1, all isolates (15) of serotype K2 and 66.7 % (16/24) of Non-K1/K2 isolates carried *rmpA* gene. This referred that *rmpA* exists in serotypes other than K2. Report by Aher *et al.* (2012) demonstrated that the *rmpA*-negative isolates are less phagocytosis resistant and/or less virulent than their *rmpA* positive counter parts of the same serotype. Yeh *et al.* (2007) reported that with an almost 90 % prevalence rate of *rmpA* in liver abscess strains, it was not surprising that all of K1 or K2 isolates and more than half of the Non-K1/K2 isolates carried this gene.

The diverse occurrence and distribution of *rmpA* and *kfu* as a virulence factors which associated with different capsule K serotypes in *K. pneumoniae* might reflect the seroepidemiology of the organisms that caused the infection (Yu *et al.*, 2006).

Ma *et al*. (2005) reported that the *kfu* operon has been documented to be associated with the development of abscesses in the livers and brains of mice. Strains possessing the *kfu* and *magA* genes were strongly associated with human infections. Moreover, they identified a 20-Kb chromosomal *kfu* region in *K. pneumoniae*, the presence of a *kfu* operon might principally or secondarily modulate virulence *in vivo* and so provide a strong competitive advantage to those strains that harbor it. It is present in most of the genomes of

the tissue-invasive *K. pneumoniae* isolates and absent from most of the noninvasive strains.

Prevalence of 16S rRNA, *magA*, *k2A*, *rmpA* and *kfu* genes within different serotypes of *K. pneumoniae* isolates were showed in appendix (6).

3-8 Evaluation of a multiplex PCR for amplification of *magA*, *k2A*, 16S rRNA and *rmpA* genes of *K. pneumoniae*:

The multiplex PCR was designed by using a primer pairs magA-F and magA-R, k2A-F and k2A-R, 16S rRNA-F and 16S rRNA-R, rmpA-F and rmpA-R specific for amplification of *magA*, *k2A*, 16S rRNA and *rmpA* genes, respectively in one reaction. Figure (3-12) illustrated that *K. pneumoniae* serotype K1 isolates show positive results (PCR products) with *magA* (1283 bp) and 16S rRNA (130 bp) genes *rmpA* (536 bp) gene. *K. pneumoniae* serotype K2 show positive results with *k2A* (543 bp) and 16S rRNA (130 bp) genes, while, *K. pneumoniae* serotype Non-K1/K2 show positive results with 16S rRNA (130 bp) gene only.

Results showed also that a total of 40 isolates of *K. pneumoniae*, 23 isolates (57.5 %) with K1 capsular serotype (*magA*), 11 isolates (27.5 %) with K2 serotype (*k2A*) and 6 isolates (15 %) with Non-K1/K2 serotype. All the isolates gave a clear band with a molecular size 130 bp. and the *rmpA* gene was amplified from 11 isolates (27.5 %) of *K. pneumoniae*.

Results of amplification of virulence genes using multiplex PCR (figure 3-12) confirmed the previous results obtained by using conventional PCR, with results showing 100 % concordance.

