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Extended-spectrum beta-lactamases among *Klebsiella pneumoniae* from Iraqi patients with community-acquired pneumonia

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SUMMARY

OBJECTIVE: Beta-lactams resistance is a major clinical problem in treating pneumonia. This study aimed to detect the extended-spectrum beta-lactamases (ESBL) genes in *Klebsiella pneumoniae* among patients with community-acquired pneumonia (CAP) in Al-Najaf City, Iraq.

METHODS: A total of 511 sputum samples were obtained from all suspected patients with CAP in Al-Najaf City, Iraq, from March 2020 to September 2020. Sputum samples were subjected to microbiological tests. The disk diffusion method was used to test antibiotic sensitivity. Production of ESBLs was identified using phenotypic and genotypic methods.

RESULTS: The total prevalence of *K. pneumoniae* was 31.9% (163/511). Using CHROM agar, 41 (25.2%) isolates were ESBL producers. The imipenem 0.0% (n=0/41) and norfloxacin 0.0% (n=0/41) were the most effective antibiotics. The multiplex polymerase chain reaction showed that 46.3% (n=19/41) of isolates harbored ESBL genes. Out of 19 ESBL producers, 47.4% and 15.8% harbored $bla_{CTX\text{-}M}$ and bla_{SHV}, respectively. While $bla_{CTX\text{-}M}$ and bla_{SHV} genes were detected in 7 (36.8%) isolates, simultaneously.

CONCLUSIONS: The imipenem and norfloxacin can be used in empirical treatment of *K. pneumoniae* isolates in Iraq. The emergence of *K. pneumoniae* strains harboring ESBL resistance genes necessitates the development of a regular surveillance program to prevent the spreading of these isolates more in Iraqi health care systems.

KEYWORDS: $bla_{CTX\text{-}M}$. CAP. ESBL. Pneumonia. Klebsiella pneumoniae.

INTRODUCTION

According to the British Thoracic Society, community-acquired pneumonia (CAP) is an acute symptomatic infection of the lung parenchyma that occurs outside a hospital or nursing home[1]. CAP is caused by various microorganisms including *Klebsiella pneumoniae*[2]. No exact information about the incidence of the CAP in Iraq has been found so far. Clinical burden of CAP in older adults has only been assessed by a few large databases, with incidence rates ranging from 7.6 to 13.4 per 1,000 individuals[3]. A previous study from Iraq revealed *K. pneumoniae* as the leading cause of pneumonia[4].

Strains of *K. pneumoniae* that can produce extended-spectrum beta-lactamases (ESBLs) become seriously active against many types of beta-lactam antibiotics. In addition, these virulent strains are capable of becoming resistant to numerous classes of non-beta-lactams, making it difficult to treat infections, and are referred to as multidrug-resistant (MDR) strains[5].

Nearly 450 forms of ESBLs enzymes have been documented worldwide, and among these types, bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$ were predominant. ESBLs are enzymes that contribute to resistance to a variety of beta-lactams[6]. ESBLs hydrolyze the beta-lactam ring of beta-lactam antibiotics, causing these antibiotics to lose their antimicrobial activity. These factors may contribute to the development of pneumonia complications[6,7].

To date, there are no studies on the prevalence of ESBL-producing *K. pneumoniae* in patients with CAP in Iraq. Therefore,

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the present research attempted to identify the ESBL-producing *K. pneumoniae* in Iraqi patients with CAP by phenotypic and molecular genotypic methods.

METHODS

Sample collection and bacterial isolation

The sputum samples of suspected patients suffering from CAP referred to Al-Sader Teaching Hospital in Al-Najaf City, Iraq, from March 2020 to September 2020 were collected in sterile containers. All patients were selected and diagnosed by a respiratory infectious disease specialist based on clinical examination and radiological and laboratory findings. Sputums were processed within 1 to 2 h of collection, using standard microbiological procedures. Sputums were initially cultured on blood agar and MacConkey agar (Merck, Germany) and incubated at 37°C for 2 days. The suspected *K. pneumoniae* colonies were further tested and identified using a panel of appropriate biochemical tests, including citrate utilization, urease, methyl red/Voges Proskauer, and triple sugar iron agar[8]. The confirmed *K. pneumoniae* isolates were stocked in tryptic soy broth containing 20% glycerol and placed at -80°C for long preservation.

Phenotypic detection of ESBL-producing *K. pneumoniae*

CHROM agar

All *K. pneumoniae* isolates were streaked on plates of CHROMagar™ ESBL agar (Pioneer, France). Chrome agar plates were aerobically incubated at 35°C overnight. Colonies of ESBL producers were appeared greenish blue.

