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COMPARISON OF THE ANTIBIOTIC DISK SENSITIVITY WITH THE ANTIMICROBIAL ACTIVITY OF LOCALLY CITRUS HONEY AGAINST *KLEBSIELLA PNEUMONIA*

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Abstract

Citrus honey has been used since a long time ago due to its therapeutic and nutritional values and presents an essential role as an anti-inflammatory, antioxidant, and anti-bacterial agent; therefore this investigation aims to estimate the antibacterial activity of citrus honey toward *Klebsiella pneumonia* isolated from pneumonic ruminant (sheep, goat and cattle). Out of (50) collected nasal discharge sample (10) strain was identified as *K. pneumonia* then confirmed by Microgen™ GnA + B-ID System. Most of *K. pneumonia* strain was sensitive for (5µg) of ciprofloxacin followed by (10µg) of imipenem that shown intermediate sensitive while resistance for (10µg) gentamicin, (10 mcg/disc) ampicillin, (30µg) cefotaxime, ceftriaxone (30µg), and more resist to tobramycin (10µg), tetracycline (30µg), (30µg) amoxicillin-clavulanic acid and amikacin (30µg). Five isolates was identified as multidrug resistance strain, the encoding of wcaG and rmpA genes were find out in 5 isolates (50%); rmpA was find out in 4 isolates (80%) and wcaG in another 4 (80%) isolates, the whole of 3 isolates possessed both virulence genes (rmpA and wcaG). Citrus honey showed varying degree of inhibition zone against *k. pneumonia* when tested in Mueller-Hinton agar with different concentration, the maximum inhibition zone was shown on *K. pneumonia* was (19mm) with 100% concentration while the minimum zone (5mm) with concentration of 25%.

Key words: Citrus honey, Antimicrobial activity, Antibiotics ensitivity.

Introduction

Around the world, the antimicrobial resistance in recent healthcare representing a growing issue. Several pathogenic bacteria strains are Multidrug-resistant (MDR), which are readily becoming widespread, that forming a severe risk to patients. Today the *Klebsiella pneumoniae* considered the most popular species of bacteria that generate health care problems. *K. Pulmonary pneumonia* can be responsible for acquired infections of the community (Nordamann *et al*., 2009).

Klebsiella pneumonia belonging to the *Enterobacteriaceae* group and recognized as one of the common important opportunistic pathogens that are frequently isolated from humans and animals with several infections (Shon *et al*., 2013).

K. pneumoniae possess different pathogenicity agents which improve the capability to produce disease. Capsules of *K. pneumoniae* own various adhesions factors and complex acidic polysaccharides that enhance its pathogenicity.

K. pneumoniae is the causative factor of severe community and hospital-acquired infections involving urinary tract infections, pneumonia, meningitis with septicemias, and soft tissue infections. Also has been recognized as a causative factor of another less common, yet serious, infections like invasive syndrome, septic arthritis, or generalized pustulosis and liver abscess (Lin *et al*., 2015).

Inflammations produced by *Klebsiella* species will lead to morbidity and mortality. As well as being considered the main reason for respiratory infections such as pneumonia, ozaena, rhinoscleroma and ear infections with sinusitis.

K. pneumoniae generate different enzymes that deactivate specific and target parts of drugs. Beta lactam

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drugs are usually the targeted one by produced enzymes, while some target other drug classes, including fluoroquinolones, aminoglycosides, trimethoprim, and sulfamethoxazoles. These enzymes such as metallo-beta-lactamases, oxacillinases, extended spectrum beta lactamases and *K. pneumoniae* carbapenemases, anda nother different enzyme. These enzymes are encoded on the *K. pneumoniae* plasmids (Goodman and Gillman's, 2010).

From all completed genomes, *K. pneumoniae* strains generally possess more than one plasmid, including; low copy number plasmids that are generally large and small high copy number.

The spread of transmissible plasmids is responsible for resistance to the antimicrobial agents, which might also be holding the virulence determinants. Pronounced polysaccharide capsule is usually expressed within *K. pneumoniae* isolates. A mucoid phenotype of Capsules is important virulence factors of *K. pneumonia*; the plasmid gene that regulator the mucoid phenotype (rmpA) gives the *K. pneumoniae* a hypermucoviscous phenotype by promoting generation of capsular polysaccharide (Bhattacharjee *et al*., 2010).

