DOI : 10.35124/bca.2019.19.2.4053

Biochem. Cell. Arch. Vol. 19, No. 2, pp. 4053-4061, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

DETECTION OF PLASMID-MEDIATED AMPC β-LACTAMASE GENES AND EVALUATION THE SYNERGISTIC EFFECT OF CLOVE VOLATILE OIL AND ANTIBIOTICS IN CLINICAL ISOLATES OF *KLEBSIELLA PNEUMONIA* IN IRAQ

Mohammed Obaid Ibrahim and Safaa Abed Latef Al Meani

Department of Biology, College of Science, University of Anbar, Iraq.
e-mail: mo.mn_m@yahoo.com

(Received 26 July 2019, Accepted 23 August 2019)

ABSTRACT : *Klebsiella pneumoniae* is an important multidrug-resistant (MDR) pathogen affecting humans and a major source for hospital infections associated with high morbidity and mortality due to limited treatment options. AmpCβ-lactamases are clinically significant because they may confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins (*e.g.*, ceftriaxone, cefotaxime and ceftazidime), cephamycins (*e.g.*, cefoxitin and cefotetan) and monobactams. AmpC β-lactamase activity is not affected by ESBL inhibitor clavulanic acid. Investigating of occurrence of Plasmid-Mediated AmpC β-lactamase genes in *Klebsiella pneumoniae* isolated from different clinical sources in Al Anbar city and study the synergistic effect of clove Volatile oilwith antibiotic (ceftazidime, ceftriaxone and Cefepime). From July 2018 to December 2018, 100 samples were collected from Al Anbar city hospitals, these samples included: wound swab, urine, abscesses and burns. AmpCβ-lactamase *Klebsiella pneumoniae* were analyzed for the presence of AmpC production. Three phenotypic AmpC confirmation assays (AmpC E test, Ceftazidime -cloxacillin double-disk synergy test (CC-DDS) and Modified cephalosporinase (in activation Methods) were able to detect the majority of AmpC-positive strains correctly. Molecular detection of plasmid-mediated AmpC genes (*bla*$_{MOX}$, *bla*$_{CIT}$, *bla*$_{DHA}$, *bla*$_{FOX}$ and *bla*$_{ACC}$) by using multiplex PCR was done. the synergistic effectof clove Volatile oil was detectedusing the checkerboard technique. The present study showed a high resistance of *Klebsiella pneumoniae* to Ceftriaxone (78%), Cefpodoxium (68%), Ceftazidimim (62%), Cefepime (62%) and Cefoxintin (16%). (CC-DDS) test were positive for 18(69.22%), (mCIM) 26(100%), AmpCE testing 24 (92.30%). Molecular screening appeared many isolates harbor AmpC genes, *bla*$_{FOX}$ 11 (42.3%), *bla*$_{ACC}$ 8 (30.7%), *bla*$_{DHA}$ 8 (30.7%), *bla*$_{CIT}$ 9(34.6%) and *bla*$_{MOX}$ 7 (26.92%). The last two genes are the first time discovered in Iraq in this study.

Clove volatile oil exhibited markedly antibacterial activity and have inhibitory effectto AmpCβ-lactamase. Clove Volatile oil exhibited synergistic effect when combined with (Ceftazidimim, Cefepime and Ceftriaxone). There is high resistance to third and fourth generation of Cephalosporins among AmpC-producing *Klebsiella pneumonia* isolates which has been increasingly recognized in Al Anbar hospitals. Thus, molecular identification of the genes encoding AmpC would be essential for a reliable epidemiological investigation of their transmission in hospitals. The combination of clove volatile oils with antibiotic (Cefepime, ceftazidime and ceftriaxone) against *Klebsiella pneumonia* showed effectiveness and could improve the susceptibility of bacteria toward these antibioticsto treat the infections resulted from drug-resistant bacteria.

Key words : *Klebsiella pneumonia*, clove volatile oil, synergism, AmpCβ-lactamases

INTRODUCTION

β-lactam antibiotics, in clinical use since the 1940s, are a major class of antibacterial agents prescribed in human medicine. Since their introduction, hundreds of different β-lactamases have evolved among enteric pathogens including *K. pneumonia*e, reaching an astonishing number (>2000) and diversity (Bush and Jacoby, 2010). A significant government health issue is resistant bacteria infection. The spread of resistance to beta lactam antibiotics in Gram negative bacteria, particularly *Klebsiella pneumonia*, is frequently the result of the manufacturing of β-lactamase enzymes that can hydrolyze β-lactam rings (Wassef *et al*, 2014).

AmpC β-lactamases are typically encoded on the chromosomes of many of the Enterobacteriaceae and other organisms, AmpC enzymes belong to class C according to the Ambler structural classification of β-lactamases, while in the functional classification scheme of Bush and Jacoby (2010), they were related to the group 1 (Li and Nikaido, 2009). AmpC β- lactamases can also

be transmitted in plasmids that present a fresh danger of spread to other bacteria in a hospital or geographic district (Wassef *et al*, 2014). Most acquired ampC genes are originated from chromosomal genes, which are mobilized by plasmids that has resulted in their wide spread (Dehghani *et al*, 2016), but are less common than extended-spectrum β-lactamases (ESBLs). The CMY-type enzymes are the most common reported plasmid mediated AmpC β-lactamases (Dehghani *et al*, 2016). The inducible chromosomal AmpC genes have been found on plasmids of *Klebsiella* spp., *Escherichia coli*, and *Salmonella* spp. AmpC is scarcely expressed in *E. coli*, while the AmpC gene is missing from the chromosome in *Klebsiella* and *Salmonella* species and found on plasmids (ALshamarti and AL-Muhnna, 2011). As there are multiple β-lactamases within one organism phenotypic recognition of the β-lactamases is difficult. Many phenotypic tests can recognize these two resistance mechanisms but are not able to discriminate the different family types of plasmid-mediated AmpC β-lactamases (Dehghani *et al*, 2016). They can be discriminated from extended- spectrum β- lactamases by their ability to hydrolyze cephamycins and not inhibited by clavulanic acid (Rudresh and Nagarathnamma, 2011). Polymerase chain reaction (PCR) technique was used to discriminate the five plasmid-mediated AmpC specific families (bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{FOX} and bla_{ACC}) in microorganisms (Wassef *et al*, 2014).