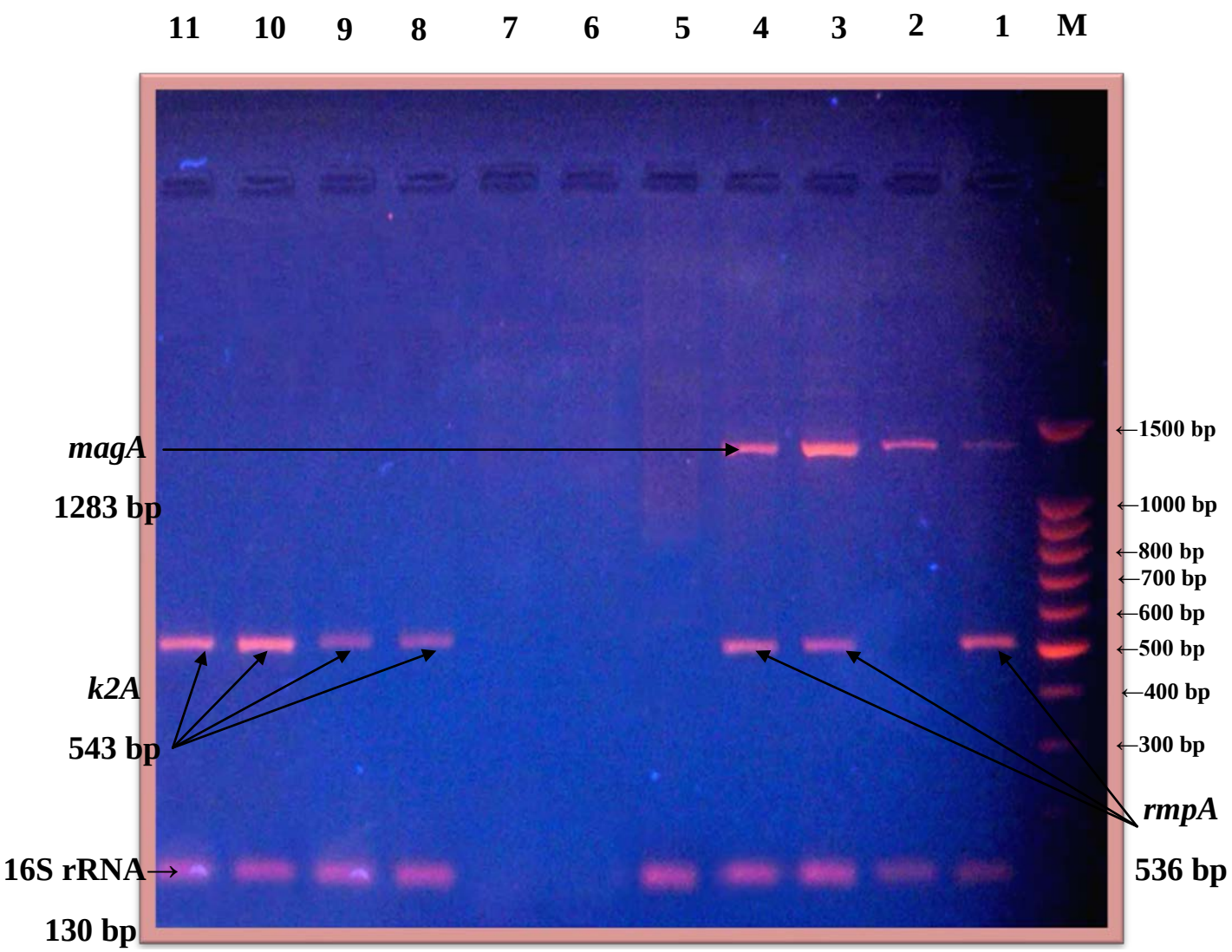


Figure (3-12) Gel electrophoresis for amplification of *mag*A, *k2A*, 16S rRNA and *rmp*A genes of *K. pneumoniae* using multiplex PCR. Electrophoresis was performed on 2 % agarose gel and run with a 70 volt/35 mAmp current for 2 hrs. Lane M is a (100 bp) ladder, Line: **1** – T1(clinical isolate K1), **2** – T8 (clinical isolate K1), **3** – T18 (clinical isolate K1), **4** – T105 (clinical isolate K1), **5** – T91 (environmental isolate Non-K1/K2), **6** – DNA-free negative control, **7** – DNA-free negative control, **8** – T57 (clinical isolate K2), **9** – T80 (clinical isolate K2), **10** – T120 (clinical isolate K2). **11**– T121(clinical isolate K2).

A multiplex PCR can be used to detects capsular types K1, K2 and Non-K1/K2, those are most associated with invasive disease or pathogenicity (Turton *et al.*, 2010), *rmpA* (virulence factor) and 16S rRNA (facilitating identification of the organism). Gierczynski *et al.* (2007) showed that result of

multiplex PCR assay for K1 and K2 was judged as a positive when two bands were present one specific for the 16S rRNA gene and the other specific for the *magA* or *k2A*, respectively. The presence of the 16S rRNA gene amplicon alone indicted the tested DNA sample contained neither *magA* nor *k2A*. Gierczynski *et al.* also revealed that the assay result was valid but negative for *K. pneumoniae* K1 and K2 geno-serotypes. Moreover, they reported that the multiplex PCR assay reduce workload of *K. pneumoniae* K1 and K2 capsular types' identification in routine diagnostic and epidemiological surveys.