Most virulence gene is situated within the chromosome transferable portion of that is responsible to the biosynthesis of *K. pneumoniae* capsule which required during the transformable of mannose into fructose that improves the capability of bacteria to escape from phagocytosis of macrophages(Cheng *et al.,* 2010).

Herbal medicine has been widely employed as traditional medicines, Honey considered one of the oldest and important herbal medicine in the treatment of diseases (Patel *et al.,* 2010).

Honey has been applied as an environmentally friendly therapy during the ages and lately regarded as a potential therapeutic for bacterial pneumonia, gastroenteritis, infected wounds, peptic ulcer and burns, and the studies have demonstrated that honey has a potent broad-spectrum with high antibacterial activity (Ahmed *et al.,* 2012) (Manisha and Shyamapada, 2011).

From the numerous important commercial fruit crops, Citrus fruits are grown in all regions of the world which belong to six genera (Citrus, Microcitrus, Eremocitrus, Fortunella, Poncirus, and Clymendia), the large commercial fruits belong to genus Citrus, which are local to the tropical and subtropical regions of Asia(Lawal *et al.,* 2013).

Lemons, oranges, grapefruits, and mandarins, are important fruits which belong to genus Citrus. A large number of biological activities are reported about Citrus honey like antioxidant, antibacterial, antiviral, antifungal, insecticidal, anti-diarrheal, in addition to anti-cancer. Citrusoil has been employed in treatment of cancer (Mandal, 2011).

In the current study, attempts have been made to isolate and characterize *Klebsiella pneumonia* from sheep, goat, and cattle with pneumonic lungs and discover the antimicrobial activity of citrus honey versus *K. pneumonia*.

Materials and methods

Collection and Transportation of Samples:

From apparently pneumonic cattle, sheep, and goats, total of (50) Samples of nasal discharge swabs were collected from different areas in Baghdad and surrounding district for microbiological culture. Swabs were submitted in tryptose soya broth media. After collection, the samples were sent to the microbiological laboratory of market researches and consumer protection center / University of Baghdad; the nasal swabs were incubated immediately at 37°C for 24 hr aerobically.

Isolation and Identification of Bacterial Species:

After the first incubation, the nasal discharge swabs were cultured on both MacConkey agars and sheep blood and incubated aerobically at 37°C within 24-48 hr. The plates were observed of the appearance of growth, formation of colony, shape, and size, after obtaining a note about the characteristics of cultural growth, the plates were submitted to Gram's stain to study staining features and form of bacterial cell below 100X objective of the microscopic. The mucoid and smooth colonies were stained by using india ink for the presence of capsule. nutrient agar-slants was used for the transportation of Pure cultures of single colony type, from both blood and MacConkey agars, a set of biochemical tests oxidase, catalase, and oxidative-fermentative (OF) tests for definitive description. The isolates were confirmed by Microgen™ GnA + B-ID System also used as an identification system for *Enterobacteriaceae* (CLSI, 2014).

Antibiotic sensitivity testing:

Nutrient agar was used for purified the confident clinical samples of *K. pneumoniae* and incubation at 37°C within 24 hours the pure colonies were inoculated in 5mL of phosphate buffer slain (PBS) to prepare 0.5 McFarland standards tube (1.5×10^8 (CFU)/ml). The organism was cultured using a sterile nontoxic swab on the dried surface of a Muller-Hinton agar plate by streaking method, then the antibiotic discs were placed on the surface of the agar plate using Kirby-Bauer disc diffusion technique with

sterile forceps as prescribed by the Clinical and Laboratory Standards Institute (CLSI). Then plates were incubated at 37°C. Following 24 hrs of incubation, several disks containing antibiotics were used (5 µg) ciprofloxacin, imipenem (10µg), (10µg) gentamicin, ampicillin (10mcg/disc), tobramycin (10µg), cefotaxime (30µg), ceftriaxone (30µg), tetracycline (30µg), (30µg) amoxicillin-clavulanic acid, (30µg) amikacin. Each plate was measured and recorded the organism to be resistant or susceptible to antibiotics. Those organisms, which exhibited resistance to at least one agent in three or more antimicrobial categories, were recognized as multidrug-resistant bacteria (CLSI, 2014).