Volatile oils (also known as essential oils) are generally oily, exceptionally colored or clear, complicated and their compounds are volatile, marked by a powerful odor and synthesized during secondary metabolism by aromatic plants. These compounds have a broad range of pharmacological activities (Rehman *et al*, 2016). Volatile oils have been reported to possess a significant antibacterial, antioxidant and antiviral activities (Vidhani *et al*, 2016). The aim of this study was to determine the occurrence of plasmid-mediated â-lactamases (AmpC) genes in *Klebsiella pneumoniae* isolated from different sources,using several phenotypic tests for detection and confirmation of AmpC production from *Klebsiella pneumonia* and investigating if there is a synergism between clove Volatile oil and antibiotics against *Klebsiella pneumonia*.

MATERIALS AND METHODS

Bacterial isolates

From July 2018 to December 2018, 100 samples were collected from Al Anbarcity hospitals and included: wound swab, urine, abscesses and burns. All isolated of *Klebsiella pneumoniae* were diagnosed by conventional microbiological methods (colonial morphology, Gram staining and biochemical tests) according to Cheesebrough (Cheesebrough, 1998) and automated diagnosis using vitek2 compact system (bioMèrieux, Marcy l'Etoile, France).

Antimicrobial Susceptibility assay

The antibiotic susceptibilities of isolates were determined using Kirby Bauer's disk diffusion method as described in the guidelines of Clinical and Laboratory Standard Institute (CLSI). Antibiotic disks (Oxoid, UK) such as Cefepime (30 ìg), ceftazidime (10ìg), ceftriaxone (30 ìg), Cefpodoxime (10ìg) and Cefoxitin (30 ìg) were used. Results were interpreted as sensitive, intermediate and resistant according to Clinical and Laboratory Standars Institute (2018).

Phenotypic detection of AmpC â-lactamases

Ceftazidime-cloxacillin double-disk synergy test (CC-DDS)

This test was conducted based on the inhibitory effect of cloxacillin on AmpC production. All isolates were inoculated on Mueller Hinton agar. Ceftazidime /cloxacillin and ceftazidime disk (30 ìg) where used in this test. If the difference is ≥4 mm in the inhibition zones of ceftazidime /cloxacillin and ceftazidime disks was indicated as AmpC production (Khari *et al*, 2016).

Modified cephalosporinase Inactivation test

Test was used to detect a cephalosporinase in Enterobacteriaceae and *P. aeruginosa* whereas eCIM is used together with mCIM to differentiate Metallo-β-lactamases from serine cephalosporinase in Enterobacteriaceae (Sfeir *et a.*, 2019).

E-test : AmpC E test (AB bio-Me´rieux, Sweden) for ceftazidime susceptibility was performed according to the manufacturer's instructions. The AmpCE test consists of a strip containing ceftazidime on one end and ceftazidime - Clavunate on the other end. The MICs of ceftazidime and ceftazidime -Clavunate when different on one side is positive for the production of β-lactamase (El-hady and Adel, 2015).

Molecular Detection of AmpC β-lactamases genes

Phenotypic tests cannot distinguish among the different families of plasmid-mediated AmpC enzymes and may also overlook chromosomally determined AmpC β-lactamases with an extended spectrum, current "gold standard" multiplex PCR for plasmid-mediated AmpC β-lactamase detection has been used by utilizing six primer pairs. Genomic DNA was extracted from the culture of bacterial isolates from different sources by using Mini Kits extraction (bio basic, Canada). Master mix was

prepared in a total volume 25 microliters, composed of Green master mix (Bioneer, Korea), 10 picomole ofprimers solution (forward and reverse primers) (Table 1), deionized water 2 ìl and 3 ìl template DNA (80 n gas a final conc.). Genes were amplified in one multiplex PCR run. Multiplex PCR program consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of DNA denaturation at 94°C for 1min, primer annealing at 53°C for 30s and primer extension at 72°C for 1 min. After the last cycle, a final extension step at 72°C for 7 min was used. Five-microliter aliquots of PCR product were analyzed by gel electrophoresis (Bioneer, Korea) with 2% agarose for 2 hours at 70 volt. Gels were stained with Red-safe DNA stainand the image was captured using In Genius gel documentation system (Syngene, England). A100bp DNA ladder (Promega, USA) was used as a size marker.

Detection of bla_{ACC} and bla_{MOX} genes by Phenylboronic acid disk

Disks were prepared as follow: 120 mg of PBA (also known as benzene boronic acid/anhydride :AlfaAesar, Ward Hill, MA, USA) was dissolved in 3 mL dimethyl sulphoxide (Alfa Aesar). Three millilitres of sterile distilled water was added to this solution to create a stock solution; 20 ìl of the stock solution was dispensed onto each of 30 ì gcefotetan (CTT), 30 ìg Cefoxitin (FOX) and blank disks (6 mm in diameter). These disks had been obtained from Mast Group/England. The disks were allowed to dry for 30 min and used either immediately or stored in airtight vials with desiccant at 4^0C. An increase of +5 mm in zone diameter in the presence boronic acid compared with either CTT or FOX tested alone was considered to represent a positive test for presence of an AmpC β-lactamase (Coudron, 2005; Yagi *et al*, 2005).

