The Incidence of Extended Spectrum β-Lactamase Enzymes and Their Connection to Virulence Genes in Community-Acquired Urinary Tract Infection

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Abstract: In community-acquired urinary tract infections, Klebsiella pneumoniae is considered one of the most common etiological agents. Multidrug resistance and virulence are common in Klebsiella pneumoniae populations. In this study, fifty Klebsiella pneumoniae isolates were isolated from urine samples and identified using a vitek 2 compact device. The Kirby–Bauer disk diffusion technique used the antibiotic susceptibility test. According to the findings, approximately [n = 46 (92%)] of Klebsiella pneumoniae isolates are multidrug-resistant (MDR). To detect the production of Extended Spectrum β-lactamase (ESBL) enzymes, the Modified Double Disc Synergy Test (MDDST) was used. The results show that approximately [n=45 (90%)] of the isolates produce ESBLs. The most common ESBL genes (TEM, SHV, and CTX-M) were investigated in isolates. The results show that the SHV gene had the highest prevalence among ESBL genes [n = 34 (68%)], followed by the CTX-M gene [n = 33 (66%)], while none of the isolates possessed the TEM gene. The virulence factor type 3 fimbriae (MrKD) gene and biofilm (BssS) gene were revealed. The results found that the isolates contain the MrKD gene at [n = 41 (82%)]. At the same time, the results found that the isolates contained the BssS gene at [n =36 (72%)]. The prevalence of Virulence genes within ESBL-producing Klebsiella pneumoniae isolates shows that only [n = 3 (6%)] of isolates that are non-ESBL producers carry one or both virulence genes, while [n=41 (82%)] of ESBL-producing isolates contain one or both virulence genes. The prevalence of ESBL-producing Klebsiella pneumoniae in community patients was high in this research. There may also be a correlation between ESBL production and some virulence factors.

Key words: Klebsiella pneumoniae, Antibiotic Resistance, Virulence Gene, ESBL, Urinary Tract Infection, CTX-M.

Introduction

Urinary tract infections (UTIs) have severe health and financial consequences for society. These infections are considered the most prevalent type of bacterial infection, and they can strike at any moment during a person's life. People in hospitals and the general public are susceptible to urinary tract infections. CAUTIs (community-associated urinary tract infections) are frequently identified in individuals with risk factors such as age, prior UTI history, sexual activity, and diabetes mellitus. Antibiotics are the most common therapy for bacterial UTIs. After E.coli, K. pneumoniae is the most medically significant species in the Enterobacteriaceae family. One of the frequent pathogens linked to both community and hospital-acquired urinary tract infections is K. pneumoniae. Because bacterial etiology is common in UTIs, broad-spectrum antibiotics are frequently used, resulting in a rise of resistant uropathogens. Antibiotic resistance among K. pneumoniae strains is a major public health concern and a significant financial burden for UTI sufferers. Antibiotic resistance of these isolates is acquired by various processes, including the creation of extended spectrum-lactamases (ESBLs). The formation of extended-spectrum beta-lactamase, an enzyme that attacks the beta-lactam ring in medicines and renders them useless, causes ESBL resistance. Several ESBL families have been identified; however, the prominent families such as SHV, TEM, and CTX-M account for the bulk of ESBLs. The rapidly increasing resistance of ESBL producers to multiple antibiotic families is a significant issue that limits the treatment options available to ESBL producers. In addition, lipopolysaccharide (LPS), capsular polysaccharide, adhesions, and siderophores are among the virulence components found in K. pneumoniae that contribute to its pathogenicity. Adhesion is an essential phase in the infection that must be strong enough to overcome the host's defensive mechanisms. Fimbriae, also known as pili, are proteinaceous structures that extend from the bacterial cell surface to a distance of 100 nm to several microns and are composed of adhesions that are thought to aid bacterial adhesion. There are two kinds of fimbriae or pili in K. pneumoniae. Types 1 and 3. Type 3 fimbriae have a helix-like structure that gives them spring-like flexibility and stretch-ability. This fimbrial type is encoded by a chromosomally borne gene cluster that has previously been demonstrated to consist of five genes in various strains of K. pneumoniae. The MrkABCDF gene cluster encodes the primary structural component (mrkA) and the fimbrial adhesin (mrkD), whereas the genes mrkB and mrkC encode the chaperone and usher proteins, respectively. Type 3 fimbriae have been shown to mediate adhesion to...
various structures in human kidney and lung tissue and epithelial cells from human urine sediments and endothelial and bladder epithelial cell lines in vitro investigations\textsuperscript{11}. K. pneumoniae biofilm development on biotic and abiotic inert surfaces is commonly linked to Type 3 fimbriae\textsuperscript{12}. Infections caused by K. pneumoniae producing biofilms are more resistant to therapy than other infections. Biofilms serve as shields for bacterial populations, allowing them to evade host defenses. The bacteria are also protected from severe circumstances such as pH changes, forces of shear, and nutritional deficiencies\textsuperscript{13}. As a result, the current study was designed to look into the distribution of extended-spectrum β-lactamase enzyme genes (TEM, SHV, CTX-M), virulence genes (type 3 fimbriae MrKD gene, biofilm BssS gene), and the relationship between them in K. pneumoniae isolates from urine samples.

