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The Occurrence of ESBL genes among Extended-Spectrum β-Lactamases Producing *Klebsiella pneumoniae* among wound and burn cases in Al-Najaf Hospitals

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ABSTRACT— Drug resistance is one of the major issues in the world, and it led to a limitation of treatment options for serious health problems. The present work was achieved to detect the existence of extended-spectrum β-lactamase (ESBL) genes among *Klebsiella pneumoniae* isolates. Wound exudates and burn swabs (a total of 1590) were collected from the general hospitals in Al-Najaf city. The recommended biochemical assays were used to detect the *Klebsiella* spp. and the API 20E system was used to confirm this detection. MIC-strip and disk diffusion methods were used to identify the antibiotics susceptibility pattern and ESBLs were detected phenotypically, while by using the PCR technique, the *bla*-genes (bla_{OXA}, bla_{CTX-M}, bla_{TEM}, and bla_{SHV}) were identified. Out of 1590 isolates, 109 (6.9%) isolates of *K. pneumoniae* were identified, and 4 (3.6%), 32 (29.3%), and 93 (85.3%) isolates were considered as pan-drug resistant (PDR), extensive drug resistant (XDR), and multi-drug resistant (MDR) respectively. ESBL producer isolates were identified in 86% of the isolates. PCR assay identified that 52.3% of the isolates holding one type of *bla*-genes at least with the predominance of the bla_{CTX-M} gene (35.7%). While the bla_{OXA} gene was detected in 36 (33%) isolates and bla_{SHV} gene was detected in 12 (11%) isolates. From the results of the present study, we concluded the presence of an alarming percentage of isolates of *K. pneumoniae* that produce ESBL in hospitals of Al-Najaf city and the existence of high rate of CTX-M-ESBL among these isolates.

KEYWORDS: *Klebsiella pneumonia*, Extended-Spectrum β-Lactamases**.**

1. INTRODUCTION

Klebsiella pneumoniae is a prominent hospital acquired pathogen that can cause outbreaks, as what happened in the previous decades especially in 1980s and 1990s [1]. Most *K. pneumoniae* infections are nosocomial and associate with a high mortality rate if the treatment was inappropriate. Antibiotic resistant phenotypes often obtained from the nosocomial infections isolates, and can spread to the community settings [2].

Broad and narrow spectrum cephalosporins, aztreonam except cephamycins and carbapenems can be hydrolyzed by extended-spectrum B-lactamases (ESBLs). CTX-M is a recently described rapidly growing family of the ESBLs. Over the last 20 years, CTX-M displaces SHV as a significant sort of ESBLs that distributes around the world [3]. There are different geographical patterns of ESBL, and according to

enormous observation programs that indicated the predominance of ESBLs among pathogens throughout the world [4]. So, the present work was achieved to detect the existence of extended-spectrum β-lactamase (ESBL) genes among *Klebsiella pneumoniae* isolates.

2. Material and methods

2.1 Collection of Specimens

In this cross-sectional study, specimens of two different clinical types were randomly collected from patients attended or admitted to Al-Sadder Medical City, and Al-Hakeem General Hospital, from November 2020 to July 2021. Informed consent was obtained for each patient. These specimens included wound exudates and burn swabs.

The Kufa Medical College Ethical Committee approved the protocol of this study.

2.2 Bacterial isolates identification

The diagnosis of the isolates of *K. pneumoniae* were performed according to [5] by using recommended biochemical tests and confirmed by using the API 20E system.

2.3 Antibiotic Susceptibility Testing

The guidelines recommended by Clinical And Laboratory Standards Institute (CLSI) [6] were followed to perform the antibiotic susceptibility testing of *K. pneumoniae* isolates. Antibiotics disks (Cypress, Belgium): Amoxicillin (25 µg), ampicillin-sulbactam (20 µg), amoxicillin-clavulanic acid (30 µg), piperacillin (25 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), aztreonam (30 µg), meropenem (10 µg), imipenem (10 µg), nalidixic acid (30 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), gatifloxacin (5 µg), amikacin (30 µg), (10 µg), kanamycin (30 µg), chloramphenicol (30 µg), sulfamethoxazole (50 µg), trimethoprim (5 µg) were used. The reference strain for quality control of the tested antibiotics in this study was *E. coli* ATCC 25922.