Detection of the Virulence Genes of wcaG and rmpA

Purifiedof *K. pneumoniae* was done according to (Karimnasab *et al.,* 2013), the evaluated for the existence each virulence genes of rmpA and wcaG within the Gene Amp PCR System using a specific primer as in table 1.

Duplex PCR was used for the amplification of the wcaG and rmpA genes. The freezing-thawing process was used through Genomic DNA preparation and the template of extracted DNA was used as during PCR reactions. PCR situations were set: 94^0C within 5 minutes was used as initial denaturation ; next, 35 cycles of 94^0C within 1 minute, 54^0C within1 minute, plus 72^0C within 1 minute, followed with the last elongation at 72^0C within 7 minutes (Lopes *et al.,* 2005).

Tests for the confirmation of the pure honey

10 samples of Citrus honey were collected from a different area in Baghdad city and surrounding district and sent to the laboratory of Iraqi quality control to confirm the purity of citrus honey such as Dissolution Test, Crystallization Test and Physico-chemical properties like pH, moisture and sugar contents were determined and Total phenolic content was measured according to (Iraqi honey Standards, 2013)**.**

Antimicrobial activity of citrus honey against *K. pneumoniae*:

A concentration of 25% (v/v) to 100% (v/v) was prepared by dilution the citrus honey with sterile distilled water. Agar well diffusion technique was used and a sterile cork-borer was used to perform identical wells in the prepared Mueller-Hinton agar that poured into sterile Petri-dishes, 100µl of the test antibacterial agent (citrus honey) was used to fill up the wells, the inoculated Petri-dishes were incubated at 37°C within 18-24 hrs.The diameters of the inhibition zones were measured with millimeters (mm) after the incubation period (Clinical and Laboratory Standards Institute, 2008).

Statistical Analysis

SAS (2012) a Statistical Analysis System was employed for conclude the various factors in study parameters. The LSD (least significant difference) analysis was applied to significant compare within the means in this investigation.

Results and Discussion

Isolation of bacteria

In the animals, the respiratory tract infections are a prevalent manifestation especially in the ruminant, and It is regarded the bacterial flora of the respiratory tract, in this study MacConky agar media was used for streaked the collected specimens directly then incubated at 37ºC within 24 hours, the data revealed that 10 isolates of *K. pneumonia* were isolated from 50 Samples of nasal discharge swabs that collected from apparently pneumonic animal and examined by the microbiological of market researches and consumer protection center laboratory/university of Baghdad. The results show in table 2, out of 10 suspected *K. pneumonia* that isolate 2(13.3%) isolate from cattle, 5(25%) isolate from sheep followed by 3(20%) from goats.

The confusion of the inter-related variables and stress factors that culminate with bacterial species is considered the reasons of pneumonia in ruminants (Boudreaux, 2004) particularly when the animals' immune system is

Table 1: The Primers Sequences and Sizes of Products.

Target	Primer Name	Primer Sequence (5-3)	Amplicon Size (bp)
rmpA	rmpAFrmpAR	ACTGGGCTACCTCTGCTTCACTTGCATGAGCCATCTTTCA	516
wcaG	wcaGFwcaGR	GGTTGGGTCAGCAATCGTAACTATTCCGCCAACTTTTGC	169

Table 2: The numbers and percentage of *K. pneumonia* isolates from nasal discharge.

Animal species	No. of samples	No. of (+) Ve (*K. pneumonia*) isolates	Percentage (%) of suspected positive results of *K. pneumonia* isolates
cattle	15	2	13.3 %
sheep	20	5	25%
goats	15	3	20%
Total	50 sample	10 sample	20%

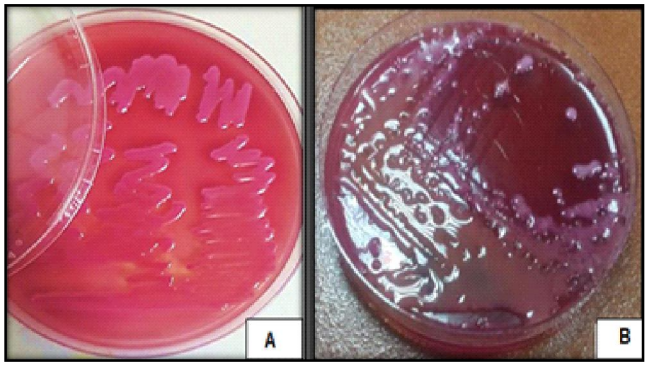
compromised by unusual another external factor.