## Materials and methods

### Bacterial Isolation and Identification

From different laboratories in Baghdad, (50) isolates of K. pneumoniae were collected from (133) bacterial isolates from urine samples from the period (2020/07/1) to (2020/09/25). For their identification, K. pneumoniae isolates were diagnosed by the vitek 2 compact (bioMérieux/France) device.

### Antibiotic Susceptibility Test

The Kirby–Bauer disk diffusion technique was used to perform Antibiotic susceptibility testing for all isolates\textsuperscript{14}. Antibiotics listed below were utilized: Ampicillin (10 μg), Piperacillin (100 μg), Amoxicillin–Clavulanate (20/10 μg), Cefazidime/Avibactam (30/20 μg), Cefotaxime (30 μg), Ceftriaxone (30 μg), Loracarbef (30 μg), Cefpodoxime (10 μg), Cefuroxime (30 μg), Cefazolin (30 μg), Cefuroxime (30 μg), Gentamicin (10 μg), Tobramycin (10 μg), Amikacin (30 μg), Doxycycline (30 μg), Tetracycline (10 μg), Ciprofloxacin (5 μg), Levofloxacin (5 μg), Nalidixic acid (30 μg), Trimethoprim–Sulfamethoxazole (1.25/23.75 μg), Chloramphenicol (30 μg), Nitrofurantoin (300 μg).

### Modified Double Disc Synergy Test (MDDST)

Detect ESBL enzymes production by using the Modified Double Disc Synergy Test through the following steps\textsuperscript{15}: After uniformly spreading the inoculum onto sterile Mueller–Hinton agar (biolab/Hungary), the disc which contained amoxicillin–Clavulanate (20/100μg) was placed in the center of the plate and the discs of third-generation cephalosporin [cefotaxime (30 μg), ceftriaxone (30 μg), and cefpodoxime (10 μg)], and fourth-generation cephalosporin [cefpime (30 μg)], were placed 15mm and 20 mm apart respectively, center to center to that the amoxicillin–Clavulanate disc. After being incubated for 18–24 h at 37 °C, any increase in the inhibition zone toward the amoxicillin–Clavulanate disc was considered positive for ESBL production.

### Detection of Antibiotic Resistance and Virulence Factor Genes

#### DNA extraction

For the isolation and purification of DNA from K. pneumoniae isolates, DNA was extracted using G-spin\textsuperscript{TM} Total DNA Extraction Kit (Intron biotechnology/Korea) according to the manufacturer’s instructions. Using Nanovue plus\textsuperscript{TM} spectrophotometer (GE Healthcare/UK), estimated concentration and purity.