Extraction of clove Volatile oil (*Syzygium aromaticum*)

It was accomplished by steam distillation using Clevenger apparatus. 500 ml of distilled water were put with 100 g of leaves in a Clevenger flask, which the lower part was linked to a heat source and the higher part to a condenser. The procedure was completed for 2.5 h, then the water vapor generated in the flask pass through the leaves loaded with volatile oil to the condenser, where it is condensed. After that, the oil separated by decantation (Lawrence and Reynolds, 1991).

Beta lactamase activity

Standard procedure for assay of hydrolyzed /3-lactam antibioticwas applied according to Sargent (1968).

$$\text{Hydrolyzed substrate (micromoles)} = \frac{\Delta Ku}{Ku - \text{Blank A}} \times \frac{40}{F}$$

Enzyme activity was calculated from the following formula: β-Lactamase activity (units per milliliter)

$$= \frac{\Delta Ku}{Ku - \text{Blank A}} \times \frac{40}{F} \times \frac{1}{T} \times \frac{1}{V}$$

In these equations, AKu = (Ku-blank B) minus (Ku-sample). Ku-blank A, Ku-blank B and Ku-sample, absorbance is expressed as Klett units

F - consumed moles of iodine per mole of the hydrolyzed substrate

T- time (minutes) for enzyme reaction

V- volume (milliliters) of enzyme solution added to the assay tube.

One unit of enzyme activity was defined as that quantity which hydrolyzes 1,umol of substrate per min under theexperiment conditions

Synergism between clovevolatileoils and antibiotics

The probable existence of synergismbetween the volatile oil and antibiotics like Cefepime, ceftazidime, and ceftriaxone was done using the checker board technique as described previously (Bhardwaj, 2017).

Determination of minimum inhibitor concentration (MIC)

Minimum inhibitory concentration (MIC) of the volatile oil and antibiotics solutions were evaluated by ResazurinMicrotitre-plate Assay (REMA) with slight modifications. In aseptic conditions, volume of 100 µl of Mueller-Hinton Broth (MHB) was added to all wells of microtitre-plates then 100 µl of 1% volatile oil transferred into the first row of the 96 well plates.Serial dilutions were performed by pipetting 100 µl of the material test from the first row to the other rows in serially decreasing concentrations (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256). 10 µl of bacterial suspension which containing 1.5×10^8 CFU/ml were added to each well. They were wrapped loosely with Para-film to ensure that bacteria did not become dehydrated, incubated for 18-24 h at 35±2°C. After incubation, 10 µl of resazurin solution (Alamar blue) was added to each well and the plate was re-incubated for 2-4 h for the observation of colour change. The results were analyzed visually by observing the changes in colour of resazurin, changes from purple to pink,or red or colourless being recorded as positive. The lowest concentration with no change of resazur in colour was taken as the MIC value (Kolareviæ *et al*, 2016; Manuel and Abdulrahman, 2017).

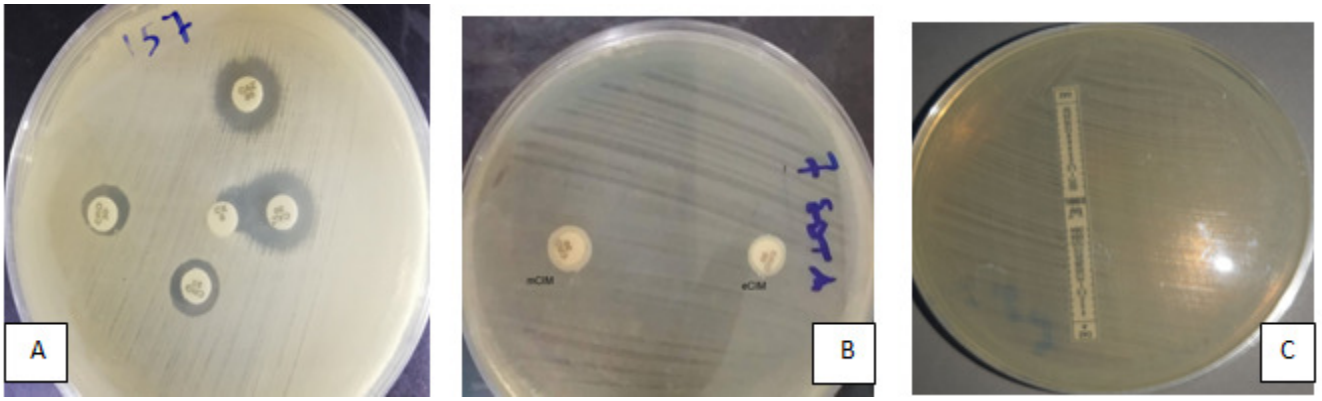


Fig. 1 : Phenotypic detection of AmpC β-lactamases with *K. pneumoniae* (A) positive Ceftazidime -cloxacillin is a double-disk synergy test, (B) left edge of plate positive modified cephalosporinase inactivation methods no growth around disk), B(right edge of plate negative EDTA cephalosporinase inactivation methods test no growth around disk and (C) negative AmpC E test no inhibition around E test strip.

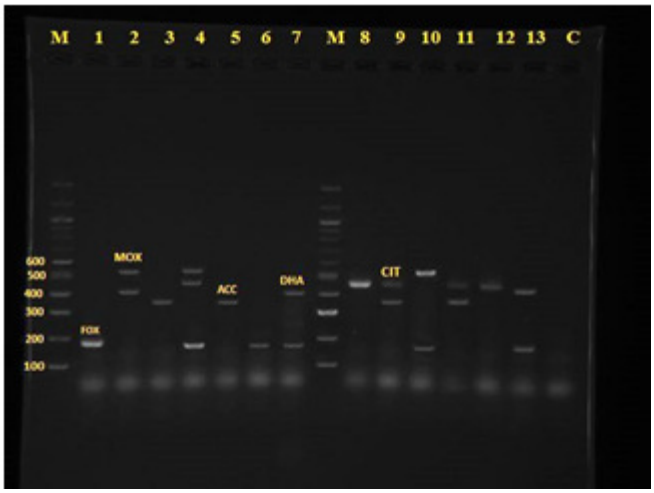


Fig. 2 : Multiplex PCR products. *K. pneumoniae* isolates from 1 to 13. Electrrophoresis was done in 2% agarose gel at 70 volt. M, 100-bp DNA ladder.

