RESEARCH ARTICLE

Bacteriological and Molecular Study Characterization of *ESBL Klebsiella pneumoniae* Isolates Collected from Different Clinical Specimens

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ABSTRACT

The increase in the incidence of both community-acquired and hospital-acquired infections caused by *Klebsiella pneumoniae*, one of the major opportunistic pathogens, poses a serious threat to public health worldwide. In this study, *K. pneumoniae* isolates were evaluated for their antimicrobial susceptibility. Samples were collected from 80 patients diagnosed with various diseases. Genomic DNA of *K. pneumoniae* isolates was extracted; extended-spectrum β-lactamase (ESBL) genes were detected in 53.75% of the isolates, with predominance for ESBL genes *blaTEM*, *blaSHV*, and *blaCTX-M* being 82.5%, 92.5%, and 70%, respectively. Of the 80 clinical isolates of *K. pneumoniae* obtained from the diverse healing centers and therapeutic research facilities in Duhok/Iraq, 80% of the isolates were from the genus *K. pneumoniae.* Ampicillin and aztreonam showed 100% antimicrobial resistance, whereas imipenem, ertapenem, and meropenem were 100% sensitive. The samples of ESBL-producing *K. pneumoniae* isolates showed different results for different clinical specimens, with 71.42% in urine, 40.90% in wound swabs, 42.10% in sputum, and 50% in blood culture. The recurrence of the ESBL production can easily be overlooked within the clinical isolates of *K. pneumoniae* when utilizing the strategies suggested by the current Clinical Laboratory Standards Institute. The identification of ESBL-producing isolates is central for formulating approaches for an experimental antimicrobial treatment.

Keywords: *Klebsiella pneumoniae,* ESBL, Antibiotic susceptibility, UTI

1. INTRODUCTION

Klebsiella pneumoniae is a member of the Enterobacteriaceae, a rod-shaped, gram-negative, lactose-fermenting bacillus with a conspicuous capsule.

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Klebsiella pneumoniae is an astute pathogen that mostly affects those with resistant systems and tends to cause nosocomial infections (Chaudhary and Aggarwal, 2004). A subset of hypervirulent *K. pneumoniae* serotypes associated with increased generation of capsule polysaccharide can influence healthy people and cause life-threatening community-acquired infections, such as pyogenic liver abscess, meningitis, necrotizing fasciitis, endophthalmitis, and extreme pneumonia (Knothe et al., 1983).

K. pneumoniae utilizes an assortment of harmful variables, particularly capsular polysaccharide, lipopolysaccharide, fimbriae, external film proteins, and determinants for press procurement and nitrogen source utilization, for survival and resistance avoidance amid disease (Bradford et al., 1994). Extended-spectrum β-lactamases (ESBLs) are called extended-spectrum because they are able to hydrolyze a broader range of β-lactam antimicrobial than the basic parent β-lactamases, from which they are derived. Such ESBLs also have the capacity to deactivate β-lactam antimicrobial containing an oxyimino group, such as oxyimino-cephalosporins (e.g., ceftazidime, ceftriaxone, or cefotaxime) or oxyimino-monobactam (Bradford, 2001). Otherwise, they are not active against cephamycins and carbapenems. Generally, they are repressed by β-lactamase inhibitors such as clavulanate and tazobactam. ESBLs have been found in a wide range of gram-negative rods. A high number of strains expressing these enzymes are a part of the Enterobacteriaceae family (Paterson and Bonomo, 2005; Winokur et al., 2000).

2. MATERIALS AND METHODS

2.1. Sample Collection

This study used 80 clinical isolates of *K. pneumoniae* from diverse clinical isolates collected from patients of both genders and different age groups. These isolates were collected from urine, wound swabs, sputum, and blood culture samples that were isolated in clinics and restorative research facilities in Duhok/Iraq between November 2017 and April 2018. Clinical isolates were cultured in brain heart infusion broth. After incubation at 37˚C for 24 h, each isolate was sub-cultured on blood agar and MacConkey agar media by streak plate strategy and incubated at 37˚C for 24 h (Golamreza and Moghadas, 2010).

