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Original article

Reveal by genotyping and phenotyping methods of some Metallo-Beta Lactamases genes among environmental *Klebsiella pneumoniae* isolates

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ABSTRACT

Background: Since carbapenems are currently the preferred treatment for severe infections brought on by multidrug-resistant bacteria which can create Extended-Spectrum β-Lactamases (ESBLs), it is extremely concerning that Gram-negative bacteria are becoming resistant to carbapenem. It has been demonstrated that *Klebsiella pneumoniae* produces a beta-lactamase that hydrolyses the β-lactam ring in antibiotics, making it one of the few bacteria which are currently exhibiting a high value of resistance because of changing in the organism's core genome. **Methods:** For the current study, 50 samples were gathered from different water sources, and based on morphological and biochemical testing, 10 isolates were determined to be *K. pneumoniae*. According to Kirby and Bauer's disc diffusion test, all of the isolates under investigation exhibited resistance to Ampicillin, Cefitrixone, Amoxicillin, Ticarcillin+Clavulanic acid, Ceftazidime, and Ticarcillin. Every specimen that had the highest percentage of resistant isolates was 100% effective against the antibiotics being studied. E-test strips were used to detect the isolates' Minimum Inhibitory Concentrations (MICs) value. **Results:** Upon using Combined Disc Test (CDT) to detect Metallo- beta lactamases, it was found that 8.3% of isolates were MBLs producers. Nevertheless, PCR technique exposed those three isolates only harbored *IMP-1* gene and no one was had *NDM-1* gene. **Conclusions:** Current study found relationship between *IMP-1* and resistant to antibiotics, when $P \leq 0.05$. For that *K. pneumoniae* isolated from water began resist to carbapenems antibiotics by horizontal way or by plasmid from clinical isolates to environmental isolates.

Introduction

Klebsiella spp. is a major source of (Nosocomial and Community-Acquired) infections and can cause opportunistic infections [1, 2]. It is rod-shaped, non-motile, and has a noticeable polysaccharide capsule. The entire cell surface is encased in this capsule, which also gives the organism its large appearance on gram stain and resistance to numerous host defense mechanisms. Most ferment lactose result in highly mucoid colonies on plates due to the luxuriant polysaccharide capsule that is produced [3]. Broad spectrum antibacterial drugs called carbapenems are reserved for use as a last resort in cases of infections brought on by isolates of *K. pneumoniae* that are resistant to multiple drugs [4, 5]. About 30% of strains are capable of fixing nitrogen in anaerobic environments, and it is found naturally in soil. If aspirated, it can damage the lungs of both humans

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and animals, particularly the alveoli, causing bloody sputum. It is present in the typical oral, skin, and intestinal flora [6].

Because water systems are exposed to high concentrations of antibiotic resistance genes (ARGs) and antimicrobial-resistant bacteria (ARBs) from human and animal waste, they are a major area of research interest. Antibiotic resistance in bacteria is encouraged by the elevated levels of antibiotic deposit in waste-water. A lot of researches have shown that waste-water acts as a source for antibiotic resistance genes, which continue to exist in wastewater treatment plant effluents even after disinfection and filtration. Depending on the phylogroup, *Klebsiella* spp. can be found in a variety of settings, such as surface waters, plants, soil, and waste-water [7].

Multiple antibiotic resistances are common in *Klebsiella* organisms, and recent research suggests that plasmids are the main source of these resistance agents. Generally speaking, *Klebsiella* infections are more common in those with compromised immune systems; middle-aged and older men with incapacitating illnesses are most frequently affected. Both pneumonia and urinary tract infections are the most prevalent illnesses brought on by *Klebsiella* [8]. *Klebsiella* can cause infections in surgical wounds, the lower biliary tract, and the urinary tract in addition to pneumonia. *Klebsiella* species that produce Extended-Spectrum β-Lactamases (ESBLs) are not sensitive to β-lactam antibiotics, with the exception of carbapenems. Other common targets for resistance include trimethoprim/sulfamethoxazole, tetracyclines, aminoglycosides, fluoroquinolones, and chloramphenicol [9].

