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DETERMINATION OF BETA LACTAM RESISTANCE OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM CLINICAL SPECIMENS AND WATER SAMPLES

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ABSTRACT : Ninety three *Klebsiella pneumoniae* isolates were collected from clinical and water samples. Sixty- six (20.625%) clinical isolates were isolated from 320 clinical samples from different hospitals at Medical City Iraq, Baghdad. Twenty-seven (18%) water isolates were collected from 150 different water sources from different water samples of Baghdad city. All isolates were diagnosed by biochemical tests and by CHROM agar. The isolates were tested for their ability to beta-lactam antibiotics (Imipenem, Meropenem, Amoxiclave, Cetazidime, Ceftriaxone and Cefotaxime) resistance and Minimum Inhibitory Concentration (MIC) determined for each isolate by using agar dilution method. The results show that the clinical isolates were more resistance to beta lactam antibiotics than water isolates and both of them were (100 %) resist to Amoxiclav. The resistance percentage of the clinical isolates was (6.06%) Meropenem, (13.63%) Imipenem, (19.69%) Ceftazidime, (53.03%) Cefotaxime and (86.36%) Ceftriaxone, while the resistance percentage for the water isolates was zero for the Meropenem, Imipenem, Ceftazidime, Cefotaxime and Ceftriaxone. The most resistance clinical and water isolates for beta-lactam antibiotics were diagnosed by PCR technique and they were documented by the National Center for Biotechnology Information (NCBI) of American and registered for the first time in this study as a new *K. pneumoniae* strain; clinical isolates (GMH1 80, GMH2 80, GMH11 80 and GMH12 80), while water isolates (GMH3 80, GMH4 80, GMH5 80, GMH6 80, GMH7 80, GMH8 80, GMH9 80, and GMH10 80).

Key words : Clinical specimens, *Klebsiella pneumonia*, biochemical species, water samples.

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INTRODUCTION

Antimicrobial resistance (AMR) is of significant concern in developing nations due to over-use of antimicrobial agents, widespread availability of counterfeit or substandard drugs and poor infection control measures (Taitt *et al*, 2017). *Klebsiella pneumoniae* is regarded globally by the World Health Organization (WHO) as a priority antimicrobial resistant (AMR) pathogen requiring new control strategies (Wyres *et al*, 2020).

K. pneumoniae is a gram-negative, encapsulated, non-motile, rod-shaped member of the Enterobacteriaceae family (Ashurst and Dawson, 2020). They are members of the normal intestinal flora of humans and animals and are also found in the normal flora of skin and mouth. Most of the nosocomial *Klebsiella* infections are caused by *K. pneumoniae* and medically, it is categorised as the most important species of the genus. Consumption of contaminated fish and water had been linked to infections by *Klebsiella* (Abd Al-Kareem *et al*, 2015).

Water pollution and the destruction of ecosystems continue to increase and it is contamination is now a major problem in the global context as a consequence of industrialization, globalization, population growth, urbanization and warfare combined with increased wealth and more extravagant lifestyles (Siri *et al*, 2011). There may be a consumption of untreated water that may be contaminated with microbial species such as *Klebsiella* and there is a need to frequently assess the levels of contamination with *Klebsiella* species in water (Vuotto *et al*, 2017).

Currently, *K. pneumoniae* strains producing Extended Spectrum Beta-Lactamases (ESBLs) and carbapenemases have spread globally. These enzymes

inactivate -lactams, a class of antibiotics that forms the basis of effective treatment for patients suffering from infections with *K. pneumonia* (Wong *et al*, 2018).

The extrajudicial use of antibacterial agents has led to antimicrobial resistance both in pathogenic and commensal organisms. There are two possible ways through which drug resistance can develop, one is horizontal gene transfer and the other is dissimilar chromosomal loci through mutations (Doorduijn *et al*, 2016).

K. pneumoniae have two major antibiotic resistance mechanisms in them. One pathway involves the expression of extended-spectrum β-lactamases (ESBLs) that contributes to produce resistance in *K. pneumoniae* against cephalosporin and monobactam. Another extremely worse resistance mechanism is that the expression of Carbapenemases by *K. pneumoniae*, which contributes to the resistance of *K. pneumoniae* against most offered β-lactams as well as the carbapenems (Ali and Malik, 2020). These organisms or superbugs are returning in new forms resistant to almost all clinically important antimicrobials. Unfortunately, the pharmaceutical pipeline merely does not have enough new medicines to maintain pace with drug-resistant bacterial infections (Effah *et al*, 2020).