The *rmpA* regulator of mucoid phenotype, which is associated with capsular types K1, K2, also detected in isolates of other capsular types (Non-K1/K2); 11 isolates of the 40 *K. pneumoniae* isolates screened were PCR-positive for *rmpA* gene. Turton *et al.* (2010) mentioned that there are many reports linking *rmpA* with virulence; *rmpA* was similarly associated with multiple capsular types (K1 and K2) and most commonly in the serotype K2.

Amplification of the 16S rRNA gene represents a highly accurate and versatile method for the identification of bacteria to the species level, even when the species in question is notoriously difficult to identify by biochemical methods (Song *et al.*, 2003). Turton *et al.* (2010) reported that these findings were confirmed with a number of clinical isolates, the former having previously been identified by biochemical testing. He demonstrated that multiplex PCR carried out on isolates of *Klebsiella* species by using primers for nine targets, 16S rRNA was used in this multiplex PCR, result showed that all the isolate gave a clear band with a molecular size 130 bp.

Turton *et al.* (2008) demonstrated that the multiplex PCR-based identification can be considered, a reliable, relatively rapid, cost-effective, easy application and repeatable and a powerful potential tool for the routine clinical identification of *Klebsiella* species.

Chapter Three *Results and Discussion*

CHAPTER FOUR

CONCLUSIONS

AND

RECOMMENDATION

4- Conclusions and Recommendations:

4-1 Conclusions:

- *K. pneumonia* isolates were isolated from clinical samples more than from environmental samples.
- *K. pneumoniae* isolated more frequently from urine sample and less from wound, ear swab, kitchen and bathrooms samples.
- *K. pneumoniae* isolated more frequently from males than females, in the age group (≤ 10) years.
- All isolates were considered as multidrug resistant, and the clinical isolates were resistant to antibiotics (except imipenem) more than the environmental isolates.
- The high ratio of resistance to B-lactam antibiotics in clinical isolates may be attributed to production of B-lactamases.
- Amplification of 16S rRNA gene of *K pneumoniae* confirmed the identification of this bacterium.
- *K. pneumoniae* serotype K1 was the most common found in clinical and environmental samples than K2 and Non-K1/K2 serotype.
- Molecular diagnosis of *K. pneumoniae* serotype K1 using *magA* gene is rapid and accurate while using *k2A* is a rapid and accurate method to molecular diagnosis of *K. pneumoniae* serotype K2.
- The distribution of *kfu* gene is more frequent than *rmpA* gene. In serotype K1 isolates *kfu* gene was more frequent than serotype K2 and Non-K1/K2 serotype, while distribution of *rmpA* gene were more frequent in serotype K1 and serotype K2 than Non-K1/K2 serotype.
- Polymerase Chain Reaction (PCR) and especially Multiplex PCR for *K. pneumoniae* considered a reliable, relatively rapid, cost-effective, and easy application and repeatable.

4-2 Recommendations:

- Studying the virulance of *K. pneumoniae* serotype K1, K2 and Non-K1/K2 *in vivo.*
- Molecular genetics study of membrane protein that encodes by *magA* and *rmpA* genes that responsible for hypermucoviscosity to use an inhibitor of cancer cells *in vivo* and *in vitro*.
- Molecular genetics study by using PCR for detection the presence of carbapeneme genes in Iraq.
- More detailed studies for other virulence factors in this bacteria (e.g. virulence genes (*iutA*, *silS*, *fur* and *terW*) located on virulence plasmid (pLVPK)) of *K. pneumoniae* as a risk factors.

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APPENDIX

Appendix

Appendix (1): Interpretation of reactions performed by API 20E

No.	Test	Substrate	Positive	Negative
1	ONPG	Orthro-nitrophenyl Galactoside	Yellow	Colorless
2	ADH	Arginine dehydrolase	Red/Orange	Yellow
3	LDC	Lysine decarboxylase	Orange	Yellow
4	ODC	Ornithine decarboxylase	Red / Orange	Yellow
5	CIT	Citrate	Blue-Green	Pale green /Yellow
6	H_2S	Sodium Thiosulphate	Black deposit	Colorless / Grayish
7	URE	Urea	Red / Orange	Yellow
8	TDA	Tryptophane deaminase	Dark brown	Yellow
9	IND	Indol	Red Ring	Yellow Ring
10	VP	Voges-proskauer	Pink / Red	Colorless
11	GEL	Kohn's Gelatin	Diffusion of Black Pigment	No diffusion
12	GLU	Glucose	Yellow	Blue / Blue green
13	MAN	Mannitol	Yellow	Blue / Blue green
14	INO	Inositol	Yellow	Blue / Blue green
15	SOR	Sorbitol	Yellow	Blue / Blue green
16	RHA	Rhamnose	Yellow	Blue / Blue green
17	SAC	Sucrose	Yellow	Blue / Blue green
18	MEL	Melibiose	Yellow	Blue / Blue green
19	AMY	Amygdalin	Yellow	Blue / Blue green
20	ARA	Arabinose	Yellow	Blue / Blue green
21	OX	____	Violet / Dark purple	Colorless / Light purple