2.2. Identification of *K. pneumoniae*

All isolates were subjected to standard corroborative tests, which included gram staining, oxidase and catalase, development on SIM (Sulfide-Indole-Motility), Simmons citrate, MR-VP (Methyl Red – Voges-Proskauer), Kligler agar, phenylalanine agar, urea agar, blood agar, and MacConkey agar (MacFaddin, 1999).

2.3. Antimicrobial Susceptibility Test and ESBL Phenotypic Confirmatory Tests

All ESBL-producing isolates were tested by a double-disk collaboration test (DDCT), in which disks containing cefotaxime (30 µg), ceftazidime (30 µg), and ceftriaxone (30 µg) were placed at a distance of 25 mm (middle to middle) from an amoxicillin-clavulanic corrosive disk (30 and 10 µg, individually), and they were brooded at 37 °C overnight. This equipment was utilized to affirm recognizable proof and for advanced characterization of the obtained K. pneumoniae isolates distinguished by routine bacteriological strategies. In this study, the susceptibility of K. pneumoniae was tested to 13 antimicrobial disks that were provided by Bioanalyse, Turkey. The susceptibility was identified using a disk dissemination strategy with a β-lactam course, which included ampicillin, aztreonam, ertapenem, imipenem, and meropenem; a course of cephalosporins, which included cefazolin, cefuroxime, ceftazidime, ceftriaxone, cefotaxime and cefepime; a quinolones course, which included ciprofloxacin and levofloxacin; and an aminoglycosides course, which included amikacin and gentamicin. All medicated susceptibility testing was performed in agreement with the Clinical Laboratory Standards Institute (CLSI) (Cheesbrough, 2006).

2.4. Bacterial DNA Extraction

Genomic DNA analyses of 50 K. pneumoniae strains were performed according to the strategy outlined by Kiesser, 1995. A single isolated K. pneumoniae colony was inculcated into 30 mL of brain heart infusion broth, incubated at 37˚C with shaking for 24 h; the bacterial cells were collected by centrifugation at 4000 rpm for 25 min. The pellet was resuspended in 3.5 mL of TE25S buffer and blended well. Thereafter, 100 µL of

lysozyme (50 mg/mL) solution was added to this suspension, incubated at 37˚C for 1 h. After that, 50 µL of Proteinase K (20 mg/mL) and 200 µL of SDS (10 %) were added to the mixture and incubated at 55˚C with shaking for 1 h. Later, 850 µL of NaCl (5 M) and 850 µL CTAB/NACL were added to the lysate and brooded at 55˚C in water bath for 10 min. The tubes were cleared out at room temperature for 5 min. At that point, an equal volume of chloroform/isoamyl (24:1) was added and blended delicately in a blender for 30 min and centrifuged at 4000 rpm for 30 min; this step was performed twice. The supernatant was exchanged into a sterile tube and 0.6 volume of cooled isopropanol was added and mixed delicately with modification. The tubes were cleared out at -20˚C for 30 min. The DNA mass was taken by the pawn class and washed with 1 mL of 70% ethanol. The DNA mass was air dried for a few minutes, and the nucleic acid was dissolved in 500 µL of TE buffer.

2.5. Detection of ESBL Genes (*blaTEM*, *blaSHV*, and *blaCTX-M*) by Polymerase Chain Reaction

The polymerase chain reaction was performed using the following primers: blaTEMprimer producing band 1080 bp (forward 5′-GAGTATCAACATTTCCGTGTC-3′, reverse 5′-TAATCAGTGGGCACCTTCTC-3′); blaSHV primer with estimated product 861 bp (forward 5′–AAGATCCACTATCGCCCAGCAG-3′, reverse 5′ AAGATCCACTATCGCCCAGCAG-3′) (Shahcheraghi et al., 2007); and blaCTX-M primer producing band 551 bp (forward 5′-ACGCTGTTGTTAGGAAGTG-3′, reverse 5′ TTGAGGCTGGGTGAAGT-3′) (Mansouri and Ramazanzadeh, 2009). The blaSHV gene was amplified under the following conditions: denaturation starting at 94˚C for 3 min, taken after 35 cycles of denaturation at 95˚C for 30 s, annealing at 56˚C for 1 min, and 72˚C for 1 min., with a last expansion at 72˚C for 10 min. The blaTEM gene was amplified under the following conditions: denaturation starting at 94˚C for 3 min, taken after 35 cycles of denaturation at 95˚C for 30 s, annealing at 45˚C for 1 min, and 72˚C for 1 min, with a last expansion at 72˚C for 10 min.

The blaCTX-M gene was amplified under the following conditions: denaturation starting at 94˚C for 3 min, taken after 35 cycles of denaturation at 95˚C for 30 s, annealing at 48˚C for 1 min, and 72˚C for 1 min, with a last expansion at 72°C for 10 min. The amplicons were run on 1% agarose gel. The gels were stained with ethidium bromide, and bands at the specified position were captured utilizing an ultraviolet light trans-illuminator.

2. RESULTS and DISCUSSION

Of the 80 clinical isolates of K. pneumoniae collected from the restorative research facilities in Duhok/Iraq, all belonged to the genus K. pneumoniae, as shown in Table 1. These results were consistent with a few of the isolates collected from Iran and Saudi Arabia (Bradford et al., 1994; Bradford, 2001). It is additionally clear from the data presented in Table 1 that the frequency of K. pneumoniae isolates was highest in urine and lowest in the blood culture tests compared with the other collected mediums.

Table 1: *Klebsiella pneumoniae* isolates collected from distinctive clinical laboratories in Duhok/Iraq among different sample types

Sample source	Total	Percentage
Urine	35	43.75
Wound swabs	22	27.5
Sputum	19	23.75
Blood culture	4	5
Total	80	100

In addition, in other parts of the world, the most noteworthy rate of K. pneumoniae isolates was isolated from urine and the least rate was from blood culture (Manikandan and Amsath, 2013). The high rates of K. pneumoniae in urine tests may be attributed to factors such as destitute cleanliness;

defilement of genital organs; the spread of disease; and bacterial virulence factors such as capsule, pili, and antimicrobial resistance genes (Kahan et al., 2006).

Table 2 shows that 43 of 80 (53.75%) K. pneumoniae isolates were ESBL makers, and these isolates were 100% safe to β-lactam antimicrobials, including ampicillin and aztreonam, and the cephalosporins, including cefazolin, cefuroxime, ceftazidime, ceftriaxone, cefotaxime, and cefepime. Cefepime is more readily hydrolyzed by ESBLs than the third-generation cephalosporins (Labombardi et al., 2006). In any case, all ESBL-producing K. pneumoniae isolates that were collected in this study were 100% resistant to cephalosporin.

Table 2: Antibiotic susceptibility of ESBL-producing *Klebsiella pneumoniae* clinical isolates

Class antibiotic	Resistant isolates	Sensitive isolates
β-lactam Ampicillin	43 (100%)	
Aztreonam	43 (100%)	
Ertapenem		43 (100%)
Imipenem		43 (100%)
Meropenem		43 (100%)
Cephalosporins Cefazolin	43 (100%)	
Cefuroxime	43 (100%)	
Ceftazidime	43 (100%)	
Ceftriaxone	43 (100%)	
Cefotaxime	43 (100%)	
Cefepime	43 (100%)	
Quinolones Ciprofloxacin	33 (76.74%)	10 (23.25%)
Levofloxacin	27 (62.79%)	16 (37.20%)
Aminoglycosides Amikacin	25 (58.13%)	18 (41.86%)
Gentamicin	23 (53.48%)	20 (46.51%)

The improvement of extended-spectrum cephalosporins within the early 1980s was considered as a major expansion to the therapeutic armamentarium on the fight against β-lactamase–mediated bacterial resistance (Bush, 2002). The information collected from clinical tests of K. pneumoniae presently indicates substantial antimicrobial resistance. The resistance to antimicrobials shown by Klebsiella has expanded worldwide (Shannon and Phillips, 1986). Carbapenems are the drugs of choice for numerous diseases caused by gram-positive and gram-negative bacteria (Yu et al., 2002). Imipenem has been found to be the most successful antibiotic (Al-Zahrani and Akhtar, 2005). The β-lactamase enzymes break down the basic β-lactam ring of β-lactam antimicrobials and cephalosporins by assaulting the amide bond within the β-lactam ring of penicillin and cephalosporin with ensuing generation of penicillanic acid and cephalosporanic acid separately, eventually rendering the compounds antibacterially inactive (Shannon and Phillips, 1986). Numerous genera of gram-negative bacteria have a chromosomally-intervening β-lactamase (Bush, 2002). Other β-lactamases are plasmid mediated (Yu et al., 2002). These enzymes are thought to have advanced from penicillin binding proteins, and the expression of these genes may be inducible, high-level constitutive, or low-level constitutive (Al-Zahrani and Akhtar, 2005).

All 43 ESBL-producing K. pneumoniae isolates appeared to have diverse resistance rates toward quinolone antimicrobials (ciprofloxacin and levofloxacin), as shown in Table 3.

The aminoglycosides, amikacin and gentamicin, also showed variable susceptibility rates. These antimicrobials interacted with nucleic acid blend by repressing the enzyme gyrase (Al-Zahrani and Akhtar, 2005). In the past, ciprofloxacin was found to be profoundly active against pathogens isolated from different clinical specimens, but, presently, the impact of this antimicrobial tends to diminish because of its visit utilization by common population (Yu et al., 2002). İn the current study, all ESBL-creating K. pneumoniae isolates were affirmed by a DDCT (Zhang et al., 2001). This strategy is considered one of the finest and simplest strategies for the phenotypic detection of ESBL generation. Worldwide, this phenotypic strategy has been utilized to detect K. pneumoniae ESBL-producing isolates of K. pneumoniae and other distinctive ESBLs by creating bacterial isolates from diverse tests; it is exceptionally vital to know the prevalence of ESBL-creating organisms in a given area so that reasonable utilization of antimicrobials might be achieved (Paterson and Bonomo, 2005). The rate of ESBL-producing isolates of K. pneumoniae isolates collected in this study was 53.75% (43/80 isolates). The high rate of ESBL makers among K. pneumoniae isolates in Duhok/Iraq is disturbing, and this may be due to the wild utilization of β-lactam and cephalosporin antimicrobials (personal communication). The prevalence of ESBLs in Iraq is high and alarming. It seems important for physicians and health care systems to be cautious about microbial ESBLs. In addition, the observation of the ESBLs is proposed to avoid dissatisfaction with treatment and to ensure adequate control of disease in Iraq. Table 3 shows that among the different clinical tests on K. pneumoniae, the ESBL movement was 71.42% in urine, 40.90% in wound swabs, 42.10% in sputum, and 50% in blood cultures.

Table 3: Distribution of *Klebsiella pneumoniae* extended-spectrum β-lactamases among different sample types

Sample source	Total no. of *K. pneumoniae*	No. of ESBL *K. pneumoniae* producers (%)
Urine	35	25 (71.42%)
Wound	22	9 (40.90%)
Sputum	19	8 (42.10%)
Blood	4	2 (50%)

In various tests conducted worldwide, rates of diversity were lower than those obtained in this study. In Iran, it was found that ESBL-producing isolates were 36%, 35%, 7%, and 5% for urine, sputum, blood, and wound specimens, respectively (Feizabadi, 2006). This variation in the predominance of ESBL makers among the isolates of K. pneumoniae in different clinical tests and distinctive locales may be due to the sample estimates being used concurrently with the wild utilization of antimicrobials and heavy medicines for antimicrobials, which may result in completely different resistance rates to antimicrobials (Zhang et al., 2001). Numerous variables such as nourishment chains, individual cleanliness, and steady development of individuals to and from diverse parts of the world—especially in developing countries—encourage the spread and movement of resistant strains (Feizabadi, 2006). Genomic DNA from 40 chosen samples of ESBL-

producing K. pneumoniae isolates from distinctive sources (urine, wound, sputum, and blood culture) were extricated utilizing the strategy depicted by Kiesser, 1995, and the normal concentration of genomic DNA isolate was 1700 ng/µL with tall virtue 1.8.10 . This strategy was found to be exceptionally effective for several reasons, including extracting a large amount of DNA that can be utilized for numerous purposes taking into account PCR enhancement, the accessibility of materials, and device required. Moreover, the advantage of this strategy lies in a few components within the extraction buffer that provide support in isolating a large amount of and immaculate DNA (Sambrook and Russell, 2001).