This study aimed to isolated *K. pneumonia* from different clinical specimens and water samples and studies the difference between their ability to resistance to beta -lactam antibiotics group by using agar dilution method.

MATERIALS AND METHODS

Bacterial isolates : A- Clinical specimens : From March to May 2019, 320 specimens (blood, burn, urine, sputum, wound, lung fluid and ear swab) were collected in sterilized containers from hospitals including: Baghdad teaching Hospital, Ghazi Al-Hariri Hospital for surgical Specialties and Teaching Laboratories at Medical city, Baghdad, Iraq. All specimens were streaked directly on blood agar and MacConkey agar at 37ºC for 18-240 hr. (Flournoy *et al*, 1990).

Water samples : From March to July 2019, 150 water samples were collected by sterile well screw cub. The samples were taken from different places (Lake, river, standing, steam, water wheal and tap water) at Baghdad city, from Al-Tarmiya, Al-Yousfiya and Al-Jadriya. Samples were taken 30 cm below the water surface, stored on ice for transportation, and processed for bacteriological analysis within 4 hr. of collection (Podschun *et al*, 2001). One ml of each water sample was added into 5 ml of MacConkey broth, incubated at 42ºC for 18-24 hr. Then one loopful of broth was cultured on MacConkey agar and EMB (Eosin Methylene Blue) agar to differentiate them from other Enterobacteriaceaes in order to obtain single isolates for used for extra identification and diagnosis tests.

All the samples (clinical and water samples) were tested for Gram staining for morphological identification, biochemical tests (Indole, Methyl red, Voges Proskauer, Citrate (IMViC)) were carried out to differentiate among *Klebsiella* species (Doorduijn *et al*, 2016; Vuotto *et al*, 2017) and CHROM agar to differentiate it from other genus of Enterobacteriaceae (Abdullah *et al*, 2009).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of antibiotics were determined by using agar dilution method (Andrews, 2001) as follow:

- Six beta lactam antibiotics were chosen, the suitable rang of antibiotic concentrations has been suggested for *Klebsiella pneumoniae*: Cefotaxime and Ceftazidime (0.004-128 mg/L), Ceftriaxone (0.001-128 mg/L), Amoxiclave (0.5-128 mg/L), Imipenem (0.06-4 mg/L) and Meropenem (0.015-4 mg/L).
- Preparation of stock solution of chosen antibiotics was done by using the formula:

$$\frac{1000}{P} \times V \times C = W$$

Where, P = potency given by the manufacturer (µg/mg), V = volume required (ml), C = final concentration of solution (multiples of 1000) (mg/L) and W= weight of antibiotic (mg) to be dissolved in volume V (ml). From the initial stock solution (10 000 mg/L), the further stock solutions were prepared.

- 20 ml of cooled molten agar (Muller Hinton agar cooled to 50ºC before adding to the antibiotic) was added to each container, including the antibiotic-free control, mix well before pouring into 90 mm Petri dishes then the agar was added and poured each concentration in turn, so agents are kept at 50ºC for minimum period of time. The plates were stored at 4-8ºC until inoculation.
- For prepare the bacterial inoculums, isolated colony were transfer by sterile loop into D.W or normal saline, incubate the broth with shaking at 35-37ºC until the visible turbidity is equal to or greater than the 0.5 McFarland standard. By using a micropipette, 1-2 µl of suspension was putting on to the surface of the agar; allow the inoculums to be absorbed into the agar before incubation at 35-37ºC in air for 18-20 hr.

Detection of isolates by 16S rRNA

DNA Extraction : Genomic DNA was isolated from bacterial growth according to the protocol of ABIOpure Extraction/ USA.

Quantitation of DNA

Quantus Florometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 ìl of DNA, 199 ìl of diluted QuantyFlour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

Primers : Primer sequence that used for 16S rRNA was 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1492R (5'-TACGGTTACCTTGTTACGACTT-3'), annealing temp. 60ºC, product size (1500 pb).

Agarose Gel Electrophoresis : After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria. Solutions used: 1 X TAE buffer, DNA ladder marker, Ethidium bromide (10mg/ml).