Appendix (2): Biochemical tests in the VITEK2 system

Weal No.	Test	abbrviation	Compound conc. (mg)
2	Ala-Phe-Pro-ARYLAMIDASE	APPA	0.0384
3	ADONITOL	ADO	0.1875
4	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	0.018
5	L-ARABITOL	IARL	0.3
7	D-CELLOBIOSE	dCEL	0.3
9	BETA-GALACTOSIDASE	BGAL	0.036
10	H2S PRODUCTION	H2S	0.0024
11	BETA-N-ACETYL-GLUCOSAMINDASE	BNAG	0.0408
12	Glutamyl Arylamidase pNA	AGLTp	0.0324
13	D-GLUCOSE	Dglu	0.3
14	GAMMA-GLUTAMYL-TRANSFERASE	GGT	0.0223
15	FERMENTATION /GLUCOSE	OFF	0.45
17	BETA-GLUCOSIDASE	BGLU	0.036
18	D-MALTOSE	dMAL	0.3
19	D-MANNITOL	Dman	0.1875
20	D-MANNOSE	Dmne	0.3
21	BETA-XYLOSIDASE	BXYL	0.0324
22	BETA-Alanine aryamidase Pna	BAlap	0.0174
23	L-Proline ARYLAMIDASE	ProA	0.0234
26	LIPASE	LIP	0.0192

27	PALATINOSE	PLE	0.3
29	Tyrosine ARYLAMIDASE	TyrA	0.0276
31	UREASE	URE	0.15
32	D-SORBITOL	dSOR	0.1875
33	SACCHAROSE /SUCROSE	SAC	0.3
34	D-TAGATOSE	dTAG	0.3
35	D-TREHALOSE	dTRE	0.3
36	CITRATE (SODIUM)	GIT	0.054
37	MALONATE	MNT	0.15
39	5-KETO-D-GLUCONATE	5RG	0.3
40	L-LACTATE alkalipisation	ILATK	0.15
41	ALPHA-GLUCOSIDASE	AGLU	0.036
42	SUCCINATE alkalipisation	SUCT	0.15
43	Beta-N-ACETYL - GALACTOSAMINIDASE	NAGA	0.0306
44	ALPHA-GALACTOSIDASE	AGAL	0.036
45	PHOSPHATASE	PHOS	0.0504
46	Glycine ARYLAMIDASE	GlyA	0.012
47	ORNITHINE DECARBOXYLASE	ODC	0.3
48	LYSINE DECARBOXYLASE	LDC	0.15
52	DECARBOXYLASE BASE	ODEC	NA
53	L-HISTIDINE assimilation	IHISa	0.087
56	COURMARATE	CMT	0.126
57	BETA-GLUCORONIDASE	BGUR	0.0378
58	O/129 RESISTANCE (comp.	O129 R	0.0105

	vibrio.)		
59	GLU-GLY-Arg-ARYLAMIDASE	GGAA	0.0576
61	L-MALATE assimilation	IMLTa	0.042
62	ELLMAN	ELLM	0.03
64	L-LACTATE assimilation	ILATa	0.186