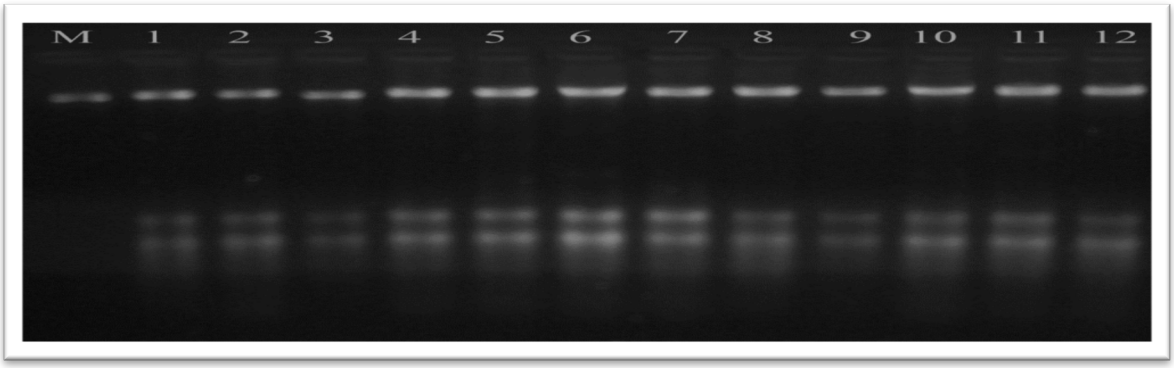

Figure 1. Genomic DNA of *Klebsiella pneumoniae* isolates running on 1% agarose gel electrophoresis at 70 V for 45 min

The results of amplified PCR-products for the three tested genes were 1080, 861, and 551 bp for *blaTEM*, *blaSHV*, and *blaCTX-M*, respectively, as shown in Figures 2, 3, and 4.

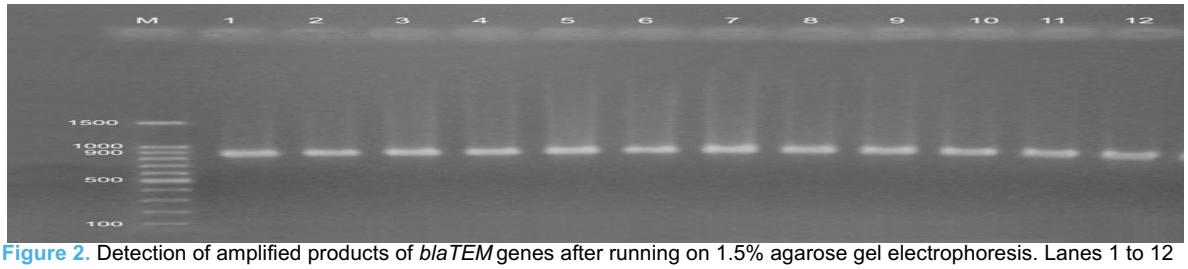

Figure 2. Detection of amplified products of *blaTEM* genes after running on 1.5% agarose gel electrophoresis. Lanes 1 to 12 represent samples that produce *blaTEM*. Lane M represents a 100-bp ladder

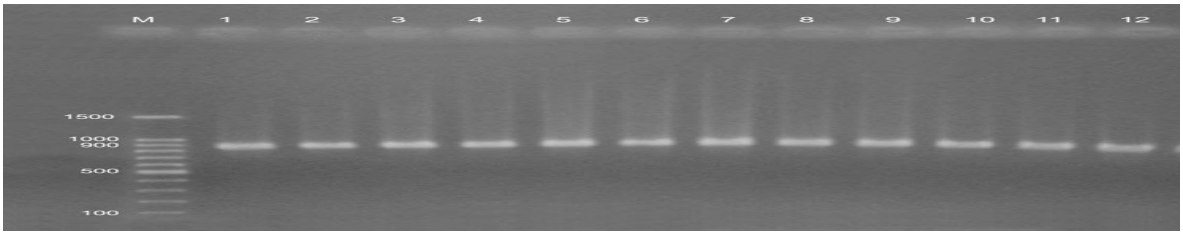

Figure 3. Detection of amplified products of *blaSHV* genes after 1.5% agarose gel electrophoresis. Lanes 1 to 12 represent *SHV* genes, Lane M represents a 100-bp ladder