Standard sequencing : PCR product was send for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation, Korea. The results were received by email then analyzed using genious software.

Statistical analysis

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

RESULTS

Bacterial isolates : Clinical specimens : Sixty six of *K. pneumoniae* isolates were isolated from 320 different clinical specimens in (20.625%) isolation percentage. The bacterial clinical isolates were named as (KPC1, KPC2, KPC3… KPC66). The percentage of isolation for each sample from the total bacterial isolates (66 *K. pneumoniae* isolates) was demonstrated in the Table 1.

Water samples : Twenty seven *K. pneumoniae* isolates were isolated from 150 water samples in (18%) isolation percentage. The bacterial water isolates were named as (KPW1, KPW2, KPW3… KPW27). The isolation percentage of each type of water source from the total *K. pneumoniae* water isolates (27 isolates), sample type and the no. of isolates of isolation was demonstrated in Table 2.

The results of biochemical that were used in diagnosing of clinical specimens and water samples show that there is no difference in identification of clinical and water *K. pneumoniae* isolates.

Antibiotics resistance and MIC determination

The results show that there are big differences between resistances against beta lactam antibiotics between clinical and water isolates. The clinical isolates are more resistance than water isolates. Also, the result had been showing that all clinical and water isolates of

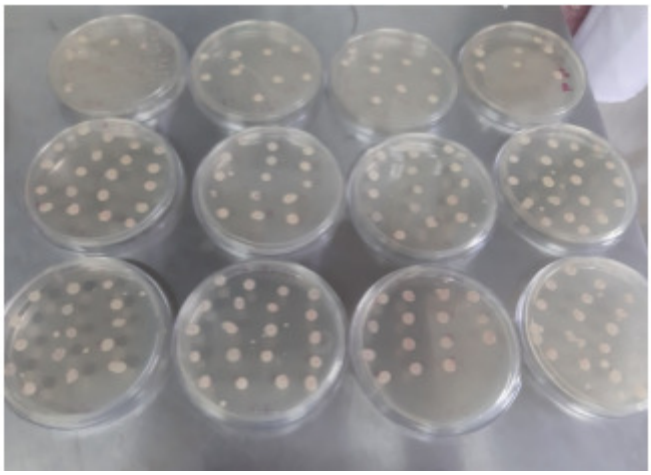

Fig. 1 : *K. pneumoniae* isolates grown on Muller-Hinton agar for sensitivity test and MIC determination of beta lactam antibiotics by agar dilution method.

Table 1 : The isolation percentage of *K. pneumoniae* for clinical specimens.

Specimen Type	Specimen No	Isolates No	%of isolation from 66 isolates	Total % of isolation from 320 specimen
Blood	65	22	33.3	6.88
Burn	60	16	24.24	5
Urine	60	14	21.21	4.38
Sputum	40	5	7.57	1.56
Wound	55	4	4.54	1.25
Lung fluid	10	3	4.54	0.94
Ear	20	2	3.03	0.63
Catheter	5	1	1.51	0.31
Pus	5	1	1.51	0.31

