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Phenotypic and Genotypic Detection of Extended Spectrum β-lactamase in MDR- *Klebsiella pneumoniae* in Anbar Governate

Shiamaa Hameed Ahmed[*] [1] , Mayada Abdullah Shehan [2]

1,2 **Department of Biology ,College of Science , University of Anbar , Iraq**

***Corresponding author. E mail: umayhm17@gmail.com**

Abstract :

Klebsiella pneumoniae is a major pathogen responsiple for nosocomial infections in world hospitals . One of the causes for the drug resistance in the *Klebsiella pneumoniae* is the production of ESBls enzymes.This research documented *CTX-M2* for the first time among *Klebsiella pneumoniae* in Al-Anbar hospitals of Iraqi. All studied clinical isolates of *K. pneumoniae* (25) resistan- cephalosporins from 75 isolates were collected from different clinicals sources such as (burned, wounds, sputum,and urine samples). The susceptibility to different antibiotics was tested by VITEK-2 system. The phenotypic detection of Extended-Spectrum Beta-lactamases enzymes by Modified Hodge test , and mCIM that all isolates are cephalosporins gene-producing, 25/25 (100%) gave positive result with mCIM ,and 4% gave positive result for modefied Hodg test. The bla $_{CTX-M2}$ and $_{OXA1}$ genes were detected by conventional PCR and the result showed 1/25(4%) strains positive for bla $_{CTXM2}$ gene and 6/25 (24%) strains harbored bla $_{OXA-1}$ genes. This results showed the coexistence of both bla $_{CTX-M2}$ and bla $_{OXA}$ genes in one strains of *K. pneumoniae*, while indicated widespread ESBLs in Anbar, Iraq.

Key words : *CTX-M2*, cephalosporins,MHT,and OXA-1.

Introduction :

Klebsiella pneumoniae is a family member of Enterobacteriaceae that causes severe infections [1][2] . After *Escherichia coli*, *K. pneumoniae* is the second most common Gram-negative pathogen in hospitals. Multidrug-resistant (MDR) species, also known as superbugs, are causing concern these days because they are getting more serious.[3] ESBLs are plasmid-mediated enzymes that confer resistance to penicillins and cephalosporins such as sulbactam and clavulanic acid combinations, as well as monobactams such as aztreonam[4]. ESBLs are most commonly present in Klebsiella pneumoniae, an opportunistic pathogen linked to serious infections in hospitalized patients, including immunocompromised patients with significant underlying diseases[5]. *K. pneumoniae*-producing ESBL was first reported in Germany in 1983, with a steady global increase in *K. pneumoniae*-mediated resistance to cephalosporins expected in the coming decades[6].

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It was first reported in the 1980s that ESBL had extended-spectrum variants, and now several classes of ESBL have been discovered, particularly Escheria coli and Klebsiella OXA-type -lactamases, which belong to molecular class D and functional group 2d, have a high hydrolytic activity against oxacillin and cloxacillin and are inhibited poorly by clavulanic acid [7]. The hydrolytic spectrum of oxacillinase has been extended to oxyimino cephalosporins in OXA-2 and OXA-10 extended-spectrum derivatives[8] .Figure 1 shows that -lactamases from classes A, C, and D use a serine at the enzyme active nucleus, while β-lactamases from class B use metal zinc ions. -lactamases are categorized into groups 1 to 3 using the Bush-Jacobi-Medeiros process, based on the degradation of β-lactam substrates and the effect of the inhibitor[4].This study aimed to determine the presence of serine ESBLs genes including OXA-1,and CTX-M2 genes among cephalosporin-resistant *K. pneumoniae* isolated from hospitalized patients in hospitals in Anbar, Iraq.

