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Determination the Relationship between Some genetic Aspects with the Capsule Formation for Pathogenic *Klebsiella pneumoniae* Serotypes K1 &K2

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Abstract

A total of 47 samples were collected from different clinical specimens (urine, wounds, burns, sputum, blood, stools) during the period from November 2013 to January 2014, only 18 isolates (38.29%) were identified as *Klebsiella pneumoniae* ,11isolates (23.4%) as *E. coli* , 9 isolates (19.1%) as *S. aureus*, 3isolates (6.3%) as *Psedomonase* spp., 2 isolates (4.2 %) as *K. terrigena* and 4 isolates (8.5%) as *K. oxytoca*. The results were shown the elevation of *K. pneumoniae* percentage among the bacterial isolates depending on cultural, microscopically, biochemically characteristics and confirmed by using the API 20E and VITEK 2 system . Also *K. pneumoniae* isolates were more frequently in sputum samples then burns, stools , urine, wounds ,and blood in different percentages (33.3%، 16.6%، 16.6%، 11.1%، 11.1%، 11.1%) respectively. The *in vitro* sensitivity tests of *K. pneumoniae* isolates against (19) antimicrobial agents were determined through disc-diffusion method. It was found that all isolates were 100% resistant to (Ampicillin /Cloxacillin, Amoxicillin, Carbencillin, Oxacillin, Penicillin G and Ceftazipime, whereas they indicated variable resistance to the other antibiotics used. While Amikacin , Gentamicin and Imipenem revealed that were the most effective drugs used in the present study. Moreover *K. pneumoniae* isolated from sputum, urine and blood samples are more resistant to antimicrobial agents at percentages (78.9%), the most resistance isolates, 6 isolates were selected .The molecular detecting of some capsular polysaccharide genes (*cps*) were investigated by using PCR specific primers. Genotyping of 6 selected *K. pneumoniae* isolates were indicated by using K1,K2 specific PCR primers. The results were shown ،that there is no isolate belong to K1 serotype, while 4 isolates (66.6%) are belonged to K2 serotype, and 2 isolates (33.3%) showed negative result for both K1/K2.

Keywords: *Klebsiella pneumoniae*, capsular polysaccharide, antimicrobial agents

تحديد العلاقة بين بعض المعاير الوراثية الخاصة بتصنيع كبسو *Klebsiella pneumonia e* المرضية ذات الأنماط المصلية K1 و K2

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الخلاصة :

جمعت 47 عينة أخذت من عينات سريريه مختلفة (الإدرار، مسحات الجروح، مسحات الحروق، القشع ،الدم، الخروج) للفترة من تشرين الثاني 2013 إلى كانون الثاني 2014 . شخصت 18 عزلة بكتيرية

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(38.29%) بكونها تعود إلى نوع *K. pneumoniae*, 11 عزلة *E. coli*(23.4%) ، 9عزلات(19.1%*aureus*,) 3عزلات(6.3%)*S.* spp.*Psedomonase*, عزلتان(2.4%) , 4عزلات(8.5%) *K. oxytoca* *K.terrigena*

النتائج بينت ارتفاع نسبة عزلة *K. pneumonia* e من بين العزلات البكتيرية الأخرى اعتمادا على الصفات المظهرية ،الكيموحيوية و تم تأكيدها باستخدام نظام التشخيص بعدة الـ API20E ونظام VITEK 2 وكانت *K. pneumonia* الأكثر ترددا في عينات القشع تليها عينات الحروق، الخروج، الإدرار ،الجروح والدم بنسب مختلفة (33.3 ،16.6 ، 16.6 ،11.1، 11.1 ،11.1) على التوالي.تم تحديد حساسية عزلات *K. pneumoniae* تجاه 19 مضادا حيويا شائعا باستعمال طريقه الانتشار بالأقراص. أظهرت النتائج جميع العزلات 100% مقاومة للمضادات Ampicillin /Cloxacillin , Amoxicillin, Oxacillin, Carbencillin , Ceftazipime Penicillin G في حين إن بعضها اظهر مقاومة متغايرة لعدد من المضادات الأخرى. بينما أظهرت العزلات ذاتها حساسية اتجاه المضادات Gentamicin, Amikacin و Imipenem. في حين عينات القشع، الإدرار والدم كانت الأكثر مقاومة للمضادات بنسبة (78.9%).وبناءا على نتائج اختبار الحساسية اختيرت (6) عزلات تمثل الأكثر مقاومة لمعظم المضادات البكتيرية المستعملة . أظهرت نتائج الكشف الجزيئي لجينات متعدد السكريد باستعمال تقنية سلسلة التفاعل لإنزيم البلمرة (PCR) وببوادئ خاصة لتصنيع كبسولة البكتريا وحسب النمط المصلي (K1,K2)،عدم وجود عزلة موجبة للنمط المصلي K1 و4 عزلات(66.6%) موجبة للنمط المصلي K2 وعزلتان (33.3%) كانت سالبة لكلا النمطين.