### Polymerase Chain reaction of Antibiotic Resistance and Virulence Genes

The PCR method detects genes encoding virulence factors (type 3 fimbriae MrKD, biofilm BssS) and extended-spectrum β-lactamase genes (TEM, SHV, CTX-M). Primers used in this study were purchased from (Alpha DNA/Canada) in lyophilized form, as shown in Table 1. The PCR amplification reaction mixture components (20μl) used to detect each gene contain the following: DNA sample, primers, nuclease-free water, and 5x FIREPol® Master Mix (Solis Bio Dyne/Europe) 16. After preparing the reaction volume in the PCR tube, the mixture was spun down via (Centrifuge/Vortex for PCR plates) and then PCR tubes were placed in the PCR thermal cycler (Bio-Rad/USA) to amplify the target DNA for (TEM, SHV, CTX-M, BssS, MrKD primers) using the following program17 as shown in Table 2.

| Gene    | Primers                                             | PCR Product (bp)$^c$ | Ref  |
|---------|-----------------------------------------------------|----------------------|------|
| TEM-F$^a$ | AT GAG TAT TCA ACA TTT CCG TT AAT CAG TGA GGC ACC TAT | 717                  | [11] |
| TEM-R$^b$ | AGGCCGTTTGGACAAAATTTAACC ATCCCGCAGATAAATCACAC   | 525                  | [12] |
| SHV-F   | AGCCGCTTGAGCAAATTTAAAC ATCCCGCAGATAAATCACAC   | 525                  | [12] |
| SHV-R   | GCT GTT GTT AGG AAG TGT GC CCA TTG CCC GAG GTG AAG | 515                  | [13] |
| CTX-M-F  | CTX-M-R GCT GTT GTT AGG AAG TGT GC CCA TTG CCC GAG GTG AAG | 515                  | [13] |
| BssS-F | GATTCATTTTGGCGATTCCCTGC TAATGAAATCTCAGACCTCACCC | 225                  | [14] |
| BssS-R | AT GGA ACC CAC ATC GAC ATT AT GAG TAT TCA AAT CTT CCG | 390                  | [15] |

**Table 1.** Primers Used for Detection Genes.
Results

In the current study, 50 *K. pneumoniae* isolates were collected from urine samples of community-acquired urinary tract infection patients. All samples were identified by vitek 2 compact device (bioMérieux/France).

Antibiotic Susceptibility Test

The antibiotic susceptibility test was done by the Kirby–Bauer disk diffusion method, then interpreted the results were using criteria published by the Clinical and Laboratory Standard Institute18. As seen in Table 3. The results showed that about \( n=46(92\%) \) of *K. pneumoniae* isolates were multidrug resistance (MDR).

The Modified Double Disc Synergy Test (MDDST) was used to detect ESBL production. As a result, any increase in the inhibition zone of cefeime, cefpodoxime, ceftriaxone, and cefotaxime toward the amoxicillin clavulanate disc was interpreted as a sign of ESBL generation. The results showed that approximately \( n=45 (90\%) \) of the isolates produced ESBLs, as shown in Figure 1.

Detection of Antibiotic Resistance and Virulence Factors Genes

The ability of *K. pneumoniae* isolates to produce ESBLs (TEM, SHV, and CTX-M) genes and the capacity of isolates to generate Virulence factor type 3 fimbriae (MrKD) genes and biofilm (BssS) genes are determined using PCR, which uses sets of primers that amplify the genes. The results showed that the SHV gene was the most prevalent among ESBL genes \( \text{n=34}(68\%) \), then the CTX-M gene \( \text{n=33}(66\%) \). While none of the isolates possessed the TEM gene, as shown in Figure 2.

The results found that the isolates contain MrKD gene at \( \text{n=41} (82\%) \) as shown in the figure A (3). It also, found that \( \text{n=36} (72%) \) of isolates contain the BssS gene as shown in the figure B (3).