Appendix (3): Result of *K. pneumoniae* Diagnosed by API 20E system

No.	Test	*K. pneumoniae*	*K. oxytoca*	*K. terrigena*	*K. ornitholytica*
1	**ONPG**	**—**	**—**	**—**	**—**
2	**ADH**	**—**	**—**	**—**	**—**
3	**LCD**	**+**	**+**	**+**	**+**
4	**ODC**	**—**	**—**	**—**	**—**
5	**CIT**	**+**	**+**	**+**	**+**
6	**H_2S**	**—**	**—**	**—**	**—**
7	**URE**	**+**	**—**	**—**	**—**
8	**TDA**	**—**	**—**	**—**	**—**
9	**IND**	**—**	**+**	**—**	**+**
10	**VP**	**+**	**+**	**+**	**+**
11	**GEL**	**—**	**—**	**—**	**—**
12	**GLU**	**+**	**+**	**+**	**+**
13	**MAN**	**—**	**—**	**—**	**—**
14	**INO**	**—**	**—**	**—**	**—**
15	**SOR**	**—**	**—**	**—**	**—**
16	**RHA**	**—**	**—**	**—**	**—**
17	**SAC**	**—**	**—**	**—**	**—**
18	**MEL**	**—**	**—**	**—**	**—**
19	**AMY**	**—**	**—**	**—**	**—**
20	**ARA**	**—**	**—**	**—**	**—**
21	**oxidase**	**—**	**—**	**—**	**—**

(+) positive result **(–) negative result**

Appendix (4): Result of *K. pneumoniae* diagnosed by VITEK 2 system

No.	Test	*K . pneumoniae*
1	APPA	—
2	ADO	+
3	PyrA	v
4	IARL	+
5	dCEL	+
6	BGAL	+
7	H2S	—
8	BNAG	—
9	AGLTp	—
10	dGLU	+
11	GGT	—
12	OFF	+
13	BGLU	+
14	dMAL	+
15	dMAN	+
16	dMNE	+
17	BXYL	+
18	BAlap	+
19	ProA	—
20	LIP	—
21	PLE	+
22	TyrA	+
23	URE	+
24	dSOR	+
25	SAC	+
26	dTAG	—
27	dTRE	+
28	CIT	+
29	MNT	+
30	5KG	—
31	ILATK	+

32	**AGLU**	**—**
33	**SUCT**	**+**
34	**NAGA**	**—**
35	**AGAL**	**+**
36	**PHOS**	**+**
37	**GlyA**	**—**
38	**ODC**	**+**
39	**LDC**	**+**
40	**IHISa**	**—**
41	**CMT**	**+**
42	**BGLUR**	**+**
43	**O129R**	**+**
44	**GGAA**	**—**
45	**IMLTa**	**—**
46	**ELLM**	**—**
47	**ILATa**	**—**

(+) positive result **(—) negative result** **V= variable result**

Appendix (5): Examination report of clinical *K. pneumoniae* with VITEK 2 system

bioMerieux Customer:

Microbiology Chart Report

Printed Mar 14, 2013 11:01 CDT

Patient Name: tamara husham
Location:
Lab ID: 47-3

Patient ID: 47-3-1
Physician:
Isolate Number: 1

Selected Organism : Klebsiella pneumoniae ssp pneumoniae

Source: urine

Collected:

Comments:	

Identification Information	Analysis Time:	4.00 hours	Status:	Final
Selected Organism	99% Probability Bionumber:	Klebsiella pneumoniae ssp pneumoniae 6607734753564010		
Organism Quantity:				
ID Analysis Messages				

Susceptibility Information	Analysis Time: 5.75 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL	POS	+	Imipenem	<= 1	S
Ampicillin	>= 32	R	Meropenem	<= 0.25	S
Ampicillin/Sulbactam	>= 32	R	Amikacin	<= 2	S
Piperacillin/Tazobactam	<= 4	S	Gentamicin	>= 16	R
Cefazolin	>= 64	R	Tobramycin	<= 1	S
Cefoxitin	<= 4	S	Ciprofloxacin	<= 0.25	S
Ceftazidime	16	*R	Levofloxacin	<= 0.12	S
Ceftriaxone	>= 64	R	Nitrofurantoin	64	I
Cefepime	4	*R	Trimethoprim/Sulfamethoxazole	>= 320	R

+= Deduced drug *= AES modified **= User modified

AES Findings		
Confidence:	Consistent	
Phenotype:	BETA-LACTAMS	EXTENDED SPECTRUM BETA-LACTAMASE

Page 1 of 1

Appendix (6):Prevalence of 16S rRNA, *magA*, *k2A*, *rmpA* and *kfu* genes within different serotypes of *K. pneumoniae* isolates