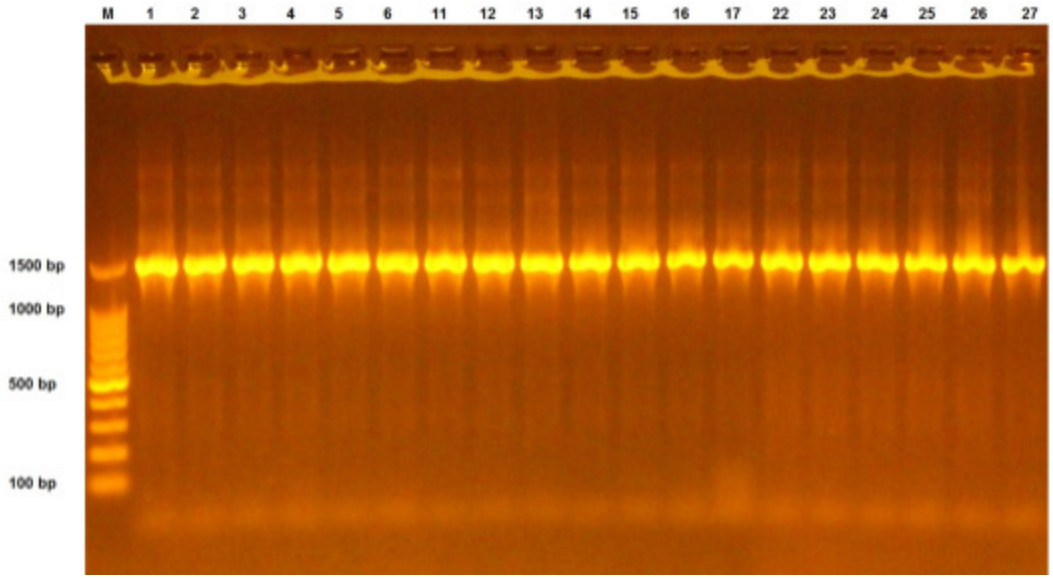

Fig. 2 : Gel electrophoresis of amplified PCR product of 16s rRNA (1500bp) of *K. pneumoniae*. 1.5% agarose gel electrophoresis stained with Ethidium Bromide. TBE buffer (1X). Lane1:100bp DNA marker. The clinical *K. pneumoniae* isolates (1-14) and water (15-27).

Table 2 : The isolation percentage of *K. pneumoniae* for water samples.

Sample type	No. of samples	No. of isolates	Percentage of isolation (%) from 27 isolate	Total percentage of isolation (%) from 150 sample
Lake	53	10	37.03	6.7
River	30	6	22.22	4
Standing	20	6	22.22	4
Steam	20	3	11.11	2
Wheel	12	2	7.4	1.3
Tap water	15	zero	zero	zero

Table 3 a : The MIC of beta lactam antibiotics of the most resistances *K. pneumoniae* clinical isolates by agar dilution method.

Clinical isolate no.	MIC of beta lactam antibiotics						P-value
	Cefotaxime (0.004-128)	Ceftazidime (0.004-128)	Ceftriaxone (0.001-128)	Amoxiclav (0.5-128)	Imipenem (0.06-4)	Meropenem (0.015-4)	
KPC1	≥128	32	≥128	≥128	1	1	0.0001 **
KPC3	≥128	≥128	≥128	≥128	0.25	≥4	0.0001 **
KPC4	≥128	≥128	≥128	≥128	≥4	≥4	0.0001 **
KPC9	≥128	≥128	≥128	≥128	≥4	1	0.0001 **
KPC10	≥128	≥128	≥128	≥128	≥4	≥4	0.0001 **
KPC14	≥128	≥128	≥128	≥128	≥4	≥4	0.0001 **
KPC22	≥128	≥128	≥128	≥128	≥4	≤ 0.015	0.0001 **
KPC26	≥128	≥128	≥128	≥128	≥4	≤ 0.015	0.0001 **
KPC27	≥128	≥128	≥128	≥128	≥4	≤ 0.015	0.0001 **
KPC38	≥128	≥128	≥128	≥128	0.5	0.125	0.0001 **
KPC39	≥128	≥128	≥128	≥128	0.5	≤ 0.015	0.0001 **
KPC48	≥128	≥128	≥128	≥128	≥4	≤ 0.015	0.0001 **
KPC55	≥128	≥128	≥128	≥128	0.5	≤ 0.015	0.0001 **
KPC62	≥128	≥128	≥128	≥128	0.5	0.125	0.0001 **

** (P≤0.01)

K. pneumoniae were resisting to Amoxiclav (128 mg/ml) and more sensitive to Imipenem and Meropenem than other beta lactam antibiotics (Ceftriaxone, Ceftazidime and Cefotaxime) and more sensitive to ceftazidme than (Ceftriaxone and Cefotaxime) and more sensitive to Cefotaxime than Ceftriaxone. The of MIC for the six beta lactam antibiotics that were used in this study by agar dilution method (Fig. 1) for clinical and water *K.*

Table 3 b : The MIC of beta lactam antibiotics of the most resistance *K. pneumoniae* water isolates by agar dilution method.