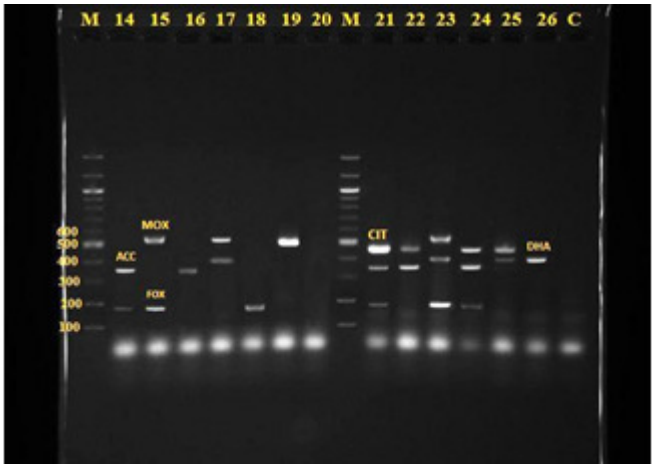


Fig. 3 : Multiplex PCR products. *K. pneumoniae* isolates from 14 to 26. Electrrophoresis was done in 2% agarose gelat 70 volt. M, 100-bp DNA ladder.

RESULTS AND DISCUSSION

Total of 100 (51%) isolatesout of 195 samples were identified as *Klebsiella pneumoniae*. The results of this study indicate that the high percentage of *Klebsiella pneumonia* distribution in urine 45 (45%) followed by 26 (26%) wound, 16(16%) abscesses and 13 (13%) burns.

Antimicrobial susceptibility test

Klebsiella pneumonia has a high resistance to Ceftriaxone (78%), Cefpodoxium (68%), Ceftazidimim (62%), Cefepeme(62%) and Cefoxintin (16%) and this resistance to third and fourth generation of Cephalosporinsin al Anbar city were mostly associated with Ampc â-lactamases. Martelius and his colleagues indicated that the resistance to the third generation of cephalosporins is the same ratio as seen *Klebsiella pneumoniae* (Martelius *et al*, 2016). Antibiotic resistance is the reduction in the effectiveness of a drug such as an antimicrobial or an antineoplastic in curing a disease or condition. When the antibiotic is not intended to kill or inhibit a pathogen, then the term is equivalent to dosage failure or drug tolerance. More commonly, the term is used in the context of resistance that pathogens have ‘‘acquired’’ that is, resistance has evolved. When an organism is resistant to more than one drug, it is said to be multidrug-resistant (Hennequin and Robin, 2016). Drug resistance poses a therapeutic problem not only in the hospital settings, but also in the community as most of the bacteria have acquired resistance to multiple antibiotics. There are various mechanisms of drug resistance in gram negative bacteria include extended spectrum β-lactamases (ESBL) production, AmpCβ-lactamase production, efflux mechanism and porindeficiency (El-hady and Adel, 2015).

Phenotypic detection of AmpC â-lactamases

A 26 isolates of *Klebsiella pneumoniae* have ability to produce AmpC â-lactamase which identified by phenotypic test as shown in Table 2.

(CC-DDS) the test appeared 18 positive isolates

Table 1 : Sequences of PCR primers.

Gene	Primers' Sequences (5'→3')	TM(^{0}C)	Amplicon size (bp)	Accession number
bla_{DHA}	F : AAC TTT CAC AGG TGT GCT GGG T	**60.3**	**405**	AJ237702
	R : CCG TAC GCA TAC TGG CTT TGC	**61.8**		
bla_{ACC}	F : CGCGGAGATTGARAAGCAAA	**56.3**	**346**	AJ133121.1
	F : AAC AGC CTC AGC AGC CGG TTA	**61.8**		
	R : TTC GCC GCA ATC ATC CCT AGC	**61.8**		
bla_{MOX}	F : GCT GCT CAA GGA GCA CAG GAT	**61.8**	**520**	D13304.2
	R : CAC ATT GAC ATA GGT GTG GTG C	**60.3**		
bla_{FOX}	F : AAC ATG GGG TAT CAG GGA GAT G	**60.3**	**190**	X77455.1
	R : CAA AGC GCG TAA CCG GAT TGG	**61.8**		
bla_{CIT}	F : TGG CCA GAA CTG ACA GGC AAA	**59.8**	**462**	HSP-2385B13
	R : TTT CTC CTG AAC GTG GCT GGC	**61.8**		

* F: Forward sequences, R: Reverse sequences.

Table 2 : The results of the phenotypic detection.

Result	CC-DDS n=26 (%)	mCIM n=26 (%)	AmpCE testing n=26 (%)
Positive	18(69.22)	26 (100)	2 (7.70)
Negative	8 (30.78)	0 (0)	24 (92.30)

Table 3 : Result of a phenylboronic acid with cefotetan and Cefoxitin to detect the bla_{ACC} and bla_{MOX} genes.

ACC Positive	AmpC disk test withFOX andPBA	AmpC disk test with CTT and PBA	MOX Positive	AmpC disktest with FOX and PBA	AmpC disk test with CTT and PBA
*2	negative	negative	*3	Negative	positive
*5	negative	negative	*4	negative	positive
*9	negative	negative	*10	Negative	positive
*11	negative	negative	*15	Negative	positive
*14	negative	negative	*17	Negative	positive
*21	negative	negative	*19	Negative	positive
*22	negative	negative	*23	Negative	positive
*24	negative	negative			

* Appearance of the gene in the phenotypic examination.