This investigation has revealed that *K. pneumonia* bacteria colonize the respiratory passageways of pneumonic ruminants and confirmed by previous numerous workers who isolated several bacterial species of pneumonic sheep and goat (Tilaye, 2010) (Megra *et al.,* 2006).

Isolation and Identification of Bacterial Species

Cultural characteristics and morphological shape and color of *Klebsiella pneumoniae* colonies that appeared on culture media was pink color on MacConkey agar, very mucoid, , circular, convex and the lactose fermenter have been taken in consideration, *Klebsiella pneumoniae.* Typically produced large, rounded, mucoid colony (due to the thick polysaccharide capsule) and pink color colonies on MacConkey agar as shown in Fig. (1 A, B), the (10) suspected isolates was Gram negative rod, mucoid non spore forming under light microscope. This characteristics and morphological shape was agreement with identification of (Hind *et al*., 2016).

Microgen™ GnA+B-ID System is a design standard kit that conducted for the identification of bacteria related to the family of Enterobacteriaceae and carried out



Photograph 1(A,B): *Klebsiella pneumoniae* on MacConkey

according to the manufacturer company and was read after incubation based on color variations with or without the addition of some reagents.

This test depend on the Oxidase test in which the reagents by using a sterile wire loop to carry a colony into a filter paper (Whatman) and a drop of oxidase reagent were combined to it. a piece of the wood stick was used to mix the reagent with the bacteria cells and the results were viewed within the 30s, purple color was developed and recorded as a positive test, while no color reported as oxidase negative. *K. pneumoniae* is oxidase negative, therefore all isolates that were oxidase negative were preserved for confirmatory biochemical tests. The Complete identification by biochemical profile with use Microgen™ GnA + B - ID System for the purpose diagnosis of *K. pneumoniae* and for the purpose to make sure final diagnosis of the bacteria with accrue isolation up 98% after the diagnosis.

Ten strains were identified as non-motile with negative results for indole, oxidase, gelatin liquefaction, and methyl red. While positive for citrate utilization, urease and catalase production, and sugars fermentation such as lactose, sucrose, glucose and mannitol, melibiose, adonitol, in addition to esculin, there was no H2S production on Triple Sugar Iron agar as shown in Fig. 2.