Water isolate no.	MIC of beta lactam antibiotics						P-value
	Cefotaxime (0.004-128)	Ceftazidime (0.004-128)	Ceftriaxone (0.001-128)	Amoxiclav (0.5-128)	Imipenem (0.06-4)	Meropenem (0.015-4)	
KPW1	64	16	64	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW5	16	8	32	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW6	1	1	4	≥128	≤ 0.06	≤ 0.015	0.0001 **
-KPW9	1	1	4	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW12	1	0.5	4	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW17	1	1	4	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW18	0.5	1	4	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW19	16	4	32	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW20	1	1	4	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW23	1	1	4	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW24	1	1	16	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW25	0.5	1	4	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW26	1	1	8	≥128	≤ 0.06	≤ 0.015	0.0001 **
KPW27	1	1	4	≥128	≤ 0.06	≤ 0.015	0.0001 **

** (P≤0.01).

Table 4 : The resistance percentage to beta lactam antibiotics for clinical and water *K. pneumoniae* isolates.

Antibiotic		Percentage for clinical isolates	Percentage for water isolates	P-value
Name	Concentration			
Imipenem	(4 mg/ml)	9 (13.63%)	Zero (0%)	0.0351 *
Meropenem	(4 mg/ml)	4 (6.06%)	Zero (0%)	0.0936 NS
Ceftazidime	(128 mg/ml)	13 (19.69%)	Zero (0%)	0.0081 **
Cefotaxime	(128 mg/ml)	35 (53.03%)	Zero (0%)	0.0001 **
Ceftriaxone	(128 mg/ml)	57 (86.36%)	Zero (0%)	0.0001 **
Amoxiclav	(128 mg/ml)	66 (100%)	27 (100%)	1.00 NS
P-value		0.0001 **	0.0001 **	—
P-value		0.0001 **	0.0001 **	—

* (P≤0.05), ** (P≤0.01)

Table 5 : DNA Concentration (ng/µl) in clinical and water *K. pneumoniae* isolates.

No. of clinical sample	Conc. (ng/µl)	No. of water sample	Conc. (ng/µl)
1	16	15	28
2	22	16	14
3	20	17	17
4	18	22	20
5	16	23	25
6	19	24	30
11	15	25	22
12	23	26	23
13	25	27	18
14	30	28	18

pneumoniae isolates were listed in Table 3a and b.

The percentage of resistance to beta lactam antibiotics are listed in Table 4 in descending arrangement of resistances from the most effective to the lowest effective of antibiotics against clinical and water isolates.

Detection of the isolates by 16S rRNA

Total 20 *K. pneumoniae* isolates (10 clinical and 10 water) that were the most resistance to the six beta lactam antibiotics that were used in this study were detected by PCR technique by using of 16S rRNA. The isolates that were chosen for detection by 16S rRNA were named as (1, 2, 3, 4, 5, 6, 11, 12, 13, 14) for clinical isolates and (15, 16, 17, 22, 23, 24, 25, 26, 27, 28) for water isolates. All the detected isolates were positive for this detection as shown Fig. 2.

DNA Concentration (ng/µl) in extracted DNA of clinical and water *K. pneumoniae* isolates was measured by using Quantus Florometer (Table 5).

Twelve (four clinical and eight water) of *K. pneumoniae* isolates were registrated as a new strain in the National Center for Biotechnology Information

(NCBI). GMH1 80 (MN749595.1), GMH2 80 (MN749597.1), GMH11 80 (MN985818.1) and GMH12 80 (MN989349.1) for clinical isolates. GMH3 80 (MN749600.1), GMH4 80 (MN749602.1), GMH5 80 (MN749603.1), GMH6 80 (MN749604.1), GMH7 80 (MN749605.1), GMH8 80 (MN749610.1), GMH9 80 (MN749614.1) and GMH10 80 (MN749618.1) for water isolates.

DISCUSSION

K. pneumoniae has become a relevant healthcare-association pathogen, being the causative agent of approximately 14-20% of the infections related to respiratory tract, lower biliary duct, surgical wounds and urinary tract (Vuotto *et al*, 2017).

Environmental isolates have been described as being indistinguishable from human clinical isolates with respect to their biochemical reactions and virulence. While the medical significance of *Klebsiella* obtained in the natural environment is far from clear, such habitats are thought to be potential reservoirs for the growth and spread of these bacteria which may colonize animals and humans (Podschun *et al*, 2001). According to Dumaru *et al* (2019), the isolation percentage of clinical *Klebsiella* spp. was (16%). Another study recorded the isolation percentage of *K. pneumoniae* (12.5%) (Shahidul *et al*, 2013), 15 (10%) in urine sample (Alcantar-Curiel *et al*, 3013). A local study by Mohammed (2018) recorded the isolation percentage for clinical isolates was 12 (60%) and for water isolates was 20(77%). Another local study by Abbas (2020) recorded that the isolation percentage from wound was 36 (60%) and from burn 24(40%). The differences between the isolation percentages of *K. pneumoniae* in this study with the other previous results may be due to the place, time, the number of specimens and samples, methods used for diagnosis and the circumstances that may contribute the isolation period.