(69.22%). The (mCIM) gave the highest positive with ratio of 100%. To further differentiate Metallo-β-Lactamase from serine cephalosporinase, mCIM has been further adapted by adding EDTA, cation chelator and inhibitor of Metallo-β-Lactamase. All of the isolates gave a negative result for Metallo-β-Lactamase. The result showed that 24 (92.30%) isolates gave a negative result for AmpC E testing with clavulanic acid because AmpC β-lactamase activity is not inhibited by clavulanic acid as shown in Table 2, Fig. 1.

Molecular detection of AmpC genes

Plasmid-mediated AmpC Beta-Lactamase genes detection using multiplex PCR. Figs. 1 and 2 showed 26 isolates of *K. pneumoniae* have AmpCβ-lactamase genes. bla_{FOX} gene was detected in 11 isolates (42.3%) which is the most widespread of the genes under study. These results are consistent with the results obtained from study conducted in Al Muthanna city in Iraq (Al-Garawyi, 2016) they find that bla_{FOX} gene was existed in (37.5%) *K. pneumoniae* isolates. The bla_{FOX} gene was first described in Argentina and Italy and believed that *Aeromonas caviae* may be thesource of this gene (Yagi *et al*, 2005). bla_{ACC} identified in 8 (30.7%) of isolates and gene bands appeared within the expected size (346bp) for all positive isolates. In our study, the bla_{ACC} gene was first time discovered in Iraq by reviewing previous studies, who found bla_{ACC} gene absent among *K. pneumoniae*, but they found this gene in *Enterobacter* spp and *E. coli* (Hassan and Manhal, 2015). bla_{ACC} was first described in Germany (Miró *et al*, 2005). With regard to bla_{DHA} gene 8 (30.7%) have this gene. Our results are consistent with the results obtained by Hassan and Manhalin in Dhi-Qar city of Iraq, they find that bla_{DHA} gene was existed in (3.57%) *K. pneumoniae* isolates (Hassan and Manhal, 2015). While bla_{CIT} was detected in 9 isolates (34.6%). In previous studies in Iraq the researchers found that

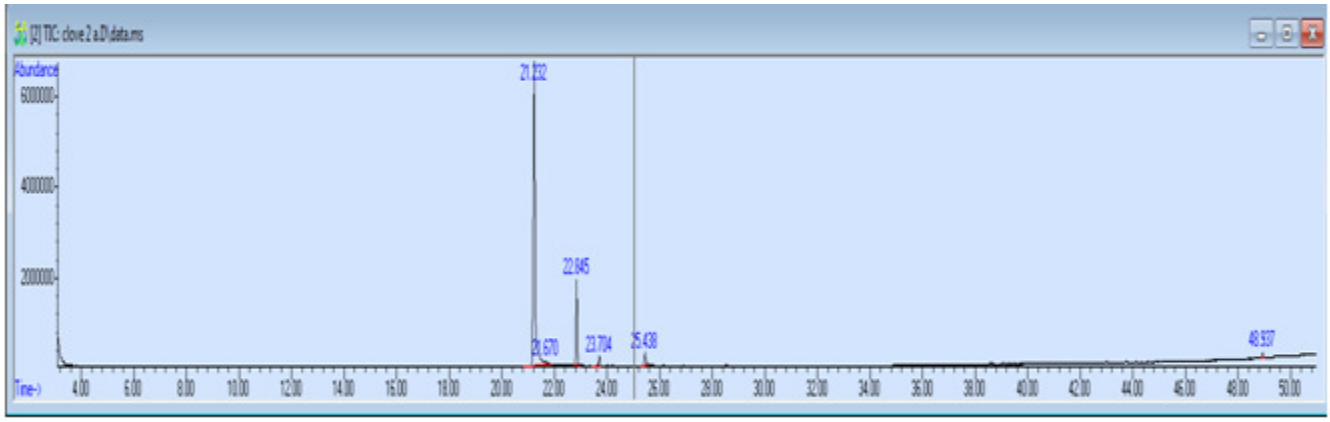


Fig. 4 : GC-MS analysis of clove volatile oil.

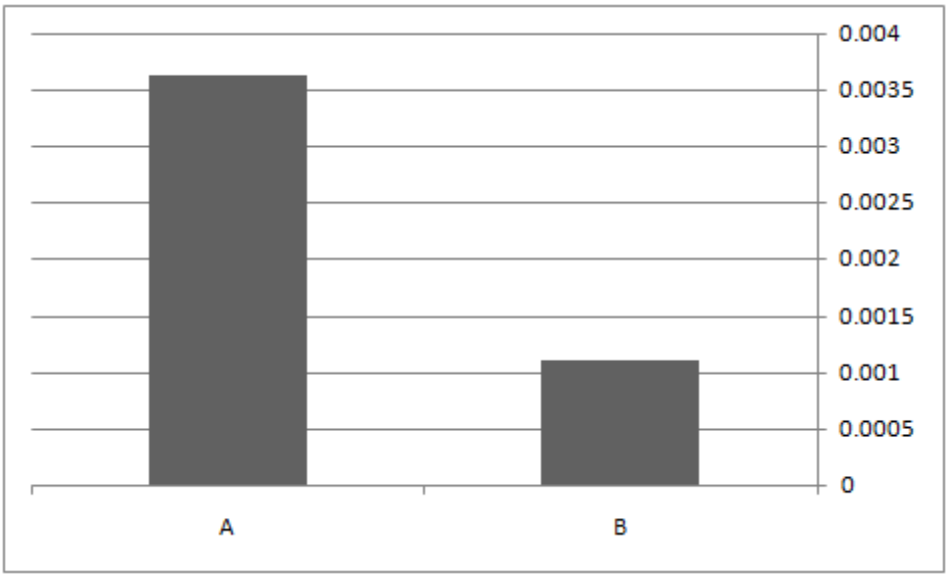


Fig. 5 : Effect ofclove volatile oil on the beta lactamase activity of *K. pneumoniae* (A)without volatile oil, (B) with volatile oil.

bla_{CIT}basically exists in *K. pneumoniae* isolates (62.5%) in Al-Muthanna city (Al-garawyi, 2016). bla_{MOX}gene was present in 7 (26.92%) of isolates, the sizebands (520 bp) that represents the bla_{MOX} gene was found in *K. pneumoniae* isolates. The bla_{MOX} gene in *K. Pneumoniae* was also the first time which discovered in our study, on other hand bla_{MOX}genewas identified in *E. coli* (Hassan and Manhal, 2015). The prevalence of resistance to cephalosporins groups by plasmid-mediated AmpC Beta-Lactamase genesis not extensively study in Iraq and for this reason there area limited control studies which looking for to AmpC β-lactamases – producing bacteria.