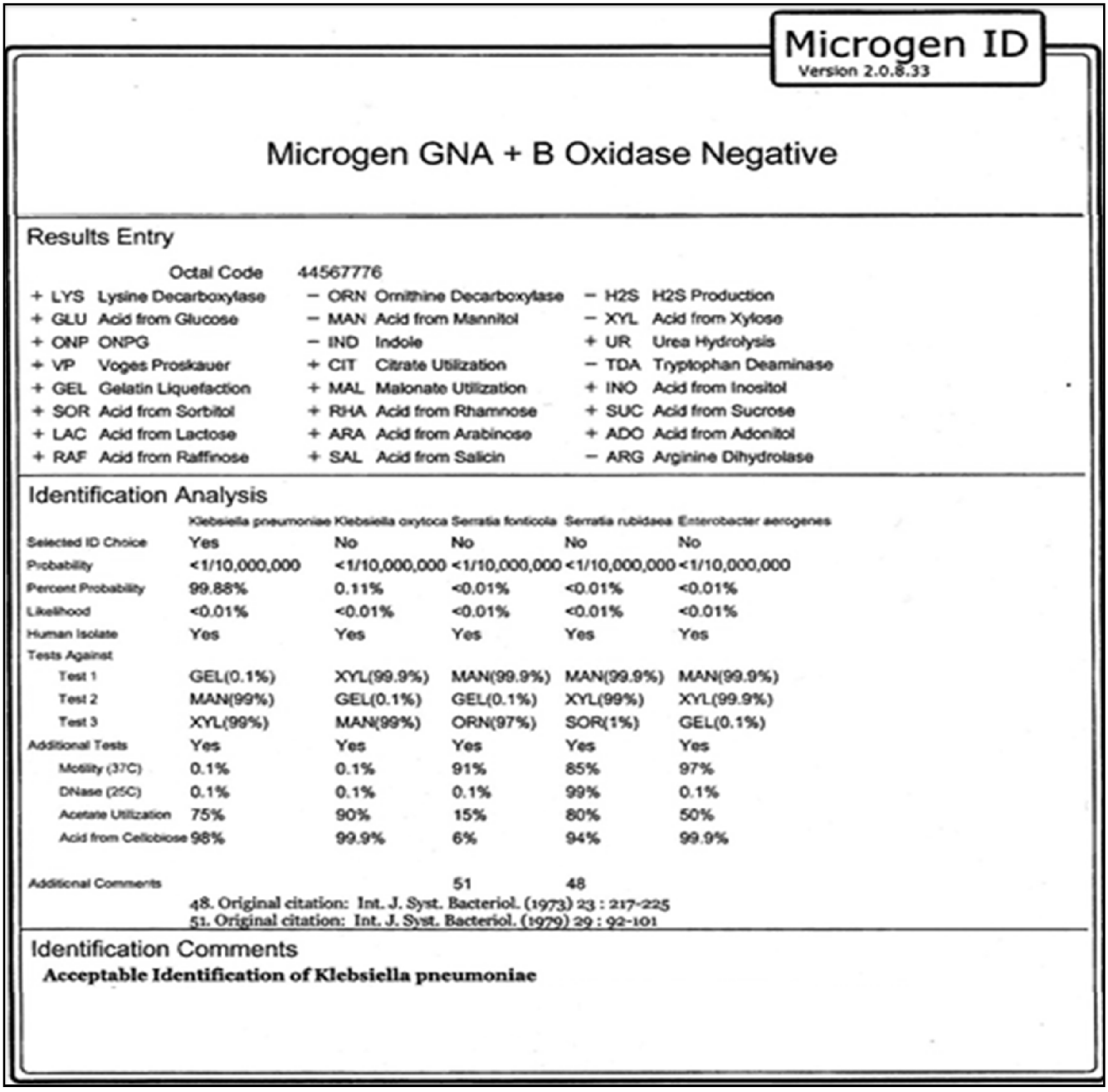
All ten isolates showing colonies and revealed a typical general biochemical reaction similar to that observed with *Klebsiella* while When tested by another biochemical reaction that involved some unusual sugars (adonitol, melibiose, esculin, with dulcitol), they displayed stability Return to the majority of *K. pneumoniae* subspecies pneumonia strain, being convinced to urease, esculin, adonitol and melibiose with citrate.

Out of 10 antibiotics tested disc the Present study showed that *Klebsiella pneumonia* (*K. pneumoniae)* is most sensitive for (5µg) ciprofloxacin followed by (10µg) imipenem that shown intermediate sensitive while resistance for (10µg) gentamicin, (10 mcg/disc) ampicillin, (30µg) cefotaxime, (30µg) ceftriaxone, and more resist to (10µg) tobramycin, (30µg) tetracycline, (30µg) amoxicillin-clavulanic acid and (30µg) amikacin as in table 3.

Within 24 hours the inhibition zones diameter was measured in a millimeter. The organism was assessed to be highly sensitive if the diameter of the inhibition zone was greater than 20 mm while intermediate if the diameter between 16-19 mm and resistant if the diameter was less than 11 mm. The intermediate readings were considered as sensitive in the evaluation of the data (Asati

Table 3: Antibiotic Sensitivity test of *Klebsiella pneumoniae*

Antibiotics	Dose	Sensitivity in %	Resistancein %
ciprofloxacin	(5 µg)	60	40
imipenem	(10 µg)	50	50
gentamicin	(10 µg)	40	60
ampicillin	(10 mcg/disc)	40	60
tobramycin	(10 µg)	30	70
cefotaxime	(30 µg)	40	60
ceftriaxone	(30 µg)	40	60
tetracycline	(30 µg)	20	80
amoxicillin-	(30 µg)	10	90
clavulanic acid amikacin	(30 µg)	10	90