Introduction:

Klebsiella pneumoniae, a member of the human intestine flora, is frequently associated with hospital-acquired pathogen causing severe respiratory tract infections such as pneumonia .Other infections caused by this organism include urinary tract infections, wound infection, abscesses, sepsis, , infections of the bloodstream , inflammation and diarrhea [1].*K. pneumoniae* bacteremia causes significant morbidity and mortality if incorrectly treated in general populations [2].Treatment of *Klebsiella* infections is complicated[3]. *K. pneumoniae* strains exhibit different virulence factors which give the bacteria the ability to invade the host, such as capsular polysaccharides, lipopolysaccharide ,serum resistance, production of urea and enterotoxin ,type 1 and type 3 adhesions, factors involved in aggregative adhesions and siderophores[4]. Extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriage of *Klebsiella* spp. and subsequently, the development of multidrug-resistant strains that produce extended- spectrum beta-lactamase that led to the orientation of the research on alternative therapies [5].PCR was performed to amplify *MagA* (mucoviscosity associated gene A) to identify the *CPS* of *K. pneumoniae* capsule K1 serotype and *RcsA* (Regulation of capsule synthesis A) to identify the *CPS* of *K. pneumoniae* capsule K2 serotype, using specific primers. All the target genes are chromosomal[6].*K. pneumoniae* contains a capsule around its cell, known as K antigen. The reason for its pathogenicity is the thick capsule layer surrounding the bacterium, it is 160 nm thick of fine fibers that protrudes out from the outer membrane at right angles[7].

This study was aimed to explore the susceptibility of local *K. pneumoniae* isolates isolated from different infected patients against mostly used antibiotics ,and the foundation of some important genes and their participation in capsule formation .

Materials and methods:

Collection of samples:

Forty-seven clinical samples were collected from different patients including both sexes with different age, who suffered from infection from different sources (urine, wound swab, burn, sputum, blood, stool), during the period of 5\11\2013-18\1\2014 from patients in hospitals in Baghdad (Child pediatric ,Al-kindi and AL-Yarmouk Teaching Hospital). These samples were taken by sterile cotton swabs then in sterile tubes while sterile container collected UTI samples directly, then stored in cooled place until transported to laboratory. Swabs were suspended in brain heart infusion broth [8] and incubated for 24 hrs. at 37ºC.

Bacterial isolation*:*

Bacterial isolation was done by streaking loopful of culture from brain heart infusion broth media on MacConky agar [9] for primary selection of pathogenic *Klebsiella pneumoniae* and on EMB agar[10]. The plates were incubated overnight at 37°C. The developed colonies which shows characteristics /growth, colors, and mucoid were transferred to new plates for further tests.

Identification of *K. pneumoniae*:

- **Gram's stain[11]:**

To examine the shape, Gram-stain reaction and arrangement of cells

Biochemical tests:

According to [8,10-12], clinical isolates those exhibiting mucoid colonies were processed for biochemical testing. The biochemical tests were employed Catalase, Oxidase, Motility, Urease, Indol, Methyl Red, Voges-Proskauer, Citrate utilization, Lactose fermentation and growth at 10 ºC tests were also carried out. Beside these tests, Api 20E system kit (Bio-Meriaux, France) and VITEK 2 system kit (Bio-Meriaux, France) were also checked for identification of *K. pneumoniae.*

Susceptibility of *K. pneumoniae* isolates to different antimicrobial agents:

Klebsiella pneumoniae subspecies strains isolated (adjusted to0.5 McFarland turbidity standards) in the present study were subjected to susceptibility testing against 19 antimicrobials commonly used. Susceptibility was determined by the disk-diffusion technique of Kirby-Bauer on Mueller-Hinton agar plates [13] .Disks containing Oxacillin OX (10 µg) , Tetracycline T (10 µg), Chloramphenicol C (10µg), Amoxicillin AX (25µg), Methicillin ME (10µg), Cefixime CFM (5µg), Erythromycin E(15µg), Setreptomycin S (10µg), Ampicillin/ Cloxacillin APX/C (25/5µg), Vancomycin VA (10µg), Piperacillin PRL(30µg), Rifampin RA (5µg), Gentamicin CN(10µg), Carbenicillin PY (25µg), Aztreonam ATA (30µg), Ceftazidime CAZ (30µg), Imipenem IPN(10µg), Pencillin G P(10 µg), Amikacin AK(30µg) were used. All disks were obtained from Bioanalyse (Turkey). After 24 hr. incubation at 37°C,organisms were classified as Sensitive (S),Intermediately resistant (I) or Resistant (R) on the basis of the size of the zone of bacteria growth inhibition according to the guidelines of the [14-15].

Extraction of DNA from the selective isolates:

Genomic DNA was extracted by using the ExiPrep™ Plus Genomic DNA kit (Bioneer Company, Korea).The protocol is designed for extraction of Genomic DNA from Gram negative and Gram-positive bacteria

Polymerase chain reaction (PCR):

Primers:

(Alpha DNA /Canada) manufactured all the primers used in this study in lyophilized form. *MagA1gene* A (F-magA5‘GGTGCTCTTTACATCATTGC3'), (R-mag A**5'**GCAATGGCCATTTGCGTTTGCGTTAG3) were produced about 1283 bp fragment [6].The other one was *magA2* gene (F-5‘CATGGCTTAGACCGATGG3') (R-**5'**CACAACAGCTGCCTGACC3') were produced about 509 bp fragment and Repeat unit exporter gene (F-5'GCATTGGCACAATTTACACG3),(R-5'TCTGCCCATAACCTCGAAAG3') were produced about 413bp fragment for K1serotype. And (F-rcsA5'CCAGGGTTTTATTTCCAGCA3'), (R-rcsA5'TGCCATAAGCAATGAACCAA3') were produced about 254 bp fragment for K2serotype.Primers were prepared by dissolving lyophilized product in nuclease free water to prepare 100 pmole of stock solution forward primers and reverse primers then were used to prepare working solution .The last three primers are designed locally by University of Baghdad/ College of Science/ Biotechnology Department.

PCR reaction:

Polymerase chain reaction was performed in a total volume of 25 µl containing 1µl of both the forward and reverse of the primer,12.5 µl master mix, 8.5 µl Nuclease free water and 2µl of the DNA , then DNA amplification was carried out with the thermal cycler.

PCR program:

The thermal cycling (Multigene TM Gradient Thermal Cycler, Labnet International, USA) was programmed as follows: initial denaturation of 5 min at 94°C and then 33 cycles of the following three steps: 94 °C for 1min, 55°C for 1 min and 72 °C for 1min and final incubation at 72 °C for 10 min [6]for *magA1*.And for *rcsA*, *magA2* and Repeat unit exporter genes(designed) were programmed as

follows: initial denaturation of 5 min at 94°C and then 33 cycles of the following three steps: 94 °C for 45 sec , 56°C for 45 min and 72 °C for 45min and final incubation at 72 °C for 5 min.

Determine the Molecular Size of Amplicons :

The PCR products were separated on 2% agarose gel electrophoresis in the presence of 100 bp DNA ladder marker (Promega, USA) and visualized under the ultraviolet light (302nm) after staining with ethidium bromide [16].