2.4 ESBL Production Detection

2.4.1 Initial Screening

The disk diffusion technique is the first method used for screening the production of ESBL to ceftazidime, ceftriaxone, cefotaxime, and aztreonam (30µg/disk each), CLSI [6].

2.4.2 Confirmatory Tests

Further determination of the potential ESBL producer isolates were achieved by the use of the disk approximation technique. This technique depend upon the clavulanic acid inhibitory effect according to the CLSI [6] criteria.

2.4.3 β-Lactamase genes detection

Monoplex PCR was used in the analyses of the isolates of *K. pneumoniae* to detect the *bla*-genes, Screening was carried out by PCR for bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{OXA} genes using primers and PCR conditions listed in Table (1). The template of DNA was prepared based on the Chang and Jiany [7] procedure. Kappa Master mix 2X (Kapa Biosystems, Massachusetts, US) was used to perform the PCR amplification based on the manufacture procedure in a T3000 thermocycler (Biometra, Germany). The agarose gel electrophoresis (Biometra, Germany) in 1.5 % (w/v) agarose gel was used to separate the PCR products and by using a gel documentation system (Biometra, Germany) these products had been visualized.

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Table (1): Genes, primers, and PCR condition used to detection *bla* $_{ESBL}$ gene

***bla*$_{ESBL}$-gene**	**Primer**	**PCR condition**	**Target bp**	**Reference**
bla$_{TEM}$	**TEM**-F 5-ATGAGTATTCAACATTTCCG-3	(94°C, 60sec; 58°C, 60sec; 72°C, 60sec) 30 cycles	867 bp	[8]
	TEM-R 5-CTGACAGTTACCAATGCTTA-3			
bla$_{SHV}$	**SHV**-F 5-GGGTTATTCTTATTTGTCGC-3	(94°C, 60sec; 56°C, 60sec; 72°C, 60sec) 30 cycles	930 bp	[8]
	SHV-R 5-TTAGCGTTGCCAGTGGTC-3			
bla$_{OXA}$	**OXA**-F 5-GGCACCAGATTCAACTTTCAAG-3	(94°C, 40 sec; 60°C, 40 sec; 72°C, 60sec) 30 cycles	554 bp	[9]
	OXA-R 5-GACCCCAAGTTTCCTGTAAGTG-3			
bla$_{CTX-M}$	**CTX-M-F** 5'- SCS ATG TGC AGY ACC AGT AA -3	(94°C, 30 sec; 63°C, 60sec; 72°C, 60sec) 30 cycles	564 bp	[10]
	CTX-M-R 5'- CCG CRA TAT GRT TGG TGG TG -3			

Statistical analysis: No statistical analysis was need in the present study.

3. Results

This study's results showed that from a total of 1590 specimens, only 109 (6.9%) isolates were identified as *K. pneumoniae* depend on the characteristics of the colonies, conventional biochemical assays and API 20E system tests.

3.1 Antibiotic Susceptibility of the Isolates of K. pneumoniae

The 109 isolates of *K. pneumoniae* were involved in the current study and investigated for sensitivity to antibiotics. The antibiotics susceptibility pattern was shown in Table (2). The results interpretation was accomplished according to CLSI guideline [6]. By using the susceptibility test, 93(85.3%) *K. pneumoniae* isolates were resistant to three or more of the 11 classes of the antibacterial agents included in the present study. According to the obtained results, all the isolates were classified as multidrug resistant (MDR) isolates according to Magiorakos et al., whereas, 32 (29.3%) isolates were considered as extensive drug resistance (XDR) organisms. While, pandrug resistance (PDR) was detected in 4 (5.4%) isolates (which revealed resistance to all the antibacterial agents that tested in this study).