No	Isolate symbol	Isolate source	16S rRNA	*magA*	*k2A*	*rmpA*	*kfu*	Serotype
1	T1	Urine	+	+	–	+	+	K1
2	T5	Urine	+	+	–	+	+	K1
3	T8	Urine	+	+	–	–	+	K1
4	T11	Blood	+	–	+	–	+	K2
5	T13	Blood	+	–	+	+	–	K2
6	T18	Urine	+	+	–	+	+	K1
7	T21	Urine	+	+	–	–	+	K1
8	T22	Blood	+	+	–	–	+	K1
9	T23	Blood	+	+	–	–	+	K1
10	T24	Blood	+	+	–	–	+	K1
11	T26	Environ.	+	–	–	+	+	K2
12	T28	Blood	+	+	–	–	+	K1
13	T31	Urine	+	+	–	–	+	K1
14	T33	Urine	+	–	+	+	+	K2
15	T37	Urine	+	–	–	–	–	Non-K1/K2
16	T38	Urine	+	–	–	–	–	Non-K1/K2
17	T39	Urine	+	+	–	–	–	K1
18	T40	Burn	+	–	+	+	+	K2
19	T48	Sputum	+	+	–	–	+	K1

20	T52	Urine	+	–	–	–	–	Non-K1/K2
21	T57	Blood	+	–	+	–	+	K2
22	T58	Urine	+	+	–	–	+	K1
23	T59	Urine	+	+	–	–	+	K1
24	T63	Urine	+	+	–	+	+	K1
25	T70	Urine	+	–	–	–	–	Non-K1/K2
26	T73	Sputum	+	–	+	+	–	K2
27	T78	Environ.	+	+	–	–	+	K1
28	T80	Urine	+	–	+	–	+	K2
29	T81	Urine	+	–	–	–	–	Non-K1/K2
30	T88	Blood	+	+	–	–	+	K1
31	T91	Environ.	+	–	–	–	–	Non-K1/K2
32	T92	Wound	+	+	–	–	+	K1
33	T93	Blood	+	+	–	–	+	K1
34	T98	Ear swab	+	–	+	+	+	K2
35	T105	Burn	+	+	–	+	+	K1
36	T108	Environ.	+	+	–	–	+	K1
37	120	Urine	+	–	+	–	+	K2
38	121	Urine	+	–	+	–	+	K2
39	122	Urine	+	+	–	–	+	K1
40	123	Urine	+	+	–	–	–	K1

Appendix

الملخص

جمعت ثلاثمائة وخمسة وعشرون عينة سريرية وبيئية من خمس مستشفيات في بغداد من مرضى (ذكور واناث) من الفئة العمرية بين 10-50 ≤ سنة في الفترة ما بين 2012/11/1 إلى 2013/1/7. عزلت 282 عزلة، شخصت 56 منها بكونها تعود الى الجنس *Klebsiella* اعتمادا على الصفات المظهرية، الفسلجية والكيموحيوية. فيما شخصت العزلات المتبقية على انها تعود الى *coli* ،*Escherichia* ،*Staphylococcus aureus* ،*Proteus* و*Pseudomonas* ،*Staphylococcus epidermidis* ،*Enterobacter* ،*Shigella*.

اظهرت النتائج الكيموحيوية والتي تم تأكيدها باستخدام نظام التشخيص بعدة ال API 20E و نظام VITEK 2 بأن عزلات ال *Klebsiella* (56 عزلة) تعود إلى اربعة انواع هي *K. pneumoniae* (40 عزلة)، *K. oxytoca* (3 عزلات)، *K. terrigena* (10 عزلات) و *K. ornitholytica* (3 عزلات). 36 عزلة (90 %) من *K. pneumoniae* عزلت من مصادر سريرية و 4 عزلات (10 %) من مصادر بيئية. وكانت عينات الأدرار اكثر مصادر عزل هذه البكتريا تليها عينات الجروح، مسحة الأذن، مطبخ وحمام المستشفيات.

وتبعا لجنس المريض فقد وجد أن الذكور لديهم فرص اكبر من الاناث للاصابة ببكتريا *K. pneumonia*، اذ كان عدد المرضى المصابين بالبكتريا من الذكور 19 (52.8 %) ومن الاناث 17 (47.2 %). كما ان المجموعة العمرية ≤ 10 سنوات كانت الاكثر تعرضا للاصابة بالبكتريا.