Antimicrobial susceptibility testing

The ESBL-producing *K. pneumoniae* isolates were tested for antimicrobial susceptibility testing (AST) using disk diffusion technique according to the Clinical and Laboratory Standards Institute (CLSI) instructions[9]. Antimicrobials were classified as follows: aztreonam (ATM, 30 µg), gentamicin (CN, 10 µg), ciprofloxacin (CIP, 5 µg), ceftazidime (CAZ, 30 µg), levofloxacin (LEV, 5 µg), amoxicillin/clavulanate (AMC, 30 µg), trimethoprim (TMP, 5 µg), norfloxacin (NOR, 10 µg), cefotaxime (CTX, 30 µg), nitrofurantoin (F, 300 µg), imipenem (IPM, 10 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), and ceftriaxone (CRO, 30 µg) (Bioanalyse, Turkey). MDR isolates were determined according to the previous definition (resistance to at least one member of three antibiotics classes)[10]. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control strains.

Molecular detection of ESBL genes among *K. pneumoniae*

The presence of ESBLs encoding genes (bla_{SHV} and bla_{CTX-M}) were investigated by multiplex polymerase chain reaction (M-PCR) using previously described primer pairs (Bioneer, Koria)[11]. The DNA was extracted using genomic DNA extraction kit (FavorPrep, USA), according to the supplier instructions. All the components of M-PCR were mixed in final volume of 20 µl as follows: 12.5 µl of Master Mix (iNtRON, Koria), 5 µl of DNA template, 1.5 µl of DNA/RNA free water, and 0.5 µl of each reverse and forward primer. M-PCR mixture was put in a thermocycler (Biosystems, USA) instrument with following program: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 50 s, annealing at 50°C for 40 s, elongation at 72°C for 60 s, and final extension at 72°C for 5 min. *E. coli* NCTC 13353 and *K. pneumoniae* ATCC 700603 were used as bla_{CTX-M}- and bla_{SHV}-positive controls, respectively.

Statistical analysis

The data for this research were analyzed statistically using the Statistical Package for Social Science (SPSS) version 20.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Bacterial isolation

In total, 511 sputum samples were taken from 302 (59.1%) male and 209 (40.9%) female patients with CAP, from which 148 (29.0%) Gram-positive bacteria and 308 (60.3%) Gram-negative bacteria (GNB) were isolated. Also, 55 (10.7%) samples showed no bacterial growth. Out of 308 GNB, 163 (52.9%) isolates were identified as *K. pneumoniae* and were recorded as a major cause for pneumonia in this study. These isolates were obtained from 102 (62.6%) males and 61 (37.4%) females, respectively. The total prevalence of *K. pneumoniae* was 31.9% (163/511).

Phenotypic detection of ESBL producers

Using CHROM agar method, 41 (25.2%) and 122 (74.8%) *K. pneumoniae* isolates were found to be ESBL producers and non-ESBL producers, respectively.

Antibiotic susceptibility testing

This test was carried out on all ESBL-producing *K. pneumoniae* isolates against 14 antibiotics (Table 1). The ceftazidime

Table 1. An antimicrobial susceptibility testing of 41 extended-spectrum beta-lactamases producing isolates of *Klebsiella pneumoniae*.

Antibiotic agent	Number (%) of isolates		
	Resistance	Intermediate	Susceptible
Amoxicillin/clavulanate	18 (43.9)	0 (0.0)	23 (56.1)
Aztreonam	39 (95.1)	0 (0.0)	2 (4.9)
Cefotaxime	40 (97.6)	0 (0.0)	1 (2.4)
Ceftazidime	41 (100.0)	0 (0.0)	0 (0.0)
Ceftriaxone	38 (92.7)	0 (0.0)	3 (7.3)
Chloramphenicol	17 (41.5)	3 (7.3)	21 (51.2)
Ciprofloxacin	11 (26.8)	0 (0.0)	30 (73.2)
Gentamicin	19 (46.3)	3 (7.4)	19 (46.3)
Imipenem	0 (0.0)	0 (0.0)	41 (100.0)
Levofloxacin	9 (22.0)	2 (4.9)	30 (73.1)
Nitrofurantoin	9 (22.0)	0 (0.0)	32 (78.0)
Norfloxacin	0 (0.0)	0 (0.0)	41 (100.0)
Tetracycline	29 (70.7)	0 (0.0)	12 (29.3)
Trimethoprim	27 (65.9)	0 (0.0)	14 (34.1)

(n=41/41; 100%), cefotaxime (n=40/41; 97.6%), ceftriaxone (n=38/41; 92.7%), and aztreonam (n=39/41; 95.1%) were among the less effective antibiotics, while imipenem (n=0/41; 0.0%) and norfloxacin (n=0/41; 0.0%) were the most effective antimicrobials. In total, 27 (65.9%) ESBL-producing *K. pneumoniae* isolates were MDR due to the resistance to at least one member of three antibiotics classes.

Molecular detection of ESBLs encoding genes

The M-PCR showed that 46.3% (n=19/41) of isolates harbored ESBL genes, while 53.7% (n=22/41) of isolates were found to be negative for these genes. Out of total 19 ESBL-positive isolates, 47.4% (n=9) harbored bla_{CTX-M} and 15.8% (n=3) harbored bla_{SHV} genes. And, bla_{CTX-M} and bla_{SHV} genes were detected in 7 (36.8%) isolates simultaneously. The bla_{CTX-M} was the most dominant gene and present either alone or in combination with bla_{SHV} gene.