The prevalence Virulence genes within ESBL-producing *K. pneumoniae* isolates show that only \( \text{n=3}(6\%) \) of isolates which are non-ESBL producing carry one or both virulence genes, while \( \text{n=41}(82\%) \) of ESBL-producing isolates contain one or both virulence genes as seen in Table 4.

Discussion

In both community and hospital settings, *K. pneumoniae* is a leading cause of serious infections such as urinary tract infection, pneumonia, skin and soft tissue infection, intra-abdominal infection, bloodstream infection, meningitis, and pyogenic liver abscesses. In humans, antimicrobials have long been used to treat *K. pneumoniae* infections19. The emergence of multidrug-resistant (MDR) *K. pneumoniae* strains worldwide is a major source of worry20. In this study, the results showed that about \( n=46(92\%) \) of *K. pneumoniae* isolates were multidrug resistance (MDR). MDR is defined as acquired non-susceptibility to at least one agent in three or more microbial categories21. Different rates of MDR were reported in many studies, such as the study done by Mohamed et al., which reported that *K. pneumoniae* had the highest level of resistance to conventional antibiotics, with 86.66% of isolates being MDR22. Also, a study done by Pishitian and Khadija concluded that most of the *K. pneumoniae* isolates were multidrug-resistant (MDR). Where 75% of *K. pneumoniae* isolates showed the MDR phenotype23. MDR obstructs disease control by increasing the risk of resistant microorganisms spreading, lowering treatment efficacy and, as a result, causing patients to be infected for more extended periods of time24. A variety of virulence factors contribute to *K. pneumoniae*’s pathogenicity25. As well as the ability to rapidly develop antibiotic resistance26. *K. pneumoniae* is, in fact, a significant ESBL host. The development of ESBLs has significantly increased bacterial resistance to beta-lactams in human infections, causing significant morbidity and death27. Extended-spectrum beta-lactamases (ESBLs) are enzymes that can give resistance towards beta-lactam antibiotics such penicillins, cephalosporins, and aztreonam. This is achieved by hydrolyzing the antibiotics, and beta-lactamase inhibitors like clavulanic acid block these enzymes28.

This study used Modified Double Disc Synergy Test (MDDST) to detect ESBL production. The results showed that approximately \( n=45(90\%) \) of the isolates produced ESBLs. These results were close to those obtained by Shakib et al., who showed that \( n=62 (88.6\%) \) of *K. pneumoniae*
**Figure 1.** Modified Double Disc Synergy Test (MDDST) (A) shows synergism of cefeime, cefpodoxime, ceftriaxone and cefotaxime with amoxicillin-Clavulanate, which indicates ESBL production, while in (B) showed no synergism with amoxicillin-Clavulanate, which indicates none of ESBL production.