The majority of ESBLs-Enterobacteriaceae infections were previously linked to medical care. According to recent reports, the epidemiology of these infections has changed, with community-associated infections accounting for nearly half of cases [10]. Bacteria produce beta-lactamases, which are enzymes that give β-lactam antibiotics like Cephalosporins, Penicillins, Cephamycins, and Carbapenems (ertapenem) multiple resistances. However, in comparison, carbapenems are resistant to β-lactamases. By disrupting the structure of antibiotics, beta-lactamase produces resistance to them. These entire antibiotics share a four-atom ring called a β-lactam in their molecular structure. By hydrolyzing the β-lactam ring, the lactamase enzyme deactivates the antibacterial properties of the molecule [11].

The synthesis of carbapenemases is the main mechanism by which Enterobacteriaceae can give carbapenem resistance. Class B of Metallo-Beta-Lactamases (MBLs) include imipenemases (IMPs), these enzymes can hydrolysis β-lactams, like carbapenems and cephalosporins. IMPs were discovered in member of Enterobacteriaceae and *Acinetobacter* species [12]. The novel MBL known as New Delhi- Metallo beta lactamase-1 (NDM-1) which can resist β-lactam antibiotics. Nonetheless, a number of strains that carry NDM-1 are also resistant to aztreonam, most likely due to an alternative resistance mechanism. Since the blaNDM-1 gene is found on plasmids, leaving few or no route for treatment. NDM-1 was primarily instituted in *Escherichia coli* and *K. pneumoniae,* with smaller amounts in *Pseudomonas* and *Acinetobacter* [13]**.**

This study aims to isolation of *Klebsiella pneumoniae* from water samples, detection the sensitivity of isolates to antibiotic discs and E-strips phenotyping and detection of *IMP-1* and bla_{NDM-1}among *Klebsiella pneumoniae* from environmental samples in Baghdad, Iraq.

Methods

Collection and identification of bacteria: 50 water samples were collected from different water sources at Baghdad governorate in clean sterilized bottled; the samples were culture in MacConkey broth using standard methods [14]. *K. pneumoniae* identified depending on the morphological features on culture media as well as under the microscope. They were inoculated onto plates of MaCconkey agar (Himedia /India) and brood at 37°C for 24hrs. Colonies of bacteria were grown on blood agar plate (Hi media /India) were tested for their shape, size, color and blood haemolysis pattern, the isolates showed gamma hemolysis. For Simmone citrate utilization and the indole tests, *K. pneumoniae* isolates were showed positive and covert of indicator color from green to blue for Simmon citrate utilization and negative for indole.

Antibiotic sensitivity test: Himedia/India's Mueller-Hinton agar (MHA) was employed. Colonies from the tested isolates' overnight culture were used after adjusting to McFarland standard. The plates were inoculated by a sterile swab that has been dipped into the inoculums [15]. The inoculated plate was covered with the chosen antibiotic disc

(table 1) and the E-test strips (table 2). After warming to room temperature, the discs should be dispensed onto the agar surface and gently pressed down with forceps, then incubated.

MIC determining by E-test method

Antibiotic strips are listed in table (2). Five antibiotics were used Cefoperazone, Cefoxitin, Cefotaxime, Gentamycin, Levofloxacillin, Meropenem+EDTA, and Amikacin (Bioanalyse). Kirby and Baures conducted a test on antibiotic sensitivity [15]. The organisms were cultivated in Mueller- Hinton broth (Himedia/India) for eighteen hours at 37ºC. Sterile swabs were used to inoculate Mueller Hinton agar MHA (Himedia/India) plates after they had been diluted to 1×10^8 cells/ml. Antibiotic strips were then put on the media and lightly pressed, incubated for the entire night at 37ºC. The results were compared with CLSI [16]. Following incubation, the zones of inhibition diameters were measured in milli-meters. By comparing it with the standard inhibition zone, the diameter was converted into sensitive (S) and resistant (R) categories.

Detection of Metallo β-lactamase:

Isolates exhibiting imipenem resistance were examined in this study in order to look for potential MBL production. Mueller-Hinton agar plates were inoculated with test organisms. Phenotypic methods CDT were used to detect MBLs [17].

Combined Disc Test (CDT)

Two discs of 10 µg imipenem were positioned 30 mm apart and inoculated with the test organism while adhering to (0.5) McFarland standards on MH- agar. And another imipenem discs with EDTA. After overnight of incubation at 37°C, imipenem disc and imipenem + EDTA discs' zones of inhibition were contrasted. Test organism may be deemed MBL positive when the zone of inhibition for imipenem + EDTA discs is larger than 4 mm when compared to imipenem alone.

