

ARTICLE / INVESTIGACIÓN

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 UTIs¹. After *E.coli*, *K. pneumoniae* is the most medically significant species in the Enterobacteriaceae family². One of the frequent pathogens linked to both community and hospital-acquired urinary tract infections is *K. pneumoniae*³. Because bacterial etiology is common in UTIs, broad-spectrum antibiotics are frequently used, resulting in a rise of resistant uropathogens¹. 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 resistance⁴. Several ESBL families have been identified; howe-

ver, the prominent families such as SHV, TEM, and CTX-M account for the bulk of ESBLs⁵. The rapidly increasing resistance of ESBL producers to multiple antibiotic families is a significant issue that limits the treatment options available to ESBL producers⁶. In addition, lipopolysaccharide (LPS), capsular polysaccharide, adhesions, and siderophores are among the virulence components found in *K. pneumoniae* that contribute to its pathogenicity⁷. 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 adhesion⁸. There are two kinds of fimbriae or pili in *K. pneumoniae*, types 1 and 3⁹. 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 mrkABCD 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, respectively¹⁰. Type 3 fimbriae have been shown to mediate adhesion to

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various structures in human kidney and lung tissue and epithelial cells from human urine sediments and endothelial and bladder epithelial cell lines in vitro investigations¹¹. *K. pneumoniae* biofilm development on biotic and abiotic inert surfaces is commonly linked to Type 3 fimbriae¹². 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 deficiencies¹³. 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 2compact (bioMérieux/France) device.

Antibiotic Susceptibility Test

The Kirby–Bauer disk diffusion technique was used to perform Antibiotic susceptibility testing for all isolates¹⁴. Antibiotics listed below were utilized: Ampicillin (10 μ g), Piperacillin (100 μ g), Amoxicillin-Clavulanate (20/10 μ g), Ceftazidime/Avibactam (30/20 μ g), Cefotaxime (30 μ g), Ceftriaxone (30 μ g), Ceftazidime (30 μ g), Cefuroxime (30 μ g), Cefazolin (30 μ g), Loracarbef (30 μ g), Cepodoxime (10 μ g), Cefixime (5 μ g), Aztreonam (30 μ g), Imipenem (10 μ g), Meropenem (10 μ g), Gentamicin (10 μ g), Tobramycin (10 μ g), Amikacin (30 μ g), Doxycycline (30 μ g), Tetracycline (10 μ g), Ciprofloxacin (5 μ g), Levofloxacin (5 μ g), Nalidixic acid (30 μ g), Trimethoprim-Sulfamethoxazole (1.25/23.75 μ g), Chloramphenicol (30 μ g), Nitrofurantoin (300 μ g).

Modified Double Disc Synergy Test (MDDST)

Detect ESBL enzymes production by using the Modified Double Disc Synergy Test through the following steps¹⁵: After uniformly spreading the inoculum onto sterile Mueller–Hinton agar (biolab/Hungary), the disc which contained amoxicillin-Clavulanate (20/10 μ g) was placed in the center of the plate and the discs of third-generation cephalosporin [cefotaxime (30 μ g), ceftriaxone (30 μ g), and cefpodoxime (10 μ g)], and fourth-generation cephalosporin [cefepime (30 μ g)], 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™ Total DNA Extraction Kit (Intron biotechnology/Korea) according to the manufacturer's instructions. Using Nanovue plus™ 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 -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 (20 μ l) 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 TEM-R ^b	AT GAG TAT TCA ACA TTT CCG TT AAT CAG TGA GGC ACC TAT	717	[11]
SHV-F SHV-R	AGCCGCTTGAGCAAATTAAC ATCCCGCAGATAAATCACCAC	525	[12]
CTX-M-F CTX-M-R	GCT GTT GTT AGG AAG TGT GC CCA TