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Detection And Prevalence of AmpC Genes in *K. pneumoniae* Isolates from Clinical Samples in Al- Basrah Governorate, Iraq.

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Abstract
AmpC beta-lactamases are clinically important cephalosporinases encoded on the chromosomes of many enteric bacteria and some other organisms. ***Objective****: detect AmpC genes in Klebsiella pneumoniae samples isolated from different sources.* ***Methods****: 100 samples were collected using sterile cotton swabs from patients in Basrah hospitals and transferred to the College of Science, University of Basrah for culture on differential media, isolation, and identification. Drug susceptibility testing was performed to detect the corresponding enzymes, and genetic testing was performed to detect the AmpC genes (CIT and DHA).* ***Results****: 79(79%) of 100 swab samples yielded positive culture results on MacConkey agar, but 54(68.4%) of 79 samples were K. pneumoniae on HiCrom K. pneumoniae agar. Drug susceptibility testing results were as follows: 12(24%) of K. pneumoniae isolates were positive for AmpC β-lactamase production, while 38 (76%) of K. pneumoniae isolates were negative for AmpC β-lactamase production, using the disk approximation test. Genetic testing results were as follows: 8(15%) K. pneumoniae isolates out of 50 sample K. pneumoniae isolates was gave the positive results for CIT gene, 9(18%) K. pneumoniae isolates out of 50 sample K. pneumoniae isolates was gave the positive results for DHA gene.* ***Conclusions****: The prevalence of AmpC genes in bacterial samples increases their resistance to many antibiotics, putting public health at risk.*

Key wards: *Antimicrobial resistance; AmpC Genes; DHA; CIT.*

INTRODUCTION:

Antimicrobial resistance is one of the greatest global health challenges, and β-lactamases, particularly the AmpC β-lactamase class, play a pivotal role in the resistance of Gram-negative bacteria to β-lactam antibiotics (Ueda *etal*., 2023). AmpC genes are naturally present in some Enterobacteriaceae genera, but their horizontal transfer via plasmids (pAmpC) to other bacteria, such as *Klebsiella pneumoniae (K. pneumoniae)*, further complicates the therapeutic profile (Karami-Zarandi *etal*., 2023). This review aims to analyze the molecular mechanisms, epidemiology, and clinical implications of AmpC genes in Enterobacteriaceae, with a focus on *K. pneumoniae* (Kurittu *etal*., 2021). The AmpC genes encode class C beta-lactamases, which confer resistance to beta-lactam antibiotics such as penicillins, cephalosporins (including third-generation ceftazidime), and beta-lactamase inhibitors (such as oxacillin) (Khan *etal*., 2024). These genes are naturally present in some bacteria, such as *Enterobacter cloacae* and *Serratia marcescens*, but they can be transferred to other bacteria, such as *Klebsiella pneumoniae*, via plasmids, leading to the emergence of drug-resistant strains ((Diela *etal*., 2017; Ueda *etal*., 2023). Relationship of AmpC genes to the Enterobacteriaceae family. Enterobacteriaceae family includes many Gram-negative bacteria (Longhi *etal*., 2022), some of which carry AmpC genes on their chromosomal surfaces (such as Enterobacter spp., *Citrobacter freundii, Morganella morganii*), while others, such as *Escherichia coli (E. coli)* and *K. pneumoniae*, acquire them through horizontal gene transfer (Khan *etal*., 2024).

AMPC EXPRESSION PATTERNS:

Normal (low-level) chromosomal expression: Some Enterobacteriaceae genera have the AmpC gene regulated by inhibitory factors, but when exposed to antibiotics such as cephalosporins, a derepression mutation may occur, leading to enzyme overproduction and high resistance (Karami-Zarandi *etal*., 2023). Alternatively, AmpC genes

can be acquired via plasmids (pAmpC): In this case, the genes are widespread among bacteria, especially in hospitals (Singh *etal*., 2021), and are acquired from bacteria such as *E. cloacae* or *C. freundii*. Several studies have shown that *K.pneumoniae* does not possess a chromosomal AmpC gene, so its resistance depends on acquiring pAmpC from other bacteria (Ueda *etal*., 2023).

KLEBSIELLA PNEUMONIAE AND AMPC GENES

K. pneumoniae typically produces ESBL enzymes (such as *TEM, SHV*, and *CTX-M*), but some strains have acquired AmpC genes via plasmids, making them resistant to broader antibiotics (Akpu *etal*., 2023), such as third-generation cephalosporins (such as ceftazidime) and beta-lactamase inhibitors (such as oxacillin) (Jomehzadeh *etal*., 2022). *K. pneumoniae* has become resistant to many antibiotics due to genes (e.g., *CMY, DHA, FOX*) are transferred via integrons or mobile plasmids (Tebano *etal*., 2024), often under the control of strong promoters (e.g., the P3 promoter in the *CMY-2* gene), leading to continuous enzyme production without the need for external stimuli (Barceló *etal*., 2024). Sometimes, it is difficult to distinguish AmpC production from ESBLs using routine methods, requiring molecular testing (such as PCR). Contradictory results may occur in susceptibility testing due to inconsistent enzyme expression (Kurittu *etal*., 2021).

Material and Methods

Sample collectionA total of 150 clinical samples were collected between Oct.-2023, 10 Mar.- 2024. Samples in this study were included 100 swab samples collected from general wounds, dialysis, abortion, ENT operating rooms, fractures, surgical operations, diabetic foot infections. These samples were obtained from multiple healthcare facilities in Al-Basrah, including Basrah Oncology Center, Al-Sader Teaching, Al-Basrah Teaching Hospital, Al-Faiha General Hospital, and Al-Mauany Hospital. The samples were collected using a sterile swab with transport media, and promptly transferred to microbiology laboratory in the Biology Department at the College of Science, where stored in a preservation box.

Isolation and Purification of Bacterial Isolates: All samples were cultured on MacConkey agar and incubated at 37°C for 24h. Following incubation, distinct colonies from the MacConkey agar were subculture onto selective media-HiCrome Agar for *K. pneumoniae* and incubated at 37°C for 24h. to obtain pure isolates. The purified bacterial colonies were stored in BHI broth supplemented with 15% glycerol for long-term storage.

Molecular Methods: DNA and plasmid Extraction**;** the Wizard® Genomic DNA purification kit and Pure Yield™ Plasmid Miniprep System (Promega, USA) was used to extract the genomic DNA and plasmid from *K. pneumoniae*.

16S ribosomal DNA (16SrDNA): the samples isolate (n=54) were reconfirmed identified by using 16SrDNA specific primers for *K. pneumonia* Table (1). According to **(Liu *et al.,*2008)**.

Table (1): The Primer Sequence Used in current Study.

Type of bacteria	Primer	Sequence of primer	Length (bp)	Product (bp)	Ref.
K. pneumonia	16SrDNA forward	5`-ATTTGAAGAGGTTGCAAACGAT-3`	22	130 bp	Liu *et al.*,2008
	16SrDNA Revers	5`-TTCACTCTGAATTTTCTTGTGTTC-3`	24		

Antimicrobial Susceptibility: the detection of susceptible bacteria isolated that produce AmpC β-lactamase enzyme by Disk approximation test according to (**Mol *etal.*, 2021**).

Detection Antibiotic Resistance plasmid-mediated AmpC Genes: Plasmid-mediated AmpC Genes (*bla*$_{DHA}$ and *bla*$_{CIT}$) were identified by using specific primers (Mol *etal.*, 2021) as in table (2).

Table (2) The specific primer used for detection of Plasmid-mediated AmpC genes:

Primers	DNA Sequences	Length	Product size bp	Ref.
bla$_{DHA}$	F: 5`-AACTTTCACAGGTGTGCTGGGT-3`	22	405bp	Mol *etal.*, 2021
	R: 5`-CCGTACGCATACTGGCTTTGC-3`	21		
bla$_{CIT}$	F: 5`-TGGCCAGAACTGACAGGCAAA-3`	21	462bp	
	R: 5`-TTTCTCCTGAACGTGGCTGGC-3`	21		

Table (3): Thermal Cycling Conditions with Modification Used in PCR to plasmid-mediated AmpC genes:

Steps	Temperature	Time	No of cycles
Initial Denaturation	94^{0}C	3 min	1
Denaturation	94^{0}C	30 sec	25
Annealing	64^{0}C	30 sec	
Extension	72^{0}C	1 min	
Final Extension	72^{0}C	7 min	1

RESULT:

Isolation and Identification of bacteria: between Oct.-2023, 10 Mar.-2024 a total of 150 clinical samples were collected from multiple healthcare facilities in Al-Basrah, including Basrah Oncology Center, Al-Sader Teaching, Al-Basrah Teaching Hospital, Al-Faiha General Hospital, and Al-Mauany Hospital. The samples in this study were distributed to 100 swab samples collected from general wounds, dialysis, abortion, E.N.T. operating rooms, fractures, surgical operations, diabetic foot infections. The 79(79%) samples from 100 swab samples were given positive results for bacterial culture in MacConkey agar while the 21(21%) samples were given negative bacterial cultures, but 54(68.4%) out of 79 sample was *K. pneumoniae* on Hicrom *K. pneumoniae* agar. Identification of bacterial growth by using MacConky agar fig.(1), and in HiCrome *K. pneumoniae* agar fig.(2).

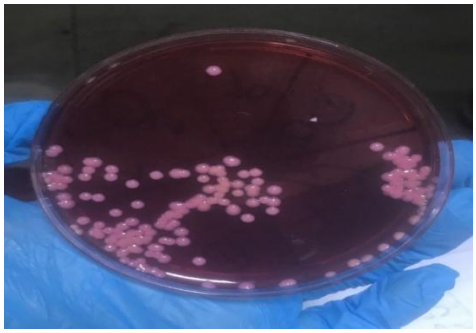

Fig (1): Colonies of *K.pneumoniae* isolate on MacConkey agar

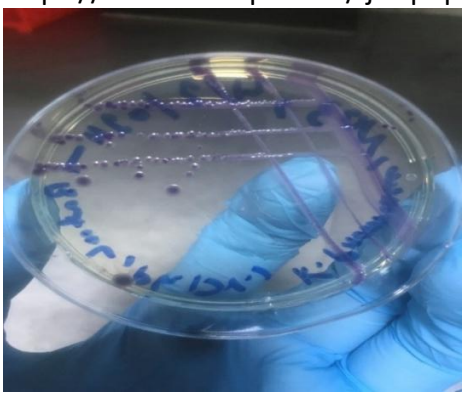

Fig (2): Colonies of *K. pneumoniae* isolate on Hi-Chrome *K. pneumoniae* agar

A high percentage of *K. pneumoniae* bacteria in the samples confirms that this bacterium is a major cause of hospital-acquired infections. These findings are consistent with previous studies showing that *K. pneumoniae* is one of the most common bacteria in hospital-acquired infections, particularly in intensive care units and patients with chronic diseases (Bazaid *etal.*, 2022). This is also attributed to the potential contamination of the hospital environment or medical instruments, particularly during surgical procedures (Lo *etal.*, 2024). The prevalence of *K. pneumoniae* is also attributed to several factors. One major factor is urinary tract infections, as *K. pneumoniae* is capable of causing urinary tract infections and nephritis in both women and men by bloodstream infections, pneumonia, and infections resulting from burns and wounds (Vidal-Cortés *etal.*, 2022; Hannoodee & Nasuruddin, 2024). The inappropriate or excessive use of antibiotics (such as carbapenems) increases selection pressure on bacteria, leading to the emergence of resistant strains such as carbapenemase-producing *K. pneumoniae* (KPC) (Karampatakis *etal.*, 2023). Poor hospital infection control and transmission in healthcare facilities: It is spread through contaminated hands, medical instruments (such as breathing tubes, catheters), or contaminated surfaces. Invasive procedures (such as mechanical ventilation and surgery) also increase the risk of infection. Overcrowding in hospitals and a shortage of healthcare workers also contribute to its spread (Ssekitoleko *etal.*, 2020; Liu *etal.*, 2022). Community-acquired infections have also been identified. Some strains (such as hypervirulent *Klebsiella* (hvKP)) cause serious infections in healthy individuals outside of hospitals, particularly in Asia. They are transmitted through direct contact or contaminated food/water (Horesh, 2020; Hu *etal.*, 2021).

We should also not forget that immunocompromised patients (such as diabetics, the elderly, and cancer patients) are more susceptible to infection (Kumar *etal*, 2022; Liu *etal.*, 2023). The spread of animals in the environment also facilitates the spread of bacterial infections. Klebsiella has been detected in soil, water, and animals, which may be a source of infection (FF *etal.*, 2023). Regarding travel and global spread, resistant strains have been observed to spread through international travel or the transfer of patients between hospitals (Theriault *etal.*, 2021). Prevention is essential by adhering to infection control measures (hand washing, sterilizing equipment), using antibiotics wisely, and consulting a doctor to avoid resistance. It also includes early detection of bacteria and isolation of patients infected with resistant strains (Effah *etal.*, 2020; Priyanka *etal.*, 2020; Tsioutis *etal.*, 2023).

Genotypic identification of *K. pneumoniae*:

The DNA extract was subjected to PCR for amplifying of 16s rDNA. The n=54 isolates were amplified by using 16s rDNA with approximately (130bp) by using specific primer. Individual band of 16s rDNA was compared with standard molecular DNA ladder (2000 bp), figure (3).

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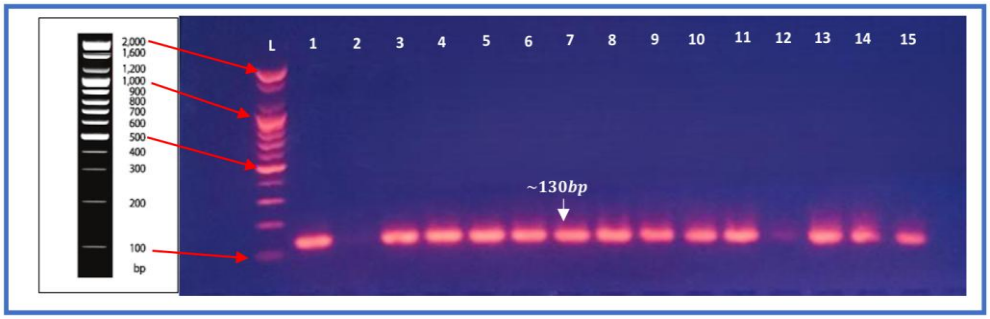

Fig (3): Agarose Electrophoresis Patterns Show PCR Amplified Products of *16s* rDNA. Lane L:(2000 bp DNA ladder), Lanes:(no. 1-15) 16S rDNA band of *K. pneumoniae* isolates. using 1.5% agarose gel, 70V, 45min.

All isolates performed molecular diagnoses utilizing PCR technique based on the diagnostic gene 16s rDNA, the results of the current study used n= 54 *K. pneumoniae* isolates the results were shown the 50 (92.6%) *K. pneumoniae* isolates out of 54 *K. pneumoniae*. And had a molecular weight of around 130 bp in comparison to the DNA ladder figure (3), while 4(7.4%) isolates were giving the negative results. The 16s rDNA gene is a suitable target for a variety of molecular investigations because of its properties. The improved identification and detection of bacteria in clinical samples has been greatly supported by 16s rDNA molecular diagnostics. When a bacterial infection is suspected, the most common method used in a typical clinical microbiological laboratory is that method (Church *etal.*, 2020). 16s rDNA gene is found in all bacteria, often in several copies (Schloss, 2021). According to Chen *etal.*, (2023), genotyping is crucial for identifying *K.* pneumoniae cases or outbreaks as well as for identifying the origin and transmission of infections. Since phenotypic approaches are more susceptible to changes in growth conditions, environmental variables, pH, and temperature, genotypic characterization techniques are often more reliable. 16s rDNA is a great diagnostic tool since it offers several advantages over biochemical and phenotypic characterizations, such as the gene's presence in all bacteria and its lack of widespread mutation (Pei *etal.*, 2023). And due to the great importance of 16S rDNA genes, they are a highly conserved region in bacterial genomes, making them a valuable tool for studying the evolutionary relationships between bacteria (Bartoš *etal.*, 2024). 16S rDNA enable the classification and identification of bacterial species based on genetic similarities and differences. They also help distinguish closely related species, such as *K. pneumoniae*, and understand their genetic diversity (Church *etal.*, 2020). 16S rDNA gene is also used to study microbial communities in different environments, their ecological roles, and their interactions with other microorganisms (Kumar *etal.*, 2021).

Detection of Plasmid-mediated AmpC (AmpC) β-lactamase:

In current study that out of n=50 *K. pneumoniae* isolates the only 12(24%) *K. pneumoniae* isolates were gave positive results for produced AmpC β-lactamase. While 38(76%) *K. pneumoniae* isolates, were shown negative results for produced AmpC β-lactamase, by using Disk approximation test (phenotypic detection of AmpC), figure (4).

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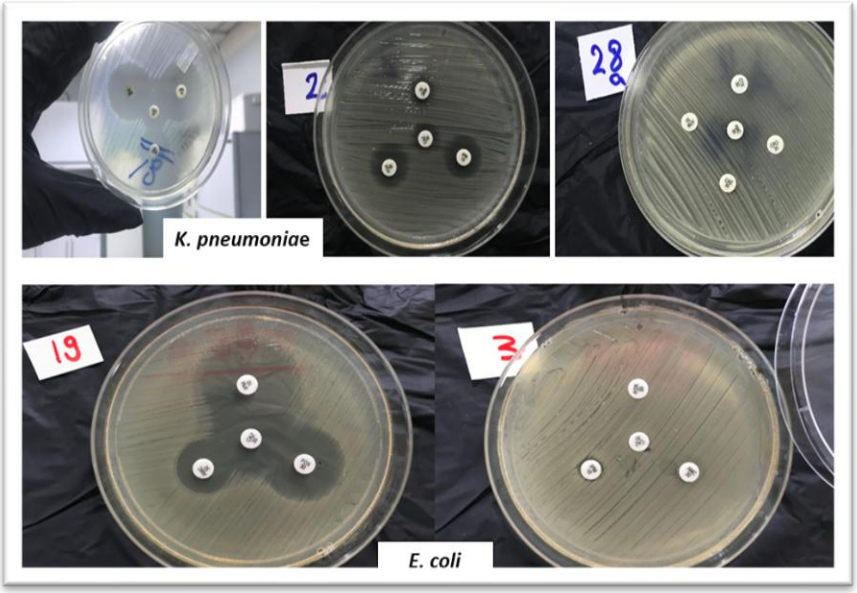

Fig (4): Disk approximation test that used to detect AmpC produced by *K. pneumonia*e and *E. coli* isolates.

About 40% of Enterobacteriaceae genus can produce high AmpC enzymes (Xue *et al.,* 2023). Strains that produce AmpC-inducing enzymes have been found to produce ESBLs at the same time, like, *E. coli* and *K. pneumoniae* can produce ESBLs and AmpC at the same time, bacteria that produce both ESBLs and plasmid-type AmpCases are called ultra-extended-β-lactamases, which are more resistant, more transmissive, and more difficult to control clinically appropriate infections (Dada-Adegbola & Abitogun, 2021). In our study is approaching study with Mohamed *etal*., (2020); Soltani *etal*., (2020); Khalifa *etal*., (2021) which explained prevalence AmpC β-lactam resistance in *K. pneumoniae* and *E. coli* isolates in hospitals due to a combination of β-lactamase activity and porin loss/alteration. The widespread resistance of *K. pneumoniae* and *E. coli* to AmpC-type antibiotics is due to several evolutionary, environmental, and usage factors. For example, the extensive and uncontrolled use of β-lactam antibiotics (such as ampicillin and cephalosporins) leads to strong selective pressure, favoring the growth of resistant strains. Some antibiotics (such as cefoxitin) also stimulate AmpC production even in bacteria that do not naturally produce it (Kurittu *etal*., 2021; Longhi *etal*., 2022; Tebano *etal*., 2024).

Also, due to genetic mutations and the transfer of resistance genes, some strains of *E. coli* and *K. pneumoniae* possess chromosomal AmpC genes (Li *etal*., 2023). Certain mutations or overexpression of AmpC (such as mutations in the promoter or repressor) lead to permanent resistance (Zhao *etal*., 2024). The transfer of AmpC plasmids (such as *CMY, DHA, FOX, MOX*) between bacteria through bacterial conjugation also accelerates the spread of resistance (Zhou *etal*., 2022). Hospitals are ideal environments for the spread of resistant strains due to the heavy use of broad-spectrum antibiotics, which encourages bacteria to develop resistance to these drugs, and the transmission of bacteria between patients (especially via contaminated hands or instruments) (Ueda *etal*., 2023). *K. pneumoniae* is known for its multidrug-resistant (MDR) pathogens and often carries AmpC plasmids (Karami-Zarandi *etal*., 2023). Poor surveillance and prevention, lack of adherence to antibiotic resistance monitoring programs (Antimicrobial Stewardship), and a lack of accurate diagnosis of AmpC-producing strains lead to inappropriate treatment. These combined factors contribute to the global spread of AmpC resistance, making it a major public health challenge (Khan *etal*., 2024).

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DETECTION OF PLASMID-MEDIATED AMPC:

1: Detection of *CIT* gene:

The plasmid DNA was extracted from 50 sample *K. pneumoniae* isolates sample isolates. The plasmid DNA were subjected to PCR to amplify the *CIT* gene. The band of the individual were amplified gene characterized approximately in (462 bp), by comparison to the standard molecular DNA ladder (2000bp) figure (4).

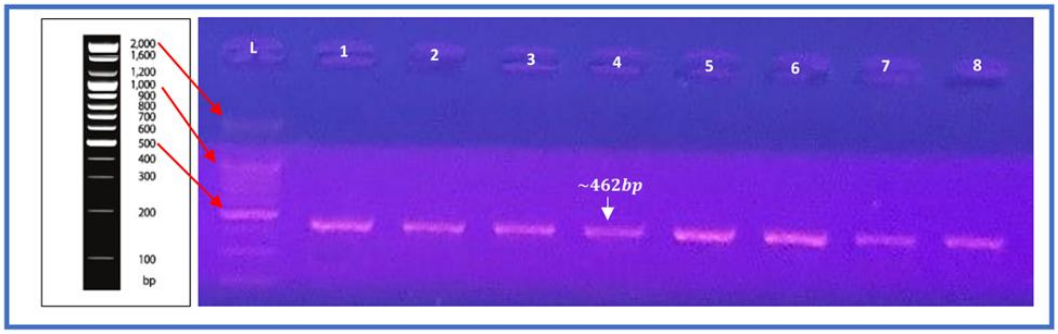

Fig (4.) Agarose electrophoresis patterns of *CIT* gene PCR amplified products. Lane1:(2000 bp DNA ladder), lane: *CIT* gene bands of *K. pneumoniae*, by using 1.5% agarose gel, 70V, 45min.

The result showed that 8(15%) *K. pneumoniae isolates* out of 50 sample *K. pneumoniae* isolates was gave the positive results for *CIT* gene, while 42(85%) *K. pneumoniae* isolates was gave the negative results for *CIT* gene.

4.2: Detection of *DHA* gene

The plasmid DNA was extracted from 50 sample *K. pneumoniae* isolates sample isolates. The plasmid DNA were subjected to PCR to amplify the *DHA* genes. The band of the individual were amplified gene characterized approximately in (405 bp bp) for *DHA* gene, by comparison to the standard molecular DNA ladder (2000bp) figure (5).

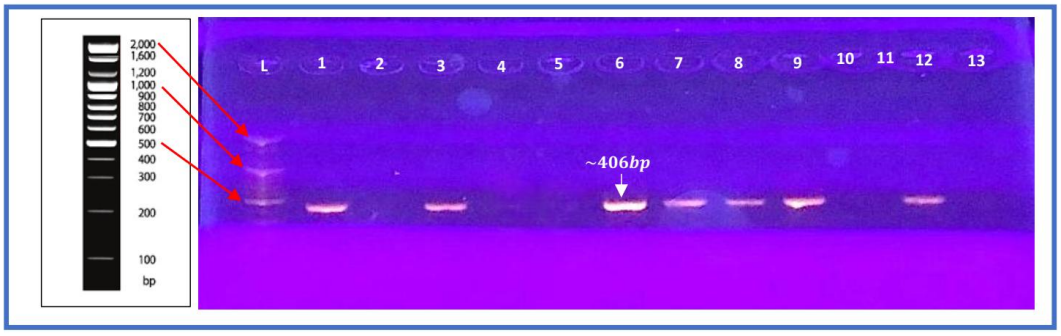

Fig (5): Agarose electrophoresis patterns of *DHA* gene PCR amplified products. Lane1:(2000 bp DNA ladder), lane: *DHA* gene bands of *K. pneumoniae*, by using 1.5% agarose gel, 70V, 45min.

the result showed that 9(18%) *K. pneumoniae isolates* out of 50 sample *K. pneumoniae* isolates was gave the positive results for *DHA* gene, while 41(82%) *K. pneumoniae* isolates was gave the negative results for *DHA* gene. *Amp*C enzymes are a type of beta-lactamase enzymes produced by some bacteria. They are characterized by their ability to break down a wide range of beta-lactam antibiotics, and include a large group of gene families, the most important of which are *DHA* &*CIT* genes (Zhou *etal*., 2022). Some studies have shown that the *Amp*C genes (*CIT, DHA*) are more prevalent due to chronic exposure to cephalosporins, which induce the expression of these genes (Barceló *etal*., 2024). Rapid horizontal gene transfer via plasmids or mobile genetic elements (such as introns or transposons) facilitates their spread between different bacterial strains (Rodríguez-Beltrán *etal*., 2021), as well as chromosomal mutations that lead to the overexpression of *Amp*C genes (Kawamura *etal*., 2022).

Robatjazi *etal*., (2021) and Akpu *etal*., (2023) which showed samples had *CIT* gene in both *K. pneumoniae* samples, but in the first study, the gene was found at a very low rate, while in the second study, the gene was found at a high rate in the samples studied. About detection of *DHA* gene Diela *etal*., (2017) and Jomehzadeh *etal*., (2022) showed in their studies the results were nearly to our study about *DHA* gene were low rate in *K. pneumoniae* sample, while Aryal *etal*., (2020) and Akpu *etal*., (2023) showed high percentage of *DHA* gene in the samples, whereas Ibrahim & Faisal, (2024) explain absent gene in sample. The difference between the results of studies may be due to differences in the number of samples studied, geographical location, contamination rates in hospitals or different environments, incorrect use of antibiotics, the amount of treatment used, and the duration of treatment. Some studies have shown that the *CIT* genes spread rapidly because they are found on mobile plasmids among Enterobacteriaceae bacteria such as *K. pneumoniae* (Akpu *etal*., 2023), which are characterized by their effective resistance to colistin (Singh *etal*., 2021). The *DHA* genes produce stable and efficient AmpC enzymes that degrade penicillins and cephalosporins, providing bacteria with strong protection without a high metabolic cost (Barceló *etal*., 2024).

CONCLUSION:

The prevalence of AmpC genes in bacterial samples is associated with resistance to multiple antibiotics, posing a threat to public health. This is because these genes can be transferred to other bacteria via plasmids, enabling other bacteria to become resistant to antibiotics.

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