Detection of *IMP-1* 1and *NDM-1* genes by PCR

The primers were lyophilized, dissolved in ddH2O to create a concentration of 100 (pmol/µl) as stock, and stored at deep freezer to create 10 (pmol/µl) concentrations as primer suspended used for working, according to IDT (Integrated DNA Technologies Company, Canada). To get the total volume of 100 µl, 10 µl of the stock was mixed with 90 µl of ddH2O water.

After conducting multiple experiments to determine the ideal conditions for initial denaturation and annealing, the temperature of each sample was adjusted using gradient PCR to determine the ideal conditions. Additionally, the concentration of DNA template was adjusted between 1.5 and 2 µl, which are significant factors in primer annealing with complement.

PCR procedure:

The DNA bacteria were extracted by using method of thermal lysis then centrifugation at 4 °C for 30 (second) at 9,000 (rpm). The amount of DNA was measured by Nanodrop. About 12.5 (µl) of Master Mix (Promega Corp., USA), mixed with 1.5 (µl) of primers listed in Table (1), 100 (ng) of DNA, and free -nuclease water was used with 25 (µl) as final volume for each one. As previously stated, PCR amplification method was used to detect *IMP-1* and *NDM-1*. The program of PCR with an initial denaturation was made to 2 min at 95°C. Then to 30 sec. at 90°C with 30 cycles, 52 °C and 72 °C for 1 min, followed by a final extension to 8 min at 65°C. As well as, PCR amplified products were done with 1.5% agarose gel in Tris-acetate-EDTA buffer. The products were run for 110 min at 75 V. The bands were observed after staining with ethidium bromide by using an UV-light [18].

Results

Identification of bacterial isolates

In Baghdad, 10 *K. pneumoniae* were obtained from various water samples. We cultivated the bacteria on MacConkey agar for detection, and the gram-negative bacteria showed up as pink colonies. The media was blue because the bacteria could use the citrate when they were cultivated on Simmon Citrate Agar. The *K. pneumoniae* indole test came back negative. Additionally, the hemolysis type on blood agar was gamma.

Sensitivity test for antibiotics

A variety of antibiotics were used to test the sensitivity in order to identify antibiotic resistance. The isolates used in the test were 100% resistant to Cefitrixone, Ampicillin, Amoxicillin, Ticarcillin, Ticarcillin+Clavulanicacid (TCC), and Ceftazidime, as shown in Figure 1 and Table 4. In contrast, the sensitivity of SPX and Ticarcillin+Cilactin (IC) was 90% and 10%, respectively. The respective dosages of ciprofloxacin, nitrofurantoin, and 70%, 20%, and 10% were administered. However, only one isolate was resistant to imipenem, but other isolates were

90% sensitive to it. However, amikacin was found to be sensitive in 50% of sensitive isolates, 10% of intermediate isolates, and 40% of the remaining isolates.

E-test strips

This study employed diffusion methods to determine the (MIC) of antibiotics employing in the E-test. According to table (5), the outcome displayed an elliptical inhibition zone surrounding the strips. Two isolates were treated with an ampicillin strip in this investigation; however, since the isolates were ampicillin-resistant, the MIC value was not provided. MIC values for azithromicin ranged from 8 to 16 µg/ml. Figure (2) illustrates the Cefitrixion strip's MIC value, which was between 19 and 20 µg/ml.

Combined Disc Test (CDT)

This test was used to detect these isolates can produce Metallo β-lactamase. And in our results were showed only one isolate can produce the enzyme from twelve, while the other isolates cannot produce the enzyme were number eleven in figure (3) and figure (4).

Identification of genes by PCR 4-

Only three of the ten isolates, according to PCR results, have the *IMP-1* gene (5,9,10), while the remaining isolates (1,2,3,4,6,7,8) do not. *NDM-1* content is none in all isolates Figure (5).

Relationship between *IMP-1* gene and resistant to antibiotics

The isolates were harbor *IMP-1* gene which was more resistant to antibiotics. While the isolates were not harbor this gene was more sensitive to antibiotics used in current study. For that, current study supposed there is relationship between *IMP-1* gene and resistant to antibiotics of *K. pneumoniae* isolated from water. Results is bellow in figure (6) showed there is correlation between *IMP-1* gene and resistant to antibiotics when $P \leq 0.05$.