Result and discussion:

Identification bacterial isolates:

Several morphological, microscopical ,physiological and biochemical tests were made to identify bacterial isolates. Moreover ,identification of bacterial isolates were confirmed by using Api System (Api20E) and VITEK 2 system. All *Klebsiella* spp. isolates, were found to be Gram negative, Non-motile, small straight rods and arranged singly or in pairs under light microscope. as described by[17]. According to the [9] characteristic parameters which depended on colonies diameters and biochemical tests, the *K. pneumoniae* identification results showed there were 18 isolates (38.2%) gave pink color because it lactose-fermenting, mucoid texture with large size , round, regular colonies on MacCkonky agar, while the numbers and percentages of *K. oxytoca* and *K. terrigena* 4 isolates(8.4%) and 2 isolates(4.2%),and the colonies diameters (3-4 mm) ,and (1.5-2.5 mm) with a weaky mucoid aspect respectively. Furthermore temperature sensitivity test which was done for different *Klebisella* species certified the previous results which elucidate *K. pneumoniae* was unable to growth at 10 ºC , which distinguish it from *K. oxytoca* and *K. terrigena* since they can live at this temperature , this result was confirmed with[18], table 1.Moreover, Api 20E system and VITEK 2 system confirmed the identification of 18 *K. pneumoniae* isolates.

Table 1-Biochemical tests of different bacterial isolates

Test		***K. pneumoniae***	***K. oxytoca***	***K. terrigena***	***E. coli***	***S. aureus***	***Pseudomonas* spp.**
Catalase		V	V	V	+	+	+
Oxidase		_	_	_	_	_	+
Indole production		_	+	_	+	_	_
Methyl red		V	V	V	V	ND	_
Voges-proskaure		+	+	+	_	V	_
Citrate utilization		+	+	+	_	ND	+
Motility		-	-	-	+	_	V
Urea hydrolysis		+	+	+	_	ND	_
TSA	H2S	-	-	-	-	ND	-
	Gas	+	+	+	+	ND	ND
	A/A	+/+	+/+	+/+	+/+	ND	K/K
Growth at 10 ºC		_	+	+	ND	ND	ND

(+) positive result, (-) negative result , V= variable result , ND=Not determined, A=acid K=alkaline

On the other hand the rest of isolates results also elucidated another genus with different percentages , which are represented as *E. coli* 11 isolates (23.4%), *S. aureus* 9 (19.1%), *Psedomonase* spp. 3 (6.3 %) .These percentages indicated to the *E. coli* became after *K. pneumoniae* at a percentage (23.4 %) ,this agreed closely with the [19] at 25.8 % , then *S. aureus* at a third position with (19.1%) , this result was agreed with the [20]study with a percentage 13.6%,table 2 and figure 1.

Table 2- Numbers and percentages of bacteria isolated from different clinical sources

Isolation Source	No. of samples	No.(%) of *K . pneumoniae*	No.(%) of *K .oxytoca*	No.(%)of *K. terrigena*	No.(%)of *E. coli*	No.(%) of *S. aureus*	No.(%)of *Pseudomonase* spp
Urine	6	2(33.3)	-	-	4(67.7)	-	-
Wound	4	2(50)	-	-	-	1(25)	1(25)
Burn	6	3(50)	1(16.6)	1(16.6)	-	1(16.6)	-
Sputum	14	6(42.8)	2(14.2)	1(7.1)	-	3(21.4)	2(14.2)
Blood	7	2(28.5)	1(14.2)	-	-	4(57.1)	-
Stool	10	3(30)	-	-	7(70)	-	-
Total	47	18(38.2)	4(8.5)	2(4.2)	11(23.4)	9(19.1)	3(6.3)

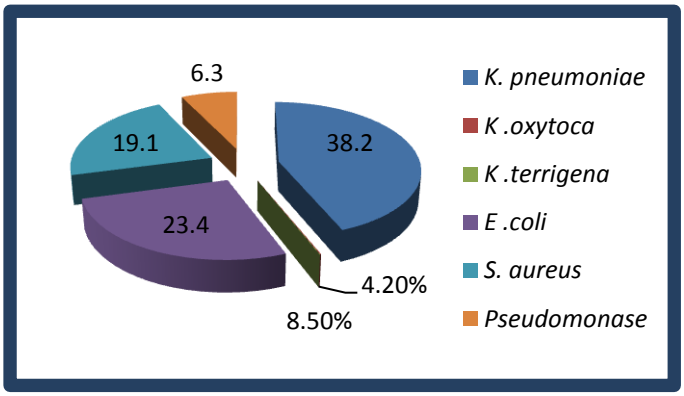

Figure 1- Percentages of bacterial isolates

Prevalence of *K. pneumoniae* according to source of samples:

Table 3 shows the presence of *K. pneumoniae* in most infections, the results indicate that the percentage of K. *pneumoniae* was (38.2%) (18/47). [21] reported that 29.1% (46/158) isolates were identified as *K. pneumoniae* . Results illustrated in table (3) show that *K. pneumoniae* more frequently from sputum which represent about (33.3%)(6/18), followed by burn swab and stool which represent about (16.6 %)(3 isolates/18).While of *K. pneumoniae* were isolated from UTI, blood and wound with percentage of (11.1%)(2 isolates/18) for each source. These results showed that the high percentage was occurred with the sputum ,and this agreed with [22].The results pointed that infection with *K. pneumonia* are more than other bacterial infection due to the results of isolations , which may be due to the multiple virulence factors of *K. pneumoniae* ,such as large capsule and resistant to antibiotics [23] that play an important role in the resistance to the immune system's [24].

Table 3- Frequency of *K. pneumoniae* according to the source of infection

Type of samples(Clinical)	Total	No. of isolates	Percentage % *	Percentage %**	Percentage %***
Urine	6	2	33.3	11.1	4.25
Wounds swab	4	2	50	11.1	4.25
Burns swab	6	3	50	16.6	6.38
Sputum	14	6	42.8	33.3	12.7
Blood	7	2	28.5	11.1	4.25
Stool	10	3	30	16.6	6.38
Total	47	18		100%	

*Percentage of *K. pneumoniae* according to source ** Percentage of *K. pneumoniae* according to total no. of *K. pneumoniae* isolate *** Percentage of *K .pneumoniae* according to total no. of total sample

Antibiotic susceptibility of *K. pneumoniae* isolates:

Depending on the National Committee for Clinical Laboratory Standards(NCCLs) guideline, 18 *K. pneumonia* were tested against 19 different antimicrobial using disc diffusion method recommended by[14].The results showed , all 18 (100%) *K. pneumoniae* isolates were resistant against Methicillin, Erythromycin, Ampicillin, Vancomycin, Rifampin, Oxacillin, Amoxicillin, Carbenicillin, Ceftazidime and Pencillin G. On the other hand all18 (100%) *K. pneumoniae* isolates were sensitive against Gentamicin , Imipenem ,and Amikacin .While some *K. pneumoniae* isolates shown varies resistant against several antimicrobial agents such as 33.3 % for both Tetracycline and Aztreonam ,38.8% for Chloramphenicol ,50% for streptomycin ,and 66.6%against both Cefixieme and Pipercillin . Moreover ,the isolates which are isolated from urine , blood ,and sputum appeared high level of resistance against most antimicrobial agents at a percentage 78.4% , while for that isolated from wound 68.4% , and from burns and stool 52.6% table 4 ,figure 2 . Depending on the obtained results 6 isolates are selected which were represent the mostly resistant isolates which are (2,4,5,10,14 ,17) for further use (detection of capsular genes). These results elicited there were elevation in resistance against most newly antibacterial agents ,that may be due to randomly used of antibacterial agents or/and some genetic mutations were occurred ,and these results are confirmed with [25-26].

Table 4-The antibiotics susceptibility results of the 18 isolates of *K. pneumoniae*