Table (2): Pattern of antibiotic susceptibility of the isolates of *Klebsiella pneumoniae* (n= 109)

Antibiotic disk	**Isolates No. (%)**	
	Resistant	**Susceptible**
Amoxicillin	109(100%)	0%
Piperacillin	98(89.9%)	11(10.0%)
Amoxicillin-clavulanic acid	103(94.4%)	6(5.5%)
Ampicillin-sulbactam	95(87.1%)	14(12.8%)
Cefotaxime	88(80.7%)	21(19.2%)
Ceftazidime	89(81.6%)	20 (18.3%)
Ceftriaxone	88(80.7%)	21(19.2%)
Cefepime	63(57.7%)	46(42.2%)
Cefoxitin	47(43.1%)	62(56.8%)
Aztreonam	72(66.1%)	37(33.9%)
Imipenem	22(20.1%)	87(79.8%)

Meropenem	26(23.8%)	83(76.1%)
Nalidixic acid	46(42.2%)	63(57.7%)
Ciprofloxacin	43(39.4)	66(60.5%)
Gatifloxacin	19(17.4%)	90(82.5%)
Levofloxacin	20(18.2%)	89(81.6%)
Amikacin	28(25.6%)	81(74.3%)
Gentamicin	38(34.8%)	71(65.1%)
Kanamycin	42(38.5%)	67(61.4%)
Chloramphenicol	29(26.6%)	83 (76.1)
Sulfamethoxazole	77(70.6%)	32(29.3%)
Trimethoprim	84(77.1%)	25(22.9%)

3.2 Screening for the Production of ESBLs

All the 109 *K. pneumoniae* isolates were investigated for the presence of ESBLs using phenotypic tests and *bla*-genes detection as follows: Out of the 109 isolates of *K. pneumoniae* for initial screening test for ESBL production, all the isolates (100%) demonstrated resistance to at least one cephalosporin item of the third-generation (potential ESBL producers). Positive resistant isolates was detected as following: 80.7% for cefotaxime, 80.7% for ceftriaxone, 81.6% for ceftazidime, and 66.1% for aztreonam. All resistant isolates were subjected to the confirmatory method by disk approximation. Based on the disk approximation test, no isolate was found to be ESBL producer.

3.3 Detection and Distribution of blaESBL genes

By the PCR technique, all the 109 isolates of *K. pneumoniae* were surveyed for the existence of bla_{ESBL} genes, and the detected *bla*-genes among isolates were demonstrated in Table (3). The frequency of *bla*-genes observed by this study was 57 (52.3%). Existence of $bla_{\text{CTX-M}}$ genes was found to be the most predominant ESBL, which observed in 39 (35.7%) isolates (Figure 1). Whereas, 36 (33%) isolates carried the bla_{OXA} gene type (Figure 2). Screening for other ESBL genes in the isolates exposed the presence of bla_{SHV} gene in 12 (11%) isolates (Figure 3). In this study, genes encoding bla_{TEM} were not identified.

The presence of a combination of bla_{ESBL} genes among the *K. pneumoniae* isolates is shown in Table (3). Three isolates carried 3 genes (bla_{OXA}, $bla_{\text{CTX-M}}$ and bla_{SHV}) and 24 isolates carried 2 genes. Nineteen isolates carried a combination of bla_{OXA} and $bla_{\text{CTX-M}}$, followed by $bla_{\text{CTX-M}}$ and bla_{SHV} in 4 isolates. While a combination of bla_{OXA} and bla_{SHV} was detected in one isolate.

Table (3): Distribution of *bla* ESBL genes and their combinations among the 109 clinical isolates of *K. pneumoniae*.