Table 1. Antibiotic discs used in current study.

ID	Antibiotics	Potency	Company
1	Ampicillin(AMP)	30µg	Bioanalyse/Turkey
2	Amoxicillin(AMC)	30µg	
3	Cefitrixon(CRO)	30µg	
4	Ticarcillin(TI)	10µg	
5	Ticarcillin/Clavulanic acid(TCC)	75/10µg	
6	Sparfloxacin (SPX)	10µg	
7	Imipenem/Cilactin(IC)	10µg/10µg	
8	Kanamicin(KF)	10µg	
9	Amikacin(AK)	10µg	
10	Ciprofloxacin(CIP)	10µg	
11	Ceftazidime(CAZ)	30µg	
12	Tetracycline(TE)	30µg	
13	N)(Nitrofurantoin	30µg	
14	Imipinem (IPM)	10µg	

Table 2. E-test antibiotic strips.

Antibiotics strips	Symbols	Company
Cefoperazone	CFP	Bioanalyse/Turkey
Cefoxitin	FOX	
Cefotaxime	CTX/CTX+	
Gentamycin	HLG	
Levofloxacillin	LEV	
Amikacin	AMK	
Meropenem+EDTA	MRP+EDTA	

Table 3. Primers of (*IMP* -1 and *NDM-1)* genes.

Primer	Sequence	GC (%)	Product size(bp.)	Reference
F- *IMP-1*	(F-5'/ CACCTCATGTTTGAATTCGCC-/3')	50.00	232 bp.	[18]
R- *IMP-1*	(R-5'/ CTCTGTCACATCGAAATCGC-/3')	50.00		
F-*NDM-1*	(F-5'/GGTTTGGCGATCTGGTTTTC-/3')	50.00	264 bp.	[13]
R-NDM-1	(R-5'/ CGGAATGGCTCATCACGATC-/3')	50.00		

Table 4. The results of Antibiotics Sensitivity test.

Antibiotics	Sensitive		Intermediate		Resistant	
	No. of isolates	Percent (100%)	No. of isolates	Percent (100%)	No. of isolates	Percent (100%)
Ampicillin(AMP)	0	0%	0	0%	10	100%
Amoxicillin(AMC)	0	0%	0	0%	10	100%
Ceftriaxone (CRO)	0	0%	0	0%	10	100%
Ticarcillin(TI)	0	0%	0	0%	10	100%
Ticarcillin/Clavulanic acid (TCC)	0	0%	0	0%	10	100%
Sparfloxcin (SPX)	9	90%	1	10%	0	0%
Ticarcillin+Cilactin(IC)	9	90%	1	10%	0	0%
Kanamycin (KF)	4	20%	0	0%	8	80%
Amikacin(AK)	5	50%	1	10%	4	40%
Ciprofloxacin(CIP)	7	70%	1	10%	2	20%
Ceftazidime (CAZ)	0	0%	0	0%	10	100%
Tetracycline(TE)	3	30%	0	0%	7	70%
Nitrofurantoin(N)	7	70%	1	10%	2	20%
Imipenem (IMP)	9	90%	0	0%	1	10%

Table 5. Results of E-test strips method.

ID	E-test strips symbol	MIC Range value µg/ml
1	**Ampicillin (AMP)**	**0**
2	**Azithromicin (AZI)**	**8-16**
3	**Cefitrixion(CTR)**	**19-20**
4	**Cefoxitin**	**4-6**
5	**Cefoperazone**	**8-12**
6	**Cefotaxime/ Cefotaxime +**	**0.32/2-1.5/0.16**

Figure 1. Sensitivity test with antibiotics discs in environmental isolates.