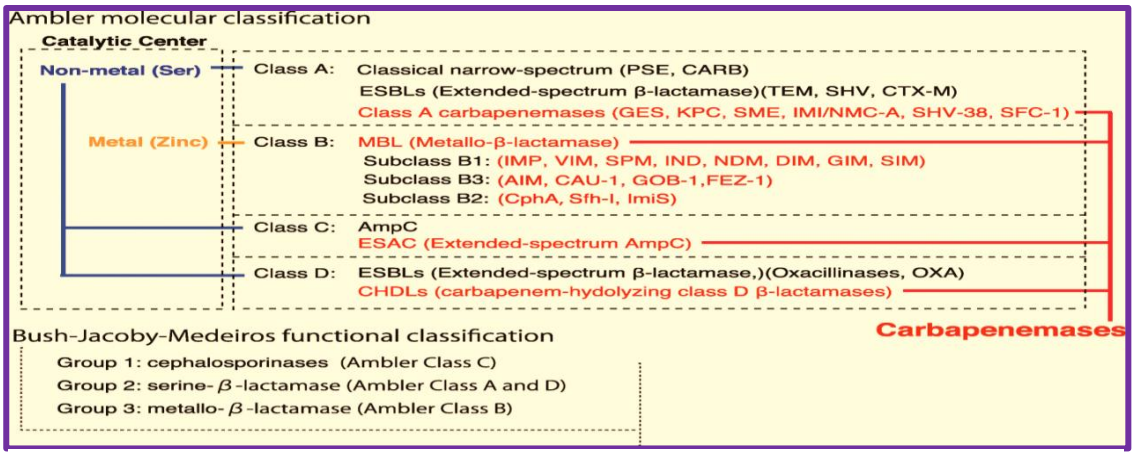

Figure1 : Classification of β - Lactamase

Materials and Methods :

Isolation and identification

In the period between August and December 2020, 75 *K. pneumoniae* clinical isolates were collected from various clinical isolates at Al-Ramadi and Al-falluja Hospitals in western Iraq. Both K. pneumoniae were collected at the bedside and immediately transported to the microbiology laboratory for inoculation and preliminary examination on appropriate culture media. Strains were removed from wounds, feces, and sputum. The identification and susceptibility profiles of K. pneumoniae were determined using the VITEK 2 method (bioMérieux) in accordance with the Clinical and Laboratory Standards Institute guidelines. [9] and

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(EUCAST)[10] . All *K. pneumoniae* was tested for their resistance against the following 15 antibiotics: piperacillin/tazobactam (TZP), cefuroxime (CXM), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP), , imipenem (IMP), and meropenem (MEM) .

Ethics and approval committee:

All study techniques that involved patients were approved by the Ethical Approval Committee, University of Anbar, Ramadi, Iraq (approval number 14, February 9, 2021). Informed, written consent was provided by all patients participating in the study.

Phenotypic detection of ESBLs :

Determination of the production of ESBls was carried out by modified Hodge test, modified Cephalosporins Inactivation Method and under the CLSI guidelines [9] and as described elsewhere[11].

Molecular Detection of ESBLs :

Using the Wizard® Genomic DNA Purification Kit, genomic DNA was extracted from an overnight culture (Promega, USA). The concentration and purity of the DNA extract were determined by measuring absorbance at 260 and 280 nm wavelengths (NanoVue Plus; United States). Electrophoresis was used to assess the integrity of genomic DNA. The primers used in this study were given in lyophilized form and then dissolved in sterile deionized distilled water (Alpha DNA, Canada). (Table I).

Table I : Sequences of OXA-1 , and CTX-M2

gene	5 ` - Oligo seq – 3`	Size bp	Refernce
OXA-1	F:ACACAATACATATCAACTTCGC	813	[12]
	R: AGTGTGTTAGAATGGTGAT		
CTX-M2	F:GCGACCTGGTTAACTAATCC	351	[13]
	R:CGGTAGTATTGCCCTTAAGCC		

*F : forward , R : Reverse.

For PCR program , the initial denaturation phase for each PCR assay with different primers was established on 95°C for 5 min also denaturation was 95°C for 32 sec.

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The annealing time was 30 sec for all primers and temperature was 60 , 65 for bla OXA-1 and bla CTX-M2 , respectively. The extension time was 30 sec in 72°C. The final extensionfor all genes was done at 72°C for 7min .

Results and disscusion :

Isolation and identification

To confirm the diagnosis, the collected isolates were initially diagnosed as *K. pneumoniae*. The bacterial isolates were cultured on Blood agar, and MacConkey agar under aerobic conditions followed by other diagnostic tests figure 2. All isolates showed pink lactose fermenter mucoid colonies on MacConkey agar (MCA). On Blood agar media they grow as nonhemolytic grey-white, mucoid colonies after 24 h of incubation. Table 1 explain other diagnostic test for *K. pneumoniaea* .

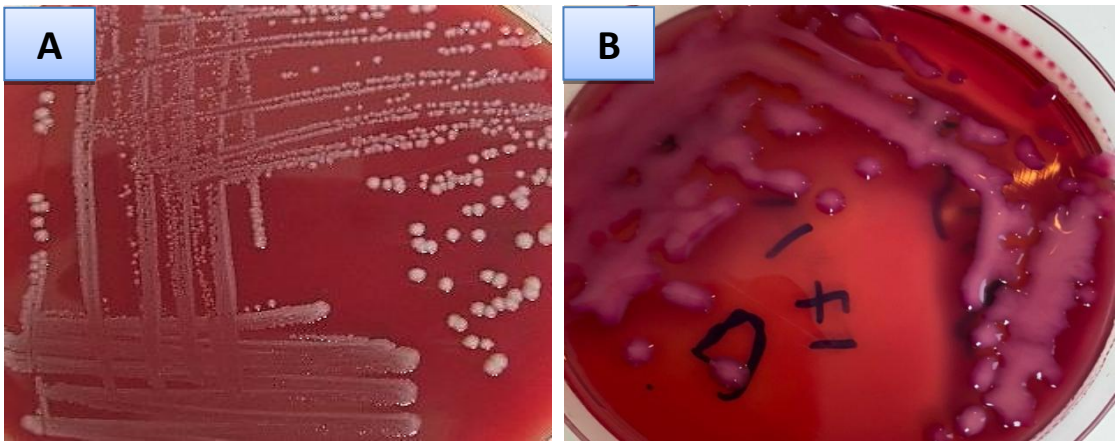

Figure 2 : Klebsiella pneumoniae on B : MacConky agar , A: Blood agar .

Table 2 : Microscopic and biochemical test .

Characteristics	Klebsiella pneumoniae
Gram stain	Negative
Shape	Rod
Catalase	+ve
Citrate	+ve
Indole	-ve
Oxidase	-ve
Urease	+ve

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Antibiotic susceptibility testing :

The antibiotic susceptibility test revealed that all of the studied *K. pneumonia* (25/75)from 75 clinical strains were resistant cephalosporins community of β- lactam and they were resistant to most antibiotics under test and it showed an elevated resistance to various groups of β- lactam and non β- lactam antibiotics.

Phenotypic detection of ESBLs:

The phenotypic detection of **ESBLs** by using : modified hodge test, modified cephalosporins inactivation methods for Suspected ESBLs . Phenotypic testing revealed the existence of the ESBL genes in all resistance isolates. Since the updated Hodge test was used as a phenotypic confirmatory procedure for ESBLs, this examination yielded a positive result of 1/25 (4 %).

In this analysis, modified carbapenem inactivation methods for suspected ESBLs development were used as phenotypic confirmatory methods for distinguishing between serine (OXA-1 and CTX-M2) and other metallo—lactamases production, with the results showing that 23/25 (92%) was serine beta lactamase and 2/25 (8%).

Molecular detection of ESBLs :

According to 3, 4, the work identified 1/25 (4%) positive strains for the bla KPC gene and 6/25 (24%) positive strains for the bla bla OXA gene. This study established the coexistence of the bla CTX-M2 and bla OXA genes in a single K. pneumoniae strain.

The frequency of occurrence of bla OXA genes in studied isolates differs considerably. In Iran, Mostatabi et al. discovered that 20.51 percent of Serratia isolates containing ESBL bore the blaOXA gene[14]. Bourouis et al. (2013) demonstrated the existence of blaOXA-1 genes among ESBL-producing E. cloacae in Tunisia[15] .In Cameroon, blaOXA-1 genes were detected in all isolates[16]. Rakotonirina et al. (2013) estimated that 14.28 percent of ESBL-producing isolates in Madagascar possessed the bla OXA-1 gene, which was lower than our finding. The antimicrobial resistance pattern of K. pneumoniae strains that cause septicemia and the prevalence of inhibitor resistant OXA-1-lactamase genes among them. Further screening for blaOXA-1 was performed on these isolates. Amplification of -lactamases genes using traditional polymerase chain reaction revealed the existence of blaOXA-1 genes in 12 K. pneumoniae isolates (20.3 percent) [17]. Ramazanzadeh (2010) reported that genes encoding TEM, OXA-1, and OXA-2 were detected in

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14.85, 14.58, and 4.17 percent of ESBL generating Klebsiella, respectively. Numerous researchers demonstrated that these genes often coexist with other genes in the same genetic environment. Combinations of blaCTX-M-15, blaOXA-1, and blaTEM-1b have been identified in 30 strains from Portugal (Mendonca et al., 2007), and a connection between bla genes has been described in the Brazilian community [18]. In Portugal [19] and the United States of America [20]., the interaction of blaCTX-M-15 and blaOXA-1 in the same strain was also identified. E. coli and K. pneumoniae have also been identified to produce CTX-M and OXA enzymes in combination [21].