Microgen ID
Version 2.0.8.33

Microgen GNA + B Oxidase Negative

Results Entry

Octal Code 44567776

+	LYS	Lysine Decarboxylase	−	ORN	Ornithine Decarboxylase	−	H2S H2S Production
+	GLU	Acid from Glucose	−	MAN	Acid from Mannitol	−	XYL Acid from Xylose
+	ONP	ONPG	−	IND	Indole	+	UR Urea Hydrolysis
+	VP	Voges Proskauer	+	CIT	Citrate Utilization	−	TDA Tryptophan Deaminase
+	GEL	Gelatin Liquefaction	+	MAL	Malonate Utilization	+	INO Acid from Inositol
+	SOR	Acid from Sorbitol	+	RHA	Acid from Rhamnose	+	SUC Acid from Sucrose
+	LAC	Acid from Lactose	+	ARA	Acid from Arabinose	+	ADO Acid from Adonitol
+	RAF	Acid from Raffinose	+	SAL	Acid from Salicin	−	ARG Arginine Dihydrolase

Identification Analysis

	Klebsiella pneumoniae	Klebsiella oxytoca	Serratia fonticola	Serratia rubidaea	Enterobacter aerogenes
Selected ID Choice	Yes	No	No	No	No
Probability	<1/10,000,000	<1/10,000,000	<1/10,000,000	<1/10,000,000	<1/10,000,000
Percent Probability	99.88%	0.11%	<0.01%	<0.01%	<0.01%
Likelihood	<0.01%	<0.01%	<0.01%	<0.01%	<0.01%
Human Isolate	Yes	Yes	Yes	Yes	Yes
Tests Against					
Test 1	GEL(0.1%)	XYL(99.9%)	MAN(99.9%)	MAN(99.9%)	MAN(99.9%)
Test 2	MAN(99%)	GEL(0.1%)	GEL(0.1%)	XYL(99%)	XYL(99.9%)
Test 3	XYL(99%)	MAN(99%)	ORN(97%)	SOR(1%)	GEL(0.1%)
Additional Tests	Yes	Yes	Yes	Yes	Yes
Motility (37C)	0.1%	0.1%	91%	85%	97%
DNase (25C)	0.1%	0.1%	0.1%	99%	0.1%
Acetate Utilization	75%	90%	15%	80%	50%
Acid from Cellobiose	98%	99.9%	6%	94%	99.9%
Additional Comments			51	48	

48. Original citation: Int. J. Syst. Bacteriol. (1973) 23 : 217-225
51. Original citation: Int. J. Syst. Bacteriol. (1979) 29 : 92-101

Identification Comments

Acceptable Identification of Klebsiella pneumoniae

Photograph 2: Microgen™ GnA + B-ID System resultes for *K. pneumoniae* isolates.

and Sadawarte, 2013).

In general, the resistance to cephalosporins (third-generation) elevates with the account of the generation of extended spectrum â-lactamases (ESBLs) by the K. pneumoniae. As well as is due to resistance to broad-spectrum, which is Plasmid-encoded cephalosporins that are becoming a broad spread phenomenon in clinical medicine.

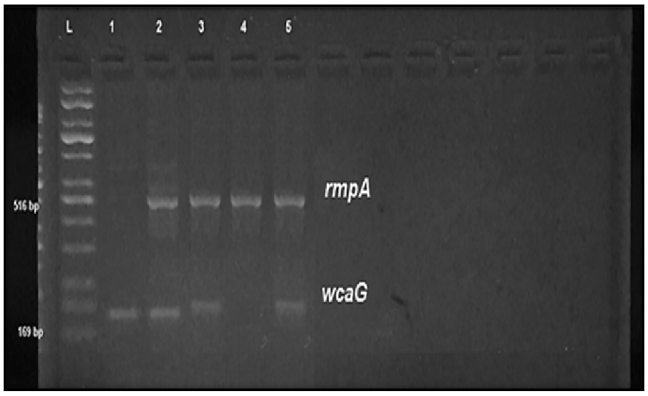
These antibiotics are inactivated by an array of differently extended-spectrum beta-lactamases (ESBLs) which have improved by mutation of TEM/SHV type beta-lactamases. The plasmid encoding these enzymes have been found in the various genus of the family Enterobacteriaceae most usually harbored by *K. pneumonia*(Sikarwar and Batra, 2011)