	Isolated	Methicillin	Gentamicin	Erythromycin	Imipenem	Ampicillin/ Cloxacillin	Vancomycin	Tetracycline	Chloramphenico	Rifampin	Oxacillin	Cefixime	Amoxicillin	Setreptomycin	Carbenicillin	Amikacin	Piperacillin	Ceftazidime	Pencillin G	0Aztreonam	Perecentage resistance of selective isolates
		E	CN	E	IPM	APX/C	VA	T	C	RA	OX	CFM	AX	S	PY	AK	PRL	CAZ	P	ATA	
Urine	1	R	S	R	S	R	R	R	S	R	R	R	R	S	R	S	R	R	R	S	
	2	R	S	R	S	R	R	R	S	R	R	R	R	R	R	S	R	R	R	R	78.9%
wound	3	R	S	R	S	R	R	S	S	R	R	R	R	S	R	S	R	R	R	S	
	4	R	S	R	S	R	R	S	R	R	R	R	R	S	R	S	R	R	R	S	68.4%
Burin	5	R	S	R	S	R	R	S	S	R	R	S	R	S	R	S	S	R	R	S	52.6%
	6	R	S	R	S	R	R	S	S	R	R	S	R	S	R	S	S	R	R	S	
	7	R	S	R	S	R	R	S	S	R	R	S	R	S	R	S	S	R	R	S	
Sputum	8	R	S	R	S	R	R	R	S	R	R	R	R	R	R	S	R	R	R	S	
	9	R	S	R	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	
	10	R	S	R	S	R	R	S	R	R	R	R	R	R	R	S	R	R	R	R	78.9%
	11	R	S	R	S	R	R	S	R	R	R	R	R	R	R	S	R	R	R	R	
	12	R	S	R	S	R	R	S	R	R	R	R	R	R	R	S	R	R	R	R	
	13	R	S	R	S	R	R	S	R	R	R	R	R	R	R	S	R	R	R	R	
Blood	14	R	S	R	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S	78.9%
	15	R	S	R	S	R	R	R	S	R	R	R	R	R	R	S	R	R	R	S	
Stool	16	R	S	R	S	R	R	S	S	R	R	S	R	S	R	S	S	R	R	S	
	17	R	S	R	S	R	R	S	S	R	R	S	R	S	R	S	S	R	R	S	52.6%
	18	R	S	R	S	R	R	S	S	R	R	S	R	S	R	S	S	R	R	S	
NO. Perecentage		(18) 100% R	(18) 100% S	(18) 100% R	(18) 100% S	(18) 100% R	(18) 100% R	(6) 33.3% R	(7) 38.8% R	(18) 100% R	(18) 100% R	(12) 66.6% R	(18) 100% R	(9) 50% R	(18) 100% R	(18) 100% S	(12) 66.6% R	(18) 100% R	(18) 100% R	(6) 33.3% R	

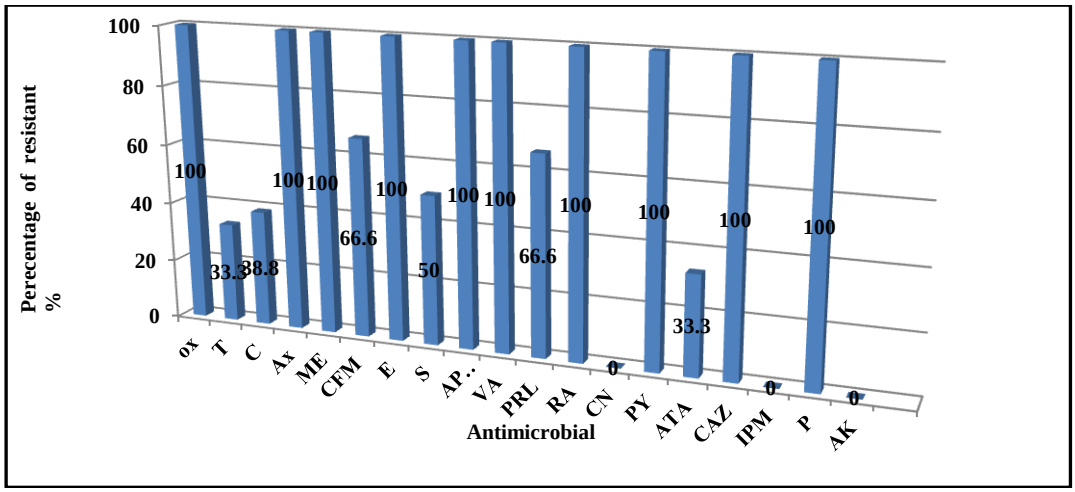

Figure2- Percentages of susceptibility of *K. pneumoniae* isolates to antimicrobials

Molecular diagnostic method for detection of *K. pneumoniae* capsule genes by PCR amplification:

The *K. pneumoniae* selective isolate were amplified with PCR for diagnosis *magA*1gene using both specific primers that depended on [11] ,and locally design(*magA*2) are tested to explored about these genes which act as indicator for K1 serotype isolates , the results appeared all isolates made negative results , and when certified this result by using other gene such as Repeat unit exporter gene using a specific primers pair , also ensured previous result ,thus these six isolates are not represent K1 serotype. While the same previous isolates were tested for *rcsA* gene(Regulation of capsule polysaccharide synthesis gene) which designated for *K. pneumoniae* serotype K2 by using a primer pair (rcs A-F and rcs A-R) specific for amplification *rcsA* gene. Result in figure (3) illustrated that PCR product was 254 bp in size, 4 isolates (66.6%) were positive for *rcsA* gene. These results referred that (pathogenic) isolates have a K2 serotype were more prevalence than K1 serotype. The rest isolates(2 isolates) were negative to amplified (*magA1, magA*2,Repeat unit exporter and *rcsA*) genes thus considered as Non –K1/K2. Also, regarding to the study, K2 was found to be more prevalent than K1 and confirmed with [27] who have indicated that K2 was more predominant in human infections.

The *K. pneumoniae rcsA* gene was responsible for a mucoid phenotype, as a result of colonic acid synthesis. Activation of colonic acid synthesis was not dependent on growth at low temperatures (30°C) [28]. On the other hand, 2 isolates where classified as non K1/K2 these isolates may be belonging to anther serotypes . Rapid detection of the virulent K1 and K2 serotypes will be helpful in diagnosis and treatment to decrease the risk of severe metastatic infection, as well as in epidemiological studies[29], therefore suggest detection of (*magA* ,Repeat unit exporter and *rcsA*)genes by PCR analysis as an easy, fast and high specific diagnostic method for identification of the *K. pneumoniae* K1 , K2 and Non K1/K2 capsule serotypes. Because the capsule act as one of virulent factor specially about antimicrobial resistance and biofilm formation thus, these genes were responsible for these resistance against most antimicrobial agent presence of cps reduces the binding of antimicrobial peptides to bacterial surface and this will promote the bacterial resistance to antibiotics [30].

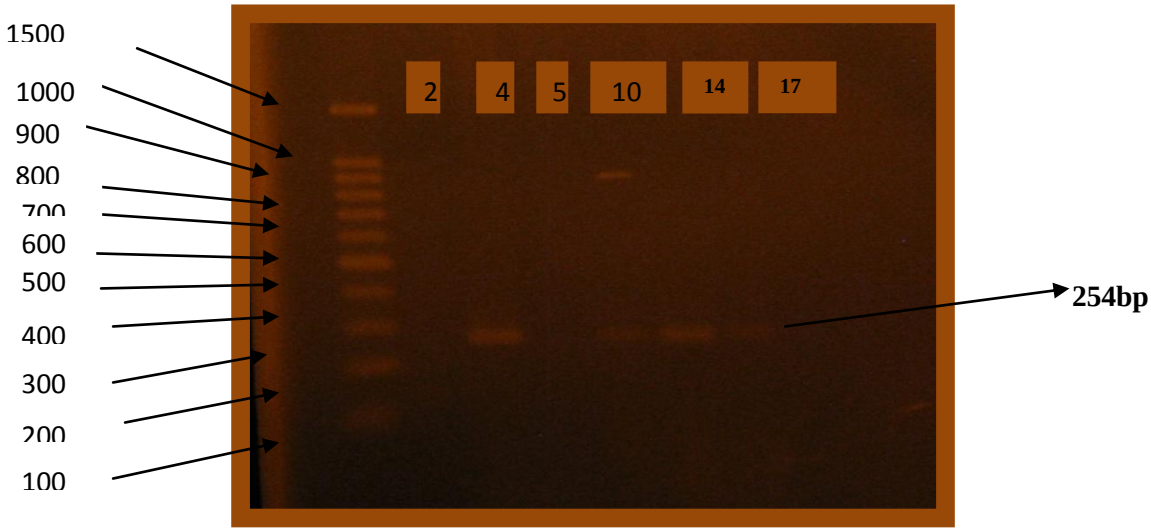

Figure 3- Gel electrophoresis for amplification of *rcsA* gene using specific primers of *K. pneumoniae* serotype K2.Electrophoresis was performed on 2% agarose gel and run with a 70 volt/35 m Amp current for 90 min. Lanes 2, 4, 5, 10, 14,17refers to isolates selected. Lane L is a(100 bp) ladder.

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