تم تحديد حساسية عزلات *K. pneumoniae* تجاه 15 مضادا حيويا شائعا بأستخدام طريقة الأقراص. اظهرت النتائج مقاومة العزلات المرضية للمضادات الحيوية المستخدمة باستثناء حساسيتها تجاه imipenem.

استخلص الحامض النووي DNA من عزلات *K. pneumoniae*، واظهرت النتائج ان التراكيز التي تم الحصول عليها كانت تتراوح بين 47.4-123.8 نانوغرام / ميكرولتر وبنقاوة مابين 1.6-2.

شخصت عزلات بكتريا *K. pneumoniae* جزيئيا باستخدام تفاعل تضخيم السلسلة (PCR) اذ ضخمت جينات 16 S rRNA وجينات *rmpA* و *kfu* ودرس مدى انتشار الانماط المصليه K ، فضلا عن تضخيم الجينات باستخدام تفاعل تضخيم السلسلة المتعدد. وأظهرت النتائج أن جميع عزلات *K. pneumoniae* أعطت حزمة واضحة بحجم 130 زوج قاعدي عند استخدام برايمر متخصص لجين

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16S rRNA في هذة البكتريا، وعند استخدام برايمرات متخصصة لجينات الكبسولة *magA* و *K2A* ، اوضحت النتائج أن 23 (57.5 %) عزلة تعود إلى النمط المصلي K1 اذ أعطت حزمة بحجم 1283 زوج قاعدي و 11 (27.5 %) عزلة تعود إلى النمط المصلي K2 أعطت حزمة بحجم 543 زوج قاعدي. وتشير هذه النتائج إلى أن *magA* و *K2A* قد يكونا مؤشرين وراثيين مفيدين لتحديد الأنماط المصلية K1 و K2، وكانت هذه الأنماط المصلية أكثر انتشارا من تلك التي ليست من النمط المصلي K1 او K2 (6 عزلات، 15 %).

ضخم جين *rmpA* و جين *kfu* واظهرت النتائج ان الجين *kfu* كان أكثر تكرارا من الجين *rmpA*، اذ ظهرت حزمه بحجم 797 زوج قاعدي تمثل الجين *kfu* في 30 عزلة بينما ظهرت حزمة بحجم 536 زوج قاعدي تمثل الجين *rmpA* في 11 عزلة. فضلا عن ذلك فأن انتشار الجين *kfu* كان في 21 عزلة من النمط المصلي K1 وهو اكثر من تكراره في عزلات النمط المصلي K2 (8 عزلات) والعزلات التي ليست ضمن النمط المصلي K1 او K2 (1 عزلة)، في حين كان جين *rmpA* أكثر شيوعا في النمط المصلي K1 والنمط المصلي K2 (5 عزلات) بينما في العزلات التي ليست ضمن النمط المصلي K1 او K2 (1 عزلة).

تم إجراء تفاعل تضخيم السلسلة المتعدد لعزلات بكتريا *K. pneumoniae* بأستخدام البوادئ الخاصة لجينات 16S rRNA، *magA* ، *k2A* و*rmpA*. أوضحت النتائج أن *K. pneumoniae* ذات النمط المصلي K1 التي تمتلك جين *rmpA* أظهرت حزم مضخمة للجينات *magA* و 16S rRNA، اما عزلات النمط المصلي K2 التي لا تمتلك الجين *rmpA* اظهرت حزم مضخمة للجين *K2A* و الجين 16S rRNA. فضلا عن ذلك فأن ال *K. pneumoniae* ذات النمط المصلي K1 التي لاتمتلك جين *rmpA* اظهرت حزمة مضخمة للجين *magA* والجين 16S rRNA. واخيرا، فأن ال *K. pneumoniae* التي ليست ضمن النمط المصلي K1 و K2 والتي لاتمتلك جين *rmpA* اظهرت فقط حزمه واحدة للجين 16S rRNA.

تعد تقنية تفاعل تضخيم السلسلة المتعدد موثوقة وسريعة نسبيا ، فعالة، سهلة التطبيق وقابلة للتكرار وممكن ان تكون أداة فعالة وقوية للتشخيص السريري الروتيني لبكتريا ال *Klebsiella*.

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قسم التقانة الاحيائية

التشخيص الجزيئي لبكتريا *Klebsiella pneumoniae* بأستخدام جينات الكبسولة

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

تمارا هشام زيدان العبيدي

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