Detection of the Virulence Genes of rmpA and wcaG with PCR

Out of 10 isolates of *K. pneumoniae* the encoding genes of rmpA with wcaG which identified in 5 isolates (50%); rmpA was identified in 4 isolates (80%) and wcaG in another 4 (80%) isolates, three of these isolates possessed two avirulence gene of (rmpA and wcaG). from 4 rmpA-positive isolates, 2 were isolated from sheep nasal discharge and 2 from goat, while The 4 wcaG-

positive strains, 2were isolated from sheep nasal discharges, one isolates from goat and other isolates from cattle results in Fig. 3.

The presence of the virulent gene with resistance may produce a survival advantage to the microorganism. Sometimes the *K. pneumoniae* isolates represent a pronounced polysaccharide capsule, which is essential virulence agents and presents a mucoid phenotype (Cheng *et al.,* 2010). The plasmid gene rmpA (regulator of mucoid phenotype) confers a hyper-mucoviscous phenotype to *K. pneumoniae* by promoting capsular polysaccharide production while the wcaG virulence gene is located in the transferable part of chromosome that responsible for K. pneumoniae capsule biosynthesis and required for the



Photograph 3: Identification the genes of rmpA with wcaG, L: 100 bp with ladder, 1-5 Clinical Isolates.

Table 4: confirmation results of the pure Citrus honey.

Test	Results	Iraqi honey Standards	Remarks
Physical test	Amber color		
1- Color			
2- Odor Without			
3- Taste sweet			
4- purity pure			
Chemical test			
1- moisture	17	21% Max	
2- Invert sugars			
a- Glucose	41	Not less than 74%	
b- Fructose	47	34% glucose	
c- F/G	88	40% fructose	
3- Sucrose	1	8 % Max	
4- Acidity	33	40ME/ Kg Max	
5- Water insoluble solid	0	0.1 Max	
6- Ash	0.06	0.3 % Max	
Microbiological test			
1- Yeast and Molds	Nil	Nil	
Source	Citrus Flower		

Table 5: Antibacterial activity of citrus honey against human *K. pneumoniae.*

NO. of *K. pneumoniae* isolates	Citrus honey concentration with diameter of inhibition zone in (mm)				LSD value
	25%	50%	75%	100%	
1	9	12	16	19	4.73*
2	9	11	15	19	4.08*
3	7	12	13	18	3.79*
4	6	13	14	17	4.18*
5	5	11	13	17	4.52*
LSD value	3.55*	3.19NS	3.07NS	2.82NS	——
	*(P<0.05), NS: Non-Significant.				

conversion of mannose to fructose, that improve the potential of bacteria to evade from the phagocytic cell (Vila *et al*., 2011) the resistance and virulence are not independent properties, and their relationship may play an essential function in the pathogenesis of K. pneumoniae infections.

The confirmation results of the pure honey

Dissolution Test: This is a process of adding some portion of honey into water. If the honey is impure, it will dissolve in the water at the top. But if the honey is pure, it will stick together and sink to the bottom of the glass.

Crystallization Test: This involves subjecting the honey to low temperature. At temperature even below 5°C, pure honey will not crystallize. Therefore, the original texture and flavor are preserved indefinitely.

Physico-chemical and microbiological properties:

The confirmation results of the purity of citrus honey as in table 4.

Antimicrobial activity of citrus honey against *K. pneumoniae*

The results of antimicrobial activity of citrus honey showed varying degree of inhibition zone against *k. pneumonia* when tested in Mueller-Hinton agar (well diffusion method) the table 5 and Fig 4 shown the results and diameter of antimicrobial activity of different concentration of citrus honey against five Multidrug resistant *k. pneumonia* isolates. The maximum zone of inhibition was shown on *K. pneumonia* was (19mm) with 100% concentration while the minimum zone (5mm) with concentration of 25%.

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