**Table 3.** Antibiotic Susceptibility of 50 *K. pneumoniae* Isolates.

| Antimicrobial Agent          | Susceptible No (%) | Intermediate No (%) | Resistant No (%) |
|------------------------------|--------------------|---------------------|------------------|
| Ampicillin                   | 0 (0.0)            | 0 (0.0)             | 50 (100)         |
| Piperacillin                 | 0 (0.0)            | 0 (0.0)             | 50 (100)         |
| Amoxicillin/Clavulanate      | 0 (0.0)            | 0 (0.0)             | 50 (100)         |
| Ceftazidime/avibactam        | 20 (40)            | 6 (12)              | 24 (48)          |
| Cefotaxime                   | 2 (4)              | 0 (0.0)             | 48 (96)          |
| Ceftriaxone                  | 3 (6)              | 1 (2)               | 46 (92)          |
| Ceftazidime                  | 3 (6)              | 0 (0.0)             | 47 (94)          |
| Cefuroxime                   | 2 (4)              | 5 (10)              | 43 (86)          |
| Cefazolin                    | 4 (8)              | 0 (0.0)             | 46 (92)          |
| Cefepime                     | 4 (8)              | 0 (0.0)             | 46 (92)          |
| Loracarbef                   | 4 (8)              | 0 (0.0)             | 46 (92)          |
| Cefixime                     | 3 (6)              | 2 (4)               | 45 (90)          |
| Cefpodoxime                  | 3 (6)              | 4 (8)               | 43 (86)          |
| Aztreonam                    | 2 (4)              | 0 (0.0)             | 48 (96)          |
| Imipenem                     | 19 (38)            | 18 (36)             | 13 (26)          |
| Meropenem                    | 26 (52)            | 14 (28)             | 10 (20)          |
| Gentamicin                   | 34 (68)            | 2 (4)               | 14 (28)          |
| Amikacin                     | 40 (80)            | 7 (14)              | 3 (6)            |
| Tetracycline                 | 2 (4)              | 0 (0.0)             | 48 (96)          |
| Doxyccycline                 | 3 (6)              | 0 (0.0)             | 47 (94)          |
| Tetracycline                 | 2 (4)              | 0 (0.0)             | 48 (96)          |
| Doxyccycline                 | 3 (6)              | 0 (0.0)             | 47 (94)          |
| Ciproflxacin                 | 36 (72)            | 1 (2)               | 13 (26)          |
| Levofoxacin                  | 37 (74)            | 0 (0.0)             | 13 (26)          |
| Nalidixic acid               | 11 (22)            | 7 (14)              | 32 (64)          |
| Trimethoprim- sulfamethoxazole | 19 (38)       | 0 (0.0)             | 31 (62)          |
| Chloramphenicol              | 27 (54)            | 8 (16)              | 15 (30)          |
| Nitrofurantoin               | 1 (2)              | 1 (2)               | 48 (96)          |
Figure 2. Agarose gel electrophoresis of PCR products of (A) TEM gene, (B) SHV gene and (C) CTX-M gene in *K. pneumoniae* isolates visualized under UV after staining by RedSafe™ nucleic acid staining solution for 2% agarose gel at 100 volts, 70 amps for 60 min. L: ladder DNA (100pb), S: Sample.
isolates were ESBL-producing\textsuperscript{29}. Pishtiwan and Khadija et al.'s research revealed that \([n=17(85\%)]\) of \textit{K. pneumoniae} isolates were positive for ESBL production\textsuperscript{29}. The prevalence of ESBL-producing clinical isolates is related to risk factors such as current antibiotic use and hospitalization\textsuperscript{29}.

The ability of \textit{K. pneumoniae} isolates to produce ESBLs (TEM, SHV, and CTX-M) genes showed that the SHV gene was the most prevalent among ESBL genes \([n=34(68\%)]\), then the CTX-M gene \([n=33(66\%)]\). At the same time, none of the isolates possessed the TEM gene. The study done by Malekjamshidi et al. showed that the most prevalent \(\beta\)-lactamase gene was SHV, followed by TEM and CTX-M\textsuperscript{30}. Also, Pishtiwan and Khadija et al. reveal that the TEM gene has the highest prevalence in \textit{K. pneumoniae} isolates at 64.7\%, followed by the CTX-M gene at 41.1\% and the SHV gene at 35.2\%\textsuperscript{23}. In addition, a study by Mbelle et al. found that all isolates contained the CTX-M gene, while (97\%) contained TEM, and (83\%) harbored SHV genes\textsuperscript{31}. There are significant geographic differences in the prevalence of resistant bacteria and their genes. Low-and middle-income countries generally have higher endemic antimicrobial resistance than high-income countries, mainly driven by antibiotic overuse in humans and animals\textsuperscript{32}. International travel can facilitate the transfer of resistant bacteria and their genes from their endemic regions to other locations\textsuperscript{33}.

Conclusions

The capacity of \textit{K. pneumoniae} isolates to generate Virulence factor type 3 fimbriae (MrKD) genes and biofilm (BssS), found that the isolates contain MrKD gene at \([n=41(82\%)]\). While the results found that \([n=36(72\%)]\) of isolates contain the BssS gene. The prevalence Virulence genes within ESBL-producing \textit{K. pneumoniae} isolates show that only \([n=3(6\%)]\) of isolates that are non-ESBL producing carry one or both virulence genes, while \([n=41(82\%)]\) of ESBL-producing isolates contain one or both virulence genes. This is consistent with the study done by Gharrah et al. that suggests a correlation between ESBL production and
Table 4. Distribution of Virulence genes within ESBLs and non-ESBLs producing *K. pneumoniae* isolates.

| No. | Isolate symbol | Virulence genes | ESBLs producing isolates |
|-----|----------------|-----------------|--------------------------|
| 1   | T969           | MrKD            | +                        |
| 2   | A1762          | MrKD, BssS      | +                        |
| 3   | T971           | BssS            | +                        |
| 4   | T973           | MrKD, BssS      | +                        |
| 5   | T975           |                 | +                        |
| 6   | T982           | MrKD, BssS      | +                        |
| 7   | A1730          |                 | +                        |
| 8   | A1553          | MrKD, BssS      | +                        |
| 9   | T985           | MrKD, BssS      | +                        |
| 10  | A1394          | MrKD, BssS      | +                        |
| 11  | A1095          |                 | –                        |
| 12  | T976           | MrKD, BssS      | +                        |
| 13  | A1789          | MrKD, BssS      | +                        |
| 14  | A1100          | MrKD, BssS      | +                        |
| 15  | T967           | MrKD, BssS      | +                        |
| 16  | A1788          | MrKD, BssS      | +                        |
| 17  | 76-8           | MrKD            | +                        |
| 18  | A1743          |                 | +                        |
| 19  | A1279          | MrKD, BssS      | +                        |
| 20  | A1794          | MrKD, BssS      | +                        |
| 21  | A1144          | MrKD            | +                        |
| 22  | A1516          | MrKD, BssS      | +                        |
| 23  | A1054          | MrKD, BssS      | +                        |
| 24  | T970           | MrKD            | +                        |
| 25  | A1557          | MrKD, BssS      | +                        |
| 26  | T979           | MrKD, BssS      | +                        |
| 27  | A1274          | MrKD, BssS      | +                        |
| 28  | A1424          | MrKD, BssS      | +                        |
| 29  | L44            | MrKD, BssS      | –                        |
| 30  | T980           | MrKD, BssS      | +                        |
| 31  | T1191          | MrKD, BssS      | +                        |
| 32  | A1984          | BssS            | +                        |
| 33  | A1993          | BssS            | –                        |
| 34  | A1994          | MrKD, BssS      | +                        |
| 35  | A1971          | MrKD, BssS      | +                        |
| 36  | A1965          | MrKD            | +                        |
| 37  | A1957          | MrKD, BssS      | +                        |
| 38  | A1946          | MrKD            | +                        |
| 39  | A1787          | MrKD, BssS      | +                        |
| 40  | T1188          | MrKD, BssS      | +                        |
| 41  | A1612          | MrKD            | –                        |
| 42  | T966           |                 | –                        |
| 43  | A1492          | MrKD            | +                        |
| 44  | A1767          | MrKD            | +                        |
| 45  | A1859          | MrKD, BssS      | +                        |
| 46  | A1875          |                 | +                        |
| 47  | A1845          | MrKD, BssS      | +                        |
| 48  | T974           | MrKD, BssS      | +                        |
| 49  | T981           | MrKD, BssS      | +                        |
| 50  | A1577          | MrKD, BssS      | +                        |
some virulence factors. In conclusion, the prevalence of ESBL-producing \textit{K. pneumoniae} in community patients was high in this research. It's also possible that ESBL production and virulence factors are linked. Antibacterial resistance is a developing problem in clinical practice. As a result, improved clinical awareness and laboratory testing are required to decrease treatment failure and prevent the spread of ESBL-producing \textit{K. pneumoniae}.

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