Detection of bla_{ACC} and bla_{MOX} genes by Phenylboronic acid disk

Phynlboronic acid test (Cefoxitin and cefotetan with boronic acid) test was conducted toconfirm the existence of bla_{ACC} and bla_{MOX} genesin *K. pneumoniae* isolates. Results were confirmed that *K. pneumoniae* isolates harbor these gens. Therefore, it is important to detect AmpC-producing organisms to ensure effective therapeutic intervention and optimal clinical outcome, as well as to control the spread of these organisms. However, most current methods for detection of these enzymes are technically demanding and time-consuming and therefore unsuitable for clinical laboratories to perform on a routine basis (Pitout *et al*, 2010).

Analysis of clove volatile oils

Clovevolatile oilanalysis was conducted by GC-MC to determine the active compounds found in it (Fig. 4).

From the Fig. 4 and Table 4, the main compounds in clove Volatileoil were Phenol, 2-methoxy-3 (2-propenyl) - Eugenol, Eugenol and Caryophyllene. In addition, we observed the presence of Humulene and Phenol, 2-methoxy-4- (2-propenyl) -acetate. Our results agree with some previous studies that noticed the same active substances clove volatile oil (Suresh *et al*, 1992; Abdullah *et al*, 2015).

Table 4 : Yield and chemical composition of clove (*Syzygium aromaticum*) volatile oil identified by GC/MS analysis.

Pk#	Retention Time	Constituents	Area%
1	21.22	Phenol, 2-methoxy-3-(2-propenyl) - Eugenol	78.60
2	21.67	Eugenol	1.52
3	22.84	Caryophyllene	13.86
4	23.70	Humulene	1.78
5	25.44	Phenol, 2-methoxy-4-(2-propenyl)- acetate	3.29
6	48.93	Squalene	0.96

Table 5 : Synergism between clove volatile oils and antibiotics against *K. pneumoniae.*

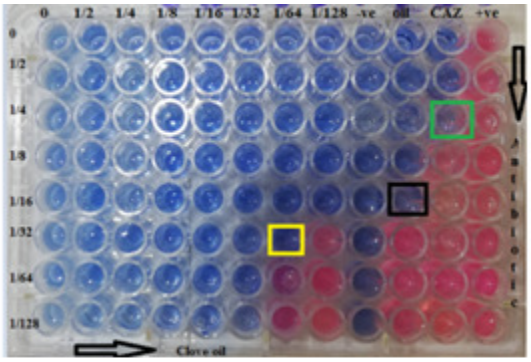
Isolat	Antibiotic	Antibiotic MIC (mg/ml) By REMA	Clove oil conc. MIC	Combined antimicrobials	Combined Antimicrobial MIC	FICI (ΣFIC)	Outcome
11	ceftriaxone	62.5	0.0625	Clove oil+CRO	0.01563.9 mg/ml	0.312	**Synergistic**
11	ceftazidime	37.5	0.0625	Clove oil+CAZ	0.0156+4.687mg/ml	0.37	**Synergistic**
11	cefepime	25	0.0625	Clove oil+CEF	0.0156+3.12 mg/ml	0.374	**Synergistic**
24	ceftriaxone	100	0.125	Clove oil+CRO	0.0156+12.5 mg/ml	0.2498	**Synergistic**
24	ceftazidime	12.5	0.125	Clove oil+CAZ	0.0156+3.12 mg/ml	0.499	**Synergistic**
24	cefepime	50	0.125	Clove oil+CEF	0.0156+3.12 mg/ml	0.187	**Synergistic**

Inhibitory activity of clove Volatile oil toward beta-lactamase

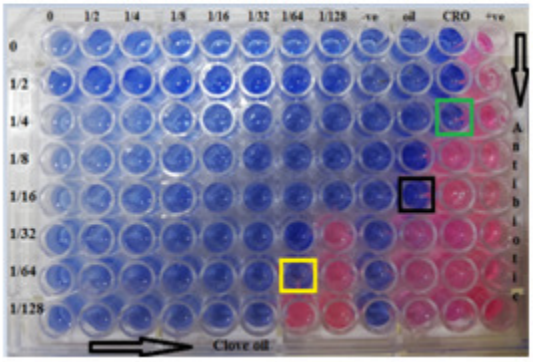
Results indicated that there was a inhibitory effect for clove volatile oil against â- Lactamase as shown in Fig. 5. *K. pneumoniae* â-Lactamase activity was (0.003633 u/mL) in all isolates without clove volatile oil, while the â-lactamase activity of *K. pneumoniae* isolates was (0.001108 U/mL) in the presence of clove volatile oil. Various essential oils have different antimicrobial activity due to their components (Verma *et al*, 2019). Antimicrobial activity clove volatile oils could be refer to Eugenol (2 methxy4 allylphenol), the main component of clove volatile oil that is already known to have antibacterial and antifungal activity (Raja *et al*, 2015).