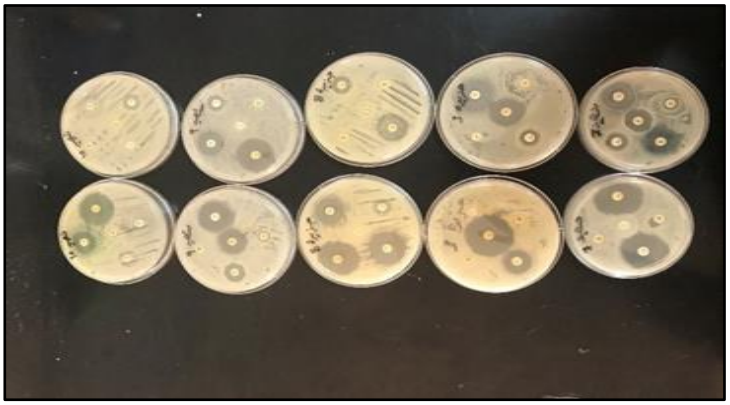


Figure 2. Identification of Metallo β-lactamase producing by *K. pneumoniae* with E-strips.

Figure 3. The presence of isolates produce Metallo β-lactamase.

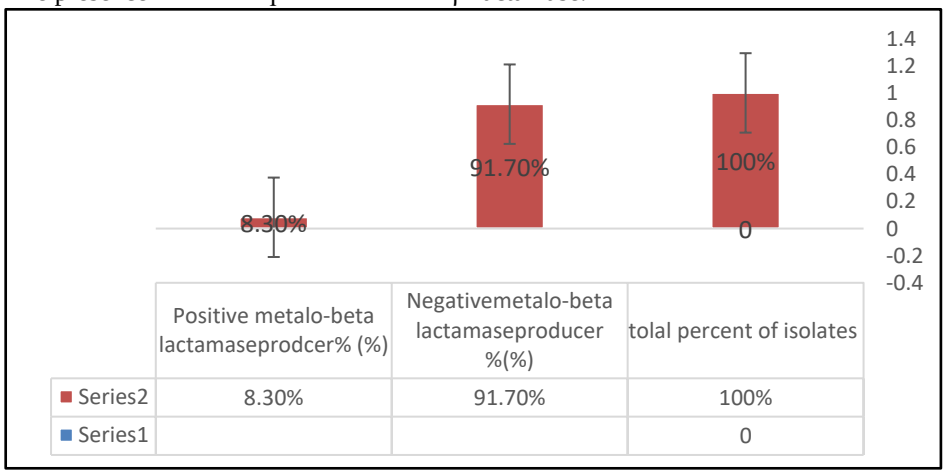


Figure 4. Detection of Metallo β-lactamase by CDT test.

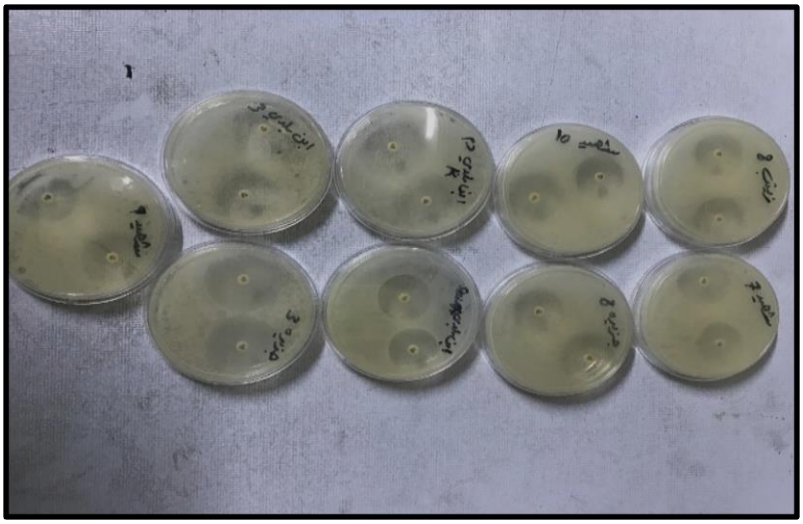


Figure 5. Results of the amplification of *IMP-1*(232 bp), *and NDM-1*(264bp) PCR product. M: DNA ladder (100bp), samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. only (5,9,10) on the left lanes were positive *IMP-1*, while the lanes (1, 2, 3, 4, 6, 7, and 8) were negative, while on the right shows negative *NDM-1* result for all isolates. N: negative control.