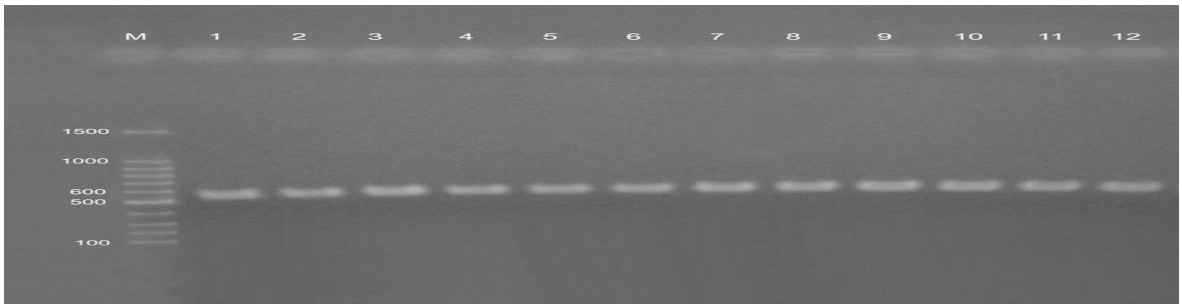

Figure 4. Detection of amplified products of *bla*CTX-M genes after running on 1.5% agarose gel electrophoresis. Lanes 1 to 12 represent *CTX-M* genes. Lane M represents 100-bp ladder

The discovery of ESBL generation cannot help define the boundaries between the diverse ESBL enzymes encoded by a few diverse genes such as *TEM*, *SHV*, and *CTX-M*. Therefore, the genotypic strategies are elective and more corroborative for the discovery and distinguishing proof of the particular genes that are capable of generating ESBLs (Falagas and Karageorgopoulos, 2009). In this respect, molecular characterization of 40 ESBL-positive isolates of *K. pneumoniae* was performed utilizing PCR for the detection of 3 diverse ESBL genes, including *TEM*, *SHV*, and *CTX-M* utilizing a particular combination of primers for each gene. The PCR items were electrophoresed by utilizing 1.5% agarose gel. Table 4 shows that the predominance of *blaTEM*, *blaSHV*, and *blaCTX-M* was 82.5%, 92.5%, and 70 %, respectively.

Table 4: Rate of ESBL genotypes among the 40 chosen isolates of *Klebsiella pneumoniae* from distinctive clinical specimens from Duhok/Iraq

ESBL genotype	No. of isolates	Percentage
TEM	33	82.5%
SHV	37	92.5%
CTX-M	28	70%

One of the major findings in this study was that SHV-type ESBL was the foremost prevailing type (Table 4). This result was in agreement with other reports from numerous countries such as Iran, Turkey, and Egypt that have affirmed that SHV type was the foremost prevailing type (94%, 74.3%, and 100%, respectively) (Amr et al., 2008; Sobhan et al., 2011; Huseyin and Hakki, 2005). The genes coding these enzymes may be harbored on the chromosome, but most of them are plasmid interceded or may be carried on integrons (Sobhan et al., 2011). Dissemination of specific clones and plague plasmids in community and nosocomial settings in our region may well be the foremost reason for the increase in ESBLs belonging to *TEM*, *SHV*, and *CTX-M* families. This may be attributed to the over-the-top utilization of antimicrobials in humans and livestock. Other components such as healing center's cross-contamination of diseases, nourishment chains, and the exchange and human relocation between regions are also considered variables that affect the spread of ESBLs (Feizabadi et al., 2006; Huseyin and Hakki, 2005).

3. CONCLUSION

Within clinical isolates of *K. pneumoniae*, the recurrence of ESBL generation can be considered small. The use of the current CLSI-prescribed techniques has shown that these organisms make numerous β-lactamases routinely. In such circumstances, the application of DDCTs in combination with amoxicillin-clavulanate with cefepime will improve the ability to discover ESBLs. An ideally recognizable confirmation of ESBL-producing isolates is important for defining

the arrangements for an experimental antimicrobial treatment, especially in high-risk units where the diseases caused by these organisms are common. This testing is also important for the evaluation of increased antimicrobial resistance and the use of legal measures to control clinical diseases.

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