DISCUSSION

In this study, the bacterial isolates were obtained from 89.2% (n=456/511) of the CAP patients, which was significantly higher than those obtained in the studies by Kishimbo et al.[1] from Tanzania (20.4%) and Regassa[12] from South Ethiopia (42.9%). These discrepancies may be due to differences in the study population, sample size, and geographical variations. This study showed more prevalence of CAP in male patients than females, which was consistent with previous report from Australia[13].

In this study, the total prevalence of *K. pneumoniae* was 31.9% among patients with CAP. This finding was lower than the previous study (54.0%) by Jaaffar et al.[4] from Iraq and higher (18.0%) than the former studies by Temesgen et al.[14] from Ethiopia.

In this study, the ESBL-producing *K. pneumoniae* showed high resistance rates against ceftazidime (100.0%), cefotaxime (97.6%), aztreonam (95.1%), ceftriaxone (92.7%), tetracycline (70.7%), and trimethoprim (65.9%), whereas all isolates were susceptible to imipenem and norfloxacin. These results were closely similar to those observed by Fils et al.[15] from France and Liu et al.[16] from China. In this research, *K. pneumoniae* isolates showed good susceptibility to fluoroquinolones and aminoglycosides. In line with our results, Zhang et al.[17] from China reported the good efficacy of ciprofloxacin and levofloxacin against *K. pneumoniae* causing community-onset infections.

Another finding of this study was the high frequency of 65.9% for MDR phenotype among ESBL-producing *K. pneumoniae* isolates. This finding was in parallel with the previous reports from Brazil (84.0%)[10] and Portugal (100%)[18]. Teklu et al.[19] concluded that the main explanation for these high resistance rates may be due to the widespread, excessive, irregular, unnecessary, and uncontrolled use of antibiotics to treat various infections. Carbapenems are still used as the best option to treat various infections, including pneumonia caused by ESBL-producing GNB. The results of this study were in agreement to the findings of most international studies, according to which imipenem has high efficacy against ESBL-producing *K. pneumoniae*[20-22].

In this study, 25.2% (n=41/163) of *K. pneumoniae* isolates showed phenotypic positive result for ESBL production using CHROM agar. Ultimately, this research was unable to validate the existence of ESBL genes by M-PCR in all isolates that had phenotypic positive test. It was found that 19 of 41 ESBL producers harbored ESBL genes using M-PCR method. These findings may presumably be due to the involvement of additional resistance pathways such as Ambler class C beta-lactamases, the presence of other mechanisms of resistance to beta-lactams, and the presence of other ESBL genes such as bla_{TEM} and bla_{PER} leading to differences between the results of phenotypic and molecular methods[23,24]. According to the CLSI, the combination disk test (CDST) is recommended for confirmation of ESBL production in Enterobacteriaceae using CAZ (30 µg) and CTX (30 µg) alone and in combination with clavulanic acid[9].

The results of this study showed that bla_{CTX-M} was the most common ESBL gene among *K. pneumoniae* isolates.

The worldwide spread of bla_{CTX-M} producing *K. pneumoniae* is a major concern in most continents. In a meta-analysis by Eskandari-Nasab et al.[25], the prevalence of bla_{CTX-M} was documented in Bahrain, Turkey, Saudi Arabia, Iran, United Arab Emirates, Pakistan, and Kuwait as 10.0, 30.0, 35.3, 56.7, 64.4, 96.9, and 100.0%, respectively. While international studies recorded varying percentages for the presence of this gene among the isolates producing ESBLs, including North Africa, America, Russia, Latin America, Brazil, and European countries, the percentages were 7.4, 26.4, 34.9, 61.1, 62.1, and 84.5%, respectively[25]. Despite the fact that TEM and SHV variants are the most universal ESBLs, it seems that they have become less common over the past decade than CTX-M. The results of this study were consistent with previous studies that found the bla_{CTX-M} gene as the most widespread ESBL type in *K. pneumoniae* isolates[16,17,25]. However, Ferreira et al.[10] from Brazil and Carvalho et al.[18] from Portugal showed a higher prevalence of bla_{SHV} compared to bla_{CTX-M} in *K. pneumoniae,* which was in contrast to our finding. Many factors, including the sample origin, sample size, studied population, and detection methods, can contribute to these differences.

Finally, our results showed the co-existence of ESBL genes in 36.8% of *K. pneumoniae* isolates. Previous studies from Brazil[10], China[16,17], and Portugal[18] reported the co-existence of various ESBL genes among clinical isolates of *K. pneumoniae*. This study had several limitations: the lack of screening of other ESBL genes such as bla_{TEM} and bla_{PER}, the lack of clinical data of patients to investigate the ESBL-related risk factors, and lack of sequencing for detected ESBL genes.

CONCLUSIONS

The emergence of MDR *K. pneumoniae* strains harboring ESBL resistance genes necessitates the development of a regular surveillance program to monitor, control, and prevent the more spread of these isolates in Iraqi health care systems.

AUTHORS' CONTRIBUTIONS

FEAR: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **EB:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **MKA:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **SA:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **MS:** Formal analysis, Writing – original draft.

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