Synergism between clove volatile oils and antibiotics

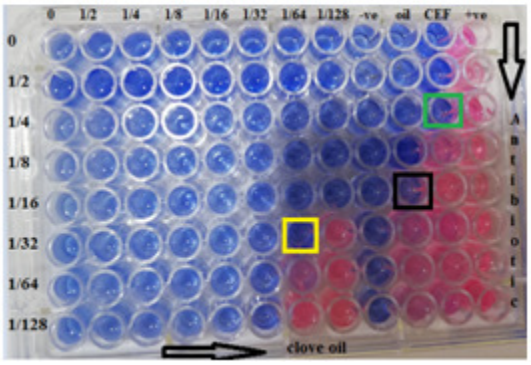
The combination of clove volatile oils with antibiotic (Cefepime, ceftazidime and ceftriaxone) against *Klebsiella pneumoniae* showed effectiveness (Table 5 and Fig. 6). Synergistic effect between clove volatile oil and antibiotics was observed. The MIC CRO decreased from 62.5 mg/ml when single to 3.9 mg/ml by effect of clove volatile oil and CEF decreased from 6.25 to 3.12 mg/ml and CAZ from 6.25mg/ml to 4.687mg/ml for isolation (11), all FICI values were less than 0.5 which indicates that the effect Synergize. All antibiotics with clove volatile oil so do isolation (24). FIC index values < 0.5 were considered as synergy and the degree of synergy increases as the value tends towards zero. Most importantly, the association of antibiotics with volatile oils targeting resistant bacteria may have a different mechanism of action and it may lead to new choices to overcome the on slaught of microbial resistance. The exploitation of volatile oils in preventing bacterial resistance is believed to be more promising because volatile oils are multi-component in nature compared to many conventional antimicrobials that only have a single target site (Yap *et al*, 2014; Al Dossary and Al Meani, 2019). In general, volatile oils act to inhibit the growth of bacterial cells and also inhibit the efflux pump and the production of toxic bacterial metabolites which facilitates the access of antibiotics to its active sites in the bacterial cell (Gutierrez *et al*, 2008). Essential oils, due to their lipophilic/hydrophobic character preferentially partitions from an aqueous phase into membrane structures of bacteria, resulting in membrane expansion, increased membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration, and alteration of ion transport processes of bacteria (Trombetta *et al*, 2005). The effect of eugenol the most common ingredient in clove Volatile oil on outer membrane permeability was evidenced by the uptake of the dye crystal violet. Generally, crystal violet penetrates the outer membrane poorly, but it easily enters when the membrane is defective. A significant enhancement in the uptake of crystal violet was observed in *Salmonella typhi* treated with eugenol when compared to control cells. This shows that eugenol alters membrane permeability and makes the cells hyperpermeable to solutes, which are generally less permeable. However, ciprofloxacin did not cause any change in permeability, as the primemechanism of action is on DNA gyrase, which leads to the termination of chromosomal replication and interferes with cell division and gene expression (Louie, 1994).



A: *Clove oil* with Ceftazidime



B: Clove Volatile oil with Ceftraxone



C: *Clove* Volatile oil with Cefepime

Fig. 6 : Representative trial of the checkerboard assay for the antimicrobial combination of Clove Volatile oil with antibiotic. The numbers on the left show the volatile oil concentrations and the numbers at the top indicate to the antibiotics concentrations, which diluted from 1/2 to 1/128. The wells represented by yellow square indicate the point at which the MIC of antibiotic combination with clove Volatile oil. The wells represented by black square indicate the point at which the MIC of clove oil. The wells represented by the green square indicate the point at which the MIC ofantibiotic. –ve control negative (media+ antibiotic), +ve control positive (media + bacterial growth).

CONCLUSION

There is high resistance to third and fourth generation of Cephalosporins among AmpC- producing *Klebsiella pneumonia* isolates, which has been increasingly recognized in Al Anbar hospitals. Thus, molecular identification of the genes encoding AmpC would be essential for a reliable epidemiological investigation of their transmission in hospitals. The combination of clove volatile oils with antibiotic (Cefepime, ceftazidime and ceftriaxone) þagainst *Klebsiella pneumonia* showed effectiveness and could improve the susceptibility of bacteria toward these antibiotics to treat the infections resulted from drug-resistant bacteria.

REFERENCES

Abdullah B H, Hatem S F and Jumaa W (2015) A comparative study of the antibacterial activity of clove and rosemary essential oils on multidrug resistant bacteria. *UK Journal of Pharmaceutical and Biosciences* **3**(1), 18–22.

Al Dossary Othman A and Al Meani Safaa A L (2019) Effect of *Mentha spicata* volatile oil on some virulence factors of *Pseudomonas aeruginosa* isolated from clinical samples. *Journal of Biotechnology* **14** (Special Issue I).

Al-Garawyi A M A (2016) Molecular expression of blaCIT and blaFOX in *Klebsiella* spp. isolates from Al-Hussein teaching hospital in Al-Muthanna Province-Iraq. *Kufa Journal For Veterinary Medical Sciences, University of Kufa* **7**(2), 33–47.

ALShamarti M J and AL-Muhnna A S H (2011) Molecular detection of AmpC Gene encoding antibiotic resistance among *Klebsiella* spp. isolated from different infections. *Al-Kufa Journal for Biology* **3**(1), 1–9.

Bhardwaj M (2017) Studies on Synergy Between Trans-Cinnamaldehyde and Selected Antibiotic (s) Using Model. *Indian Veterinary Research Institute.*

Bush K and Jacoby G A (2010) Updated functional classification of â-lactamases. *Antimicrobial Agents and Chemotherapy. Am Soc Microbiol.* **54**(3), 969–976.

Cheesebrough M (1998) District laboratory practice in tropical countries, part II (Microbiology). *Cambridgeshire Tropical Health Technology, Cambridge*, UK, 231.

Clinical and Laboratory Standars Institute (2018) M100 Performance Standards for Antimicrobial Susceptibility Testing. *Journal of Services Marketing* **doi:** 10.1108/08876049410065598.