***bla* gene type**	**Isolates No. (%)**
CTX-M	13 (11.9%)
OXA	12 (11.1%)
SHV	4 (3.7%)
OXA and *SHV*	1 (0.9%)
CTX-M and *SHV*	4 (3.7%)
OXA, CTX-M and *SHV*	3 (2.7%)

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*OXA*and *CTX-M*	19 (17.4%)
Total	57 (52.3%)

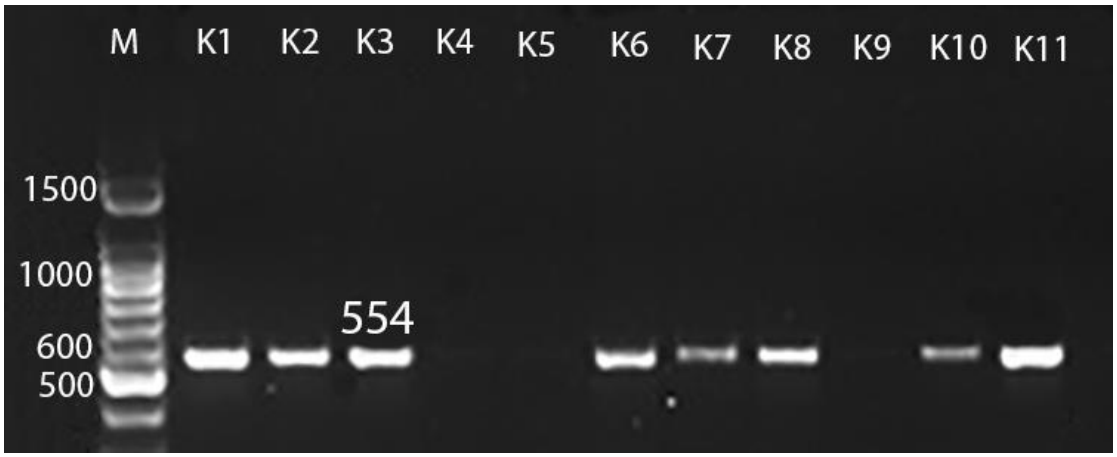

Figure (1): Ethidium bromide-stained agarose gel of monplex PCR amplified products from extracted DNA of *Klebsiella pneumoniae* isolates and amplified with bla_{CTX-M} genes primers. The electrophoresis was performed at 70 volt for 2 hr. Lane (M), DNA molecular size marker (100 bp ladder Qiagen), Lane 15 negative control. Lanes (K 1, 2, 3, 6, 7, 8, 10, 11and 14) show positive results with bla_{CTX-M} (554bp).

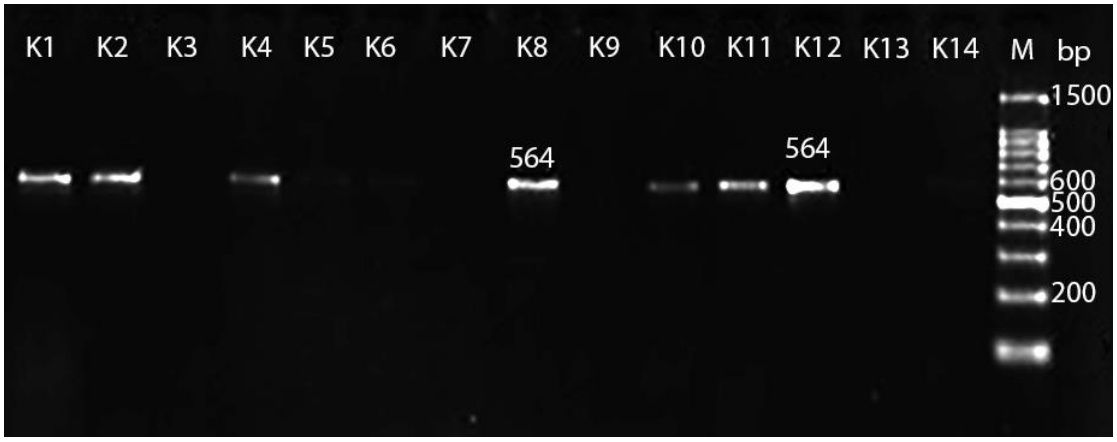

Figure (2): Ethidium bromide-stained agarose gel of monplex PCR amplified products of the extracted DNA of isolates of *Klebsiella pneumoniae* that amplified by bla_{OXA} genes primers. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (M), DNA molecular size marker (100 bp ladder Qiagen), Lane 14 negative control, Lanes (K 1, 2, 4, 5, 6, 8, 10, 11and 12) show positive results with bla_{OXA} (564bp).