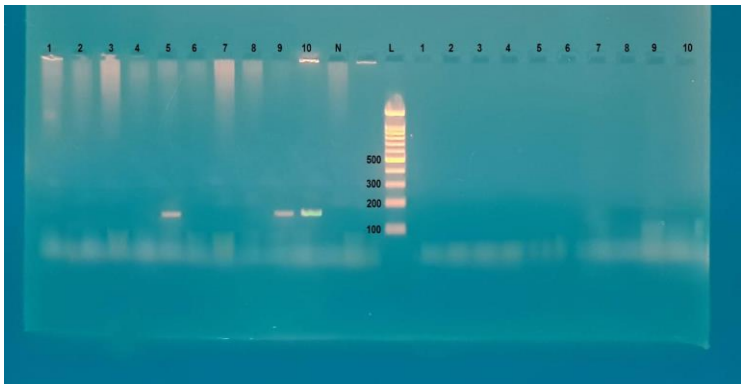
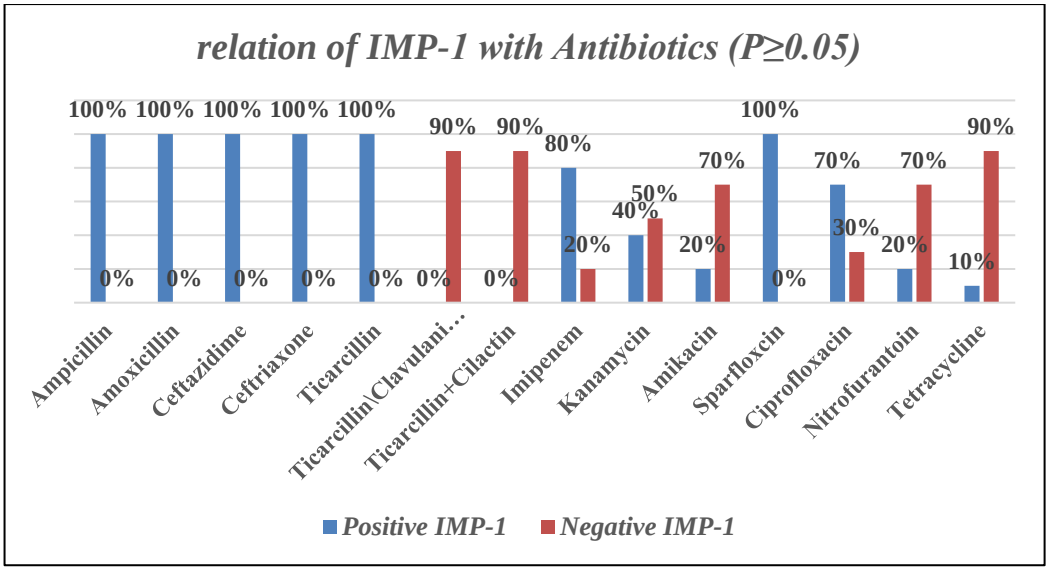


Figure 6. Relationship between *IMP-1* gene and resistant to antibiotics.



Discussion

An ecological problem, antimicrobial resistance is defined by complex relationships between various microbial populations that affect the health of people, animals, and the environment. Hasan and Aburesha in (2021) [19] reported that the isolation rate of *K. pneumoniae* was 18%, with 27 isolates obtained from 150 distinct water samples in Baghdad city and the resistance percentage was 100 % for amoxiclav and zero for the imipenem, ceftazidime, meropenem, ceftriaxone, and cefotaxime for *K. pneumoniae* isolated from different water samples. Another local study documented that out of 65 water samples collected from various surface locations in Baghdad city, 26 were identified, with 20 classifieds as *K. pneumoniae*, 4/26 [20]. *K. pneumoniae* from Taiwan was discovered in another study that concentrated on isolates from the community. Their data showed reduced susceptibilities to fluoroquinolones and the majority of β-lactam antibiotics [21].

The E-strips test MBL was given positive result for all isolate's MBL production in another article. For every *K. pneumoniae* isolate that tested positive for bla_{IMP-1}, Similarly, according to its methodology, the Direct MBL test showed an extension of the zone of inhibition surrounding the imipenem disk toward the TE (Tris-EDTA disk), indicating that the isolates were generating an MBL [17]. Another study was improved that all *Klebsiella* positive isolates showed resistance to (cefoxitin,ceftazidime, cefotaxime, and cefepime), combinations of β-lactam antibiotics with β-lactamase–inhibitor (Amoxicillin-clavulanic acid, Piperacillin-Tazobactam), and penicillin's group (ampicillin and piperacillin), according to another research that used the combined test disc method with carbapenem MICs. Furthermore, Klebsiella producers demonstrated resistance to trimethoprim, amikacin, colistin, gentamicin, tigecycline, and ciprofloxacin [22].

Since the study [13] showed that *NDM-1* was isolated from clinical isolates of Gram-negative bacteria in Kashmir, and these isolates were multi-drug resistant, the frequency of NDM-1 producers might be affected the therapeutic options. Additionally, [23] discovers that every strain of IMPKsp exhibits multidrug-resistant (MDR) characteristics. The findings demonstrate that the spread of *blaIMP* genes among *Klebsiella* species is largely influenced by multi-clonal transmission, which involves a variety of genetic environments and plasmid types. Another study [24] reported that MBL *K. pneumoniae* had emerged in Iran, and about (33.7%) of the isolates were resistant to carbapenems, of which (30.8%) were deemed MBL positive. This study also indicate a relationship between *IMP-1* gene and resistant to antibiotics of *K. pneumoniae* isolated from water On the other hand, another study was improved the correlation between IMP-1 gene and resistant to antibiotics especially Imipenem [25]. While another study was identified of *K. pneumoniae* which is capable of producing MBL was essential to control using antibiotics by physician because this gene can transfer by horizontal way or by plasmid from pathogenic bacteria to environmental isolates [26]**.** The results are concerning and demonstrate how urgently infection control measures must be implemented [27]**.**

River samples have higher prevalence of *K. pneumoniae* than other surface water types, suggesting that the surface of water may serve as sources of the bacteria. They might point to a believable exchange of antibiotic resistance genes between waters and human. As a result, the study emphasized the necessity of incorporating these pathogen sources into the single health endeavor as well as the necessity of keeping an eye out for *Klebsiella spp.* in lotic with lentic water bodies to determine the distribution of them and spread within same environments [28].

Antibiotic resistance due to genomic evolution, evolutionary traits, and selective pressures may be spread as a result of the interactions between the human, animal, and environmental sectors. Specifically, the overuse of antibiotics in cattle invariably increased development of particular resistance determinants of human strains, which is linked to fewer options for clinical therapy treatment [29, 30, 31]. However, study in Egypt was observed much higher resistance to cefepime, meropenem, ciprofloxacin and levofloxacin in *K. pneumoniae*, this result agree with current study [32, 33].

In order to stop these organisms from spreading from Iraqi water to the community, infection control measures are crucial. Our findings indicate that the *IMP-1* gene plays a significant role in bacterial resistance to antibiotics, which is a critical stage in development of infections. As a result, they are crucial in the transmission of *K.*

pneumoniae infections from water to the community.

The limitations of this study are illustrated by the following: type and size of water samples, depth of water samples, the clarity and pollutants that were found in the water samples, environmental factors and nutrients that affect microbial growth, the methods used in taking samples and in bacterial isolation, and the number of obtained bacterial isolates. All these factors lead to differences between the data recorded in this study and other studies.

Conclusions

All *K. pneumoniae* isolate that was obtained from water samples exhibited resistance to nearly all of the antibiotics used in this investigation. Metallo-beta lactamase carbpemenase genes were presented in *K. pneumoniae;* had *IMP-1* but did not detect *NDM-1* genes in Environmental isolates by PCR. As well as ,there was correlation between *IMP-1* and resistant to antibiotics when P- value *≤0.05* .For that *K. pneumoniae* isolated from water began resist to carbapenems antibiotics, according that need further study to other types of Metallo-beta lactamase and these genes because spread the resistant to antibiotics especially carbpemens in these environmental bacteria.

Conflicts of interest

None declared.

Financial disclosures

None declared.

Availability of data and materials

The raw data required to reproduce these findings are available in the body and illustrations of this manuscript.

Author's contribution

The authors confirm contribution to the paper as follows: study conception and design: EGS and JMH, theoretical calculations and modeling: EGS and JMH; data analysis and validation: EGS; draft manuscript preparation: JMH, EGS, and DNF. All authors reviewed the results and approved the final version of the manuscript.

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Ethics Approval and Consent to Participate

There is no need for ethical approval because isolates are environmental and not clinical in the present study.

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