Coudron P E (2005) Inhibitor-based methods for detection of plasmid-mediated AmpC β-lactamases in *Klebsiella* spp., *Escherichia coli* and *Proteus mirabilis. Journal of Clinical Microbiology. Am Soc Microbiol*. **43**(8), 4163–4167.

Dehghani M, Haddadi A Z and Shavandi M (2016) Multiplex PCR Study of Plasmid-Mediated AmpC Beta-Lactamase Genes in Clinical Isolates of *Escherichia coli*. *Journal of Medical Bacteriology* **5**(5–6), 21–28.

El-Hady S A and Adel L A (2015) Occurrence and detection of AmpC β-lactamases among *Enterobacteriaceae* isolates from patients at Ain Shams University Hospital. *Egyptian Journal of Medical Human Genetics*. *The Egyptian Society of Human Genetics* **16**(3), 239–244.

Gutierrez J, Barry-Ryan C and Bourke P (2008) The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*. **124**(1), 91–97.

Hassan H G and Manhal F S (2015) Phenotypic & molecular characterization of ampc â-lactamases among *Escherichia coli*, *Klebsiella* spp. & *Enterobacter* spp. Isolated from al-hussein teaching hospital in thi-qar governorate. *Al-Taqani*. *Foundation of technical education* **28**(2), E20–E32.

Hennequin C and Robin F (2016) Correlation between antimicrobial resistance and virulence in *Klebsiella pneumoniae*. *European Journal of Clinical Microbiology & Infectious Diseases* **35**(3), 333–341.

Khari F I M (2016) Genotypic and phenotypic detection of AmpC â-lactamases in *Enterobacter* spp. isolated from a teaching hospital in Malaysia. *PloS One* **11**(3), e0150643.

Kolareviæ S (2016) Optimisation of the microdilution method for detection of minimum inhibitory concentration values in selected bacteria. *Botanica Serbica* **40**, 29–36.

Langeveld W T, Veldhuizen E J A and Burt S A (2014) Synergy between essential oil components and antibiotics: a review. *Critical Reviews in Microbiology* **40**(1), 76–94.

Lawrence B M and Reynolds R J (1991) Progress in essential oils Perfumer & flavorist.

Li X-Z and Nikaido H (2009) Efflux-mediated drug resistance in bacteria. *Drugs* **69**(12), 1555–1623.

Louie T J (1994) Ciprofloxacin: an oral quinolone for the treatment of infections with gram-negative pathogens. Committee on Antimicrobial Agents. Canadian Infectious Disease Society. *CMAJ: Canadian Medical Association Journal*. **150**(5), 669.

Manuel A and Abdulrahman N (2017) Determination of Minimum Inhibitory Concentration of Liposomes: A Novel Method. *Int. J. Curr. Microbiol. App. Sci*. **6**(8), 1140–1147.

Martelius T (2016) Nosocomial bloodstream infections caused by *Escherichia coli* and *Klebsiella pneumoniae* resistant to third-generation cephalosporins, Finland, 1999–2013: Trends, patient characteristics and mortality. *Infectious Diseases* **48**(3), 229–234.

Miró E (2005) *Escherichia coli* producing an ACC-1 class C β-lactamase isolated in Barcelona, Spain. *Antimicrobial agents and chemotherapy*. *American Society for Microbiology (ASM)* **49**(2), 866.

Mutambuze J W (2014) *In Vitro* Antimicrobial Activity of BTZ043 and PNU-100480 Against *Mycobacterium ulcerans*. Kent State University.

Pitout J D D (2010) Detection of AmpC â-lactamases in *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp. and *Proteus mirabilis* in a regional clinical microbiology laboratory. *Clinical Microbiology and Infection* **16**(2), 165–170.

Raja M R C (2015) Versatile and synergistic potential of eugenol: a review. *Pharm Anal Acta* **6**(5), 367.

Rehman R (2016) Biosynthesis of essential oils in aromatic plants: A review. *Food Reviews International* **32**(2), 117–160.

Rudresh S M and Nagarathnamma T (2011) Two simple modifications of modified three-dimensional extract test for detection of AmpC β-lactamases among the members of family *Enterobacteriaceae*. *Chronicles of young Scientists* **2**(1), 42.

Sargent M G (1968) Rapid fixed-time assay for penicillinase. *Journal of Bacteriology* **95**(4), 1493.

Sfeir M M (2019) EDTA-Modified Carbapenem Inactivation Method: a Phenotypic Method for Detecting Metallo-β-Lactamase-Producing Enterobacteriaceae. *Journal of Clinical Microbiology* **57**(5), e01757-18.

Suresh P, Ingle V K and Vijayalakshmi V (1992) Antibacterial activity of eugenol in comparison with other antibiotics. *Journal of food Science and Technology* **29**(4), 254–256.

Trombetta D (2005) Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial agents and chemotherapy* **49**(6), 2474–2478.

Verma R S (2019) Chemical composition of leaves, inflorescence, whole aerial-parts and root essential oils of patchouli {*Pogostemon cablin* (Blanco) Benth.}. *Journal of Essential Oil Research* **31**(4), 319–325.

Vidhani S I (2016) Evaluation of some chemical composition, minerals fatty acid profiles, antioxidant and antimicrobial activities of Tulsi (*Ocimum sanctum*) from India. *Am J Food Sci Technol*. **4**(2), 52–57.

Wassef M (2014) Genotypic identification of AmpC β-lactamases production in Gram-negative bacilli isolates. *Jundishapur Journal of Microbiology* **7**(1).

Yagi T (2005) Practical methods using boronic acid compounds for identification of class C β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. *Journal of Clinical Microbiology* **43**(6), 2551–2558.

Yap P S X (2014) Essential oils, a new horizon in combating bacterial antibiotic resistance. *The Open Microbiology Journal* **8**, 6.