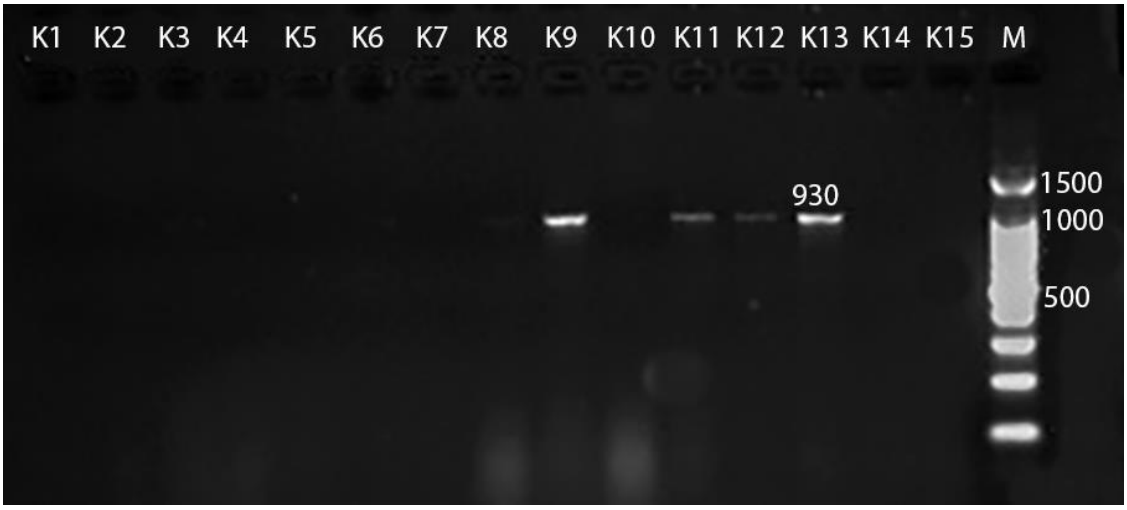

Figure (3): Ethidium bromide-stained agarose gel of monplex PCR amplified products from extracted DNA of *Klebsiella pneumoniae* isolates and amplified with bla_{SHV} genes primers. The electrophoresis was performed at 70 volt for 2 hr. Lane (M), DNA molecular size marker (100 bp ladder Qiagen), Lane 15 negative control, Lanes (K 9, 10, 11, 12, and13) show positive results with bla_{SHV} (930bp).

4. Discussion

The *K. pneumoniae* multidrug resistant strains (MDR) had been excited recently and are considered as the foremost threat to the community health [8]. In this study we found that the majority of isolates were identified as MDR. As this percentage is relatively high, although it is similar to what was reported by other studies in different places [9], and higher than studies conducted in Pakistan [10] and Southwest China [11]. This high rate of XDR, and MDR isolates of *K. pneumoniae* may be due to the irresponsible use of antibiotics and this will lead to strong limitation in the use of the antibiotics of choice for empiric therapies in farther treatments.

The antimicrobial susceptibility profile against the third generation cephalosporins gave a higher resistance rate in initial screening test. The present result comparatively higher than another previous local study by [12], as well as, another study conducted in Abidjan[1]. The results of the disk approximations test were negative to all the tested *K. pneumoniae* isolates for confirmation the production of ESBL. While in Philippines, the confirmatory tests were variable depend on the sours of isolates [13]. The ESBL-producer is difficult to detect, therefore CLSI recommended more than one confirmatory test of ESBL-producer detection. The present result is in line with what reported by [12]. The existence of an ESBL-producer isolates that were not confirmed, may be due to the simultaneous production of other type of β-lactamases that could conceal the existence of the ESBL (AmpC β-lactamase or inhibitor resistant cephalosporinases).