This explains the isolates' high resistance to third- and fourth-generation cephalosporins. Additional studies conducted in Iran have shown discrepancies in the frequency of CTX-M. CTX-M2 and CTX-M3 were found to have a frequency of 0.0 in a study conducted in Kashan [22]. However, in a study conducted in Shiraz, CTX-M2 was found to be present at a frequency of 13.5 percent [23]. This discrepancy may be explained by the sample forms. Other countries have identified varying prevalence rates of ESBL. According to studies conducted in Brazil (2010), the highest prevalence of ESBL enzymes was associated with CTX-M2 (19.6 percent) [24]. The frequency of the CTX-M enzyme in K. pneumonieae strains was reported to be 0.0 percent in studies conducted in France (2010) and Japan (2003), which is probably due to the appropriate use of beta-lactam antibiotics, especially cephalosporins, in these countries[25]. Another study by[26], OXA-48 was documented in Anbar governate.

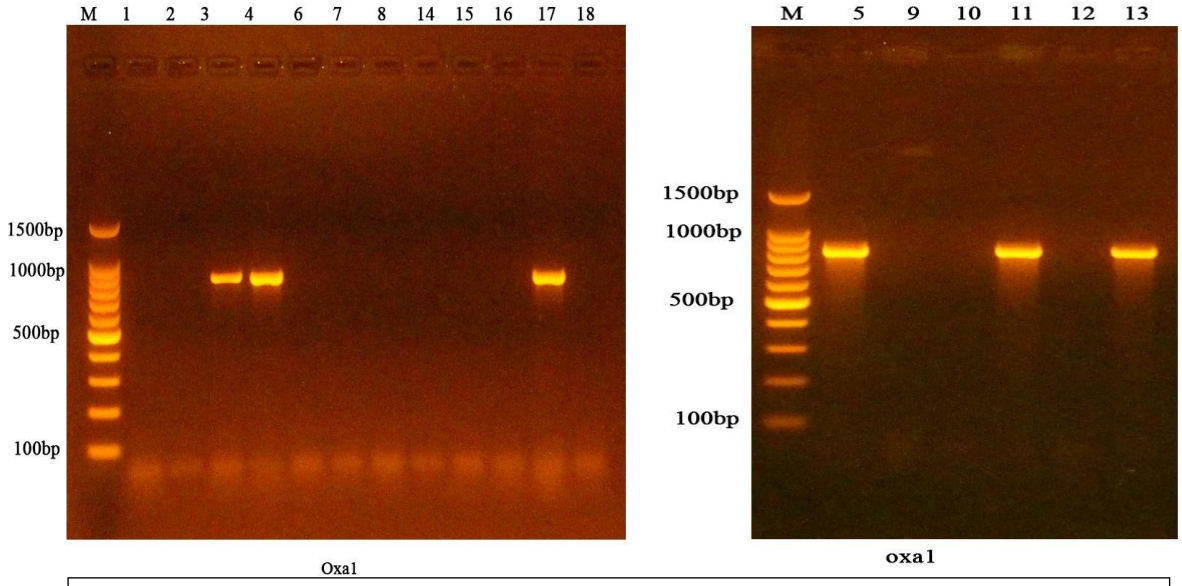

Figure 3 : Results of the amplification of *OXA1* gene of *Klebsiella pneumoniae* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-18 resemble PCR products.

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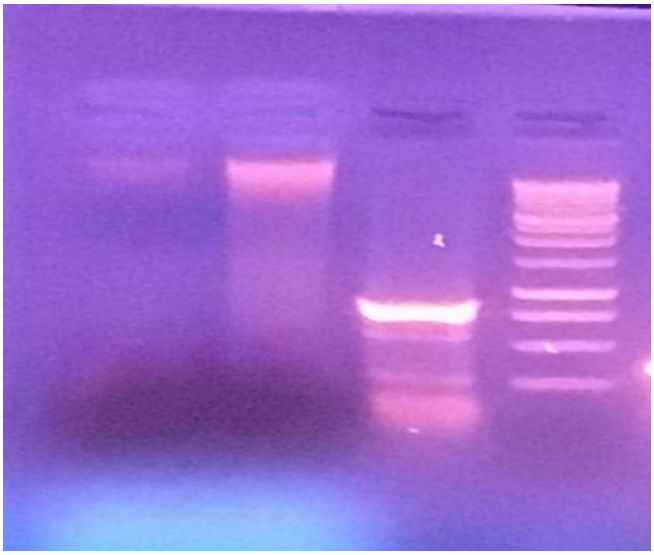

Figure 2 : Results of the amplification of IMP gene of Klebsiella pneumoniae samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-18 resemble PCR products.

Conclusion :

High rate of occurrence of bla OXA-1 genes among identified Klebsiella which might indicated the high level of pressure obtained from the use of related antibiotics.

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