According to Effah *et al* (2020), it can be seen that there is an increasing trend in the isolation rate of *K. pneumoniae* (from 9.8% in 2005 to 13.3% in 2012) in China. Interestingly, the isolation rate decreased in 2007 but increased in the preceding years. In contrast, the resistant trends of *K. pneumoniae* in China were not congruent to the isolation rate as there were decreasing resistance trends from 2005 to 2014. Imipenem recorded the lowest resistance rate but its resistance trends tend to increase steadily from 2005 to 2014. It can be seen that *K. pneumoniae* has a great resistance rate to most of the commonly used antimicrobials. Cefotaxime recorded the highest prevalence (79.2%).

Among the 168 *K. pneumoniae* isolates studied, 121 (72.0%) were resistant to cefazidime, 115 (68.5%) to cefotaxime. The MIC to cefotaxime and ceftazidime in these strains was >128 mg/mL. All cefazidime and cefotaxime resistant isolates were positive for ESBL production. All isolates were susceptible to imipenem (Alcantar-Curiel *et al*, 2013). The antibiotics resistances to ceftazidime was 75.51% and for Imipenem 53.1% among *K. pneumoniae* that isolated from urine (Dumaru *et al*, 2019).

Another study recorded that A total of 167 (17.36%) *K. pneumoniae* isolates, most of them were extensively resistant to antibiotics. A more favorable profile was found towards meropenem showing 20% resistance (Nirwati *et al*, 2019). Antibiotic resistance profiles of the isolates using disk diffusion tests demonstrated that the isolates were highly resistant to ceftriaxone, ceftazidime, augmentin (Sasikala *et al*, 2015).

Local study by Mohammed (2018) have show that the resistance of clinical *K. pneumoniae* isolates was 80% to Cefotaxime and 30% to Ceftazidime and 10% for Imipenem and Meropenem while the resistance of water *K. pneumoniae* isolates was 35% Cefotaxime, 15% Ceftazidime and no resistance to Imipenem and Meropenem. Another study show that the resistance of water *K. pneumoniae* isolates was 5% for Cefotaxime and 12% for Ceftazidime (Njugu, 2011). Also in 2015 it has recorded that water isolates were resisting 52% to Cefotaxime (Abd Al-Kareem *et al*, 2015).

Also, another local study by Aljanaby and Alhasani (2016) show that 97.75% resistance of clinical *K. pneumoniae* isolates to Cefotaxime and 71.87% to Ceftazidime. Kumar (2013) recorded 45.1% resistance to Ceftazidime. Babakhani *et al* (2014) recorded 1.2% to Meropeneme and 67.5% to Imipenem while Manikandan and Amsath (2013), recorded that resistance to Imipenem was 13.9%. A local study recorded that all clinical and environmental isolates that used in the study were multidrug resistance for β-lactam antibiotics except carbapenemes and the clinical isolates were more resistance than environmental isolates to multiple antibiotics that used in the study (Ibrahim and Hameed, 2015).

The 16S rRNA of each species of bacteria has stable (conserved) portions of the sequence. Many copies are present in each organism. Labeled probes specific for the 16S rRNA of a species are added, and the amount of label on the doublestranded hybrid is measured. This technique is widely used for the rapid identification of many organisms (Brooks *et al*, 2013).

While, there are several new therapeutic approaches

under investigation including the use of bacteriophages, enzybiotics (phage-derived lytic enzymes) and antibodies against *Klebsiella* surface molecules; unfortunately, at present, we cannot identify candidate compounds in late-stage development for treatment of multidrug *Klebsiella* infections (Bengoechea and Pessoa, 2019).

CONCLUSION

The results is agreed with the previous results that recorded that there is no differences in biochemical identification of clinical and water *K. pneumonia* isolates and the clinical isolates is more resistance to beta lactam antibiotics than water isolates.

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