Molecular methods have points of interest over phenotypic methods in that they precisely and quickly distinguish the resistant genes. The present study demonstrated that 52.3% of *K. pneumoniae* isolates yielded positive results to at least one of the *bla*-genes, this results are much higher than the results of previous studies performed in Abidjan (26%)[1] and Pakistan (8.6%) [10].

The present results revealed that the $bla_{CTX\text{-}M}$ gene was the commonest gene (35.7%) in the positive isolates. Similar result was found in local previous study by [12], and is in agreement with numerous studies conducted in Philippines, China and Pakistan [9], [10], [13]. The high mobilization of $bla_{CTX\text{-}M}$ gene among the other bla_{ESBL} genes may responsible for the higher frequencies of CTX-M among the other ESBL enzymes, with a frequency of ten times higher than other β-lactamases of class A. The *bla*-genes are

mobilized to plasmids according to Barlow *et.al*. study [14].

Results of the current study revealed that bla_{OXA} β-lactamase gene was detected in 36% of *K. pneumoniae* isolates. This result is relatively lower than the results of other previous studies in Philippines, Morocco, and China [13], [15], [16], as well as the local previous study in Al-Najaf that was carried out by [12]. However, OXA β-lactamases characterized as one of the most prevalent plasmid-encoded β-lactamase. The widespread distribution of the bla_{OXA} gene is associated with integrons that frequently carry other ESBL determinants [17].

Moreover, the present results indicated that 11.% of *K. pneumoniae* isolates possessed bla_{SHV} gene and these results were significantly lowers than that reported worldwide [11], [12], [18]. Most isolates of *K. pneumoniae* have plasmid or chromosomally-mediated SHV-1 β-lactamase, and these β-lactamases are with a narrow-spectrum to a broad-spectrum activity, active against monobactams and carbapenems and present in high copy numbers in bacteria [19].

This study's result also demonstrated that no amplification product specific to bla_{TEM} gene in *K. pneumoniae* isolates was detected, and this finding is inconsistent with earlier reports in Al-Najaf [12], [20]. While, other studies in India and Philippines gave higher rate of bla_{TEM} by PCR [13], [21].

The present study detected that among 57 isolates carrry *bla*-genes, 30 (63.8%) isolates carry more than one β-lactamases genes within a single isolate of *K. pneumoniae*. Current result is in line with several previous studies reported the simultaneous expression of different β-lactamases genes in a single isolate [20], [22]. Moreover, the co-production of SHV and CTX-M enzymes in the strains of *K. pneumoniae* appears to be very common nowadays. The simultaneous production of OXA and CTX-M enzymes by *K. pneumoniae* aids the production of β-lactamase inhibitors resistant strains [14].

The high occurrence of ESBL-producing *K. pneumoniae* with multiple β-lactamases (CTX-M, OXA, TEM, SHV) poses major problems in the hospitals in Al-Najaf. Therefore, close monitoring and antimicrobial stewardship program must act a crucial role in controlling the spread of ESBL in Al-Najaf. These results consolidate the crucial need of a routine bacterial screening for ESBL in Al-Najaf to provide fast and truthful results.

5. Conclusion

From the results of the present study, we concluded the presence of an alarming percentage of isolates of *K. pneumoniae* that produce ESBL in hospitals of Al-Najaf city and the existence of high rate of CTX-M-ESBL among these isolates.

6. Acknowledgments

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7. Ethical Permissions

All procedures were performed in compliance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The current study has been approved by the ethics committee of the Faculty of Medicine, University of Kufa. Informed consent was obtained for each patient and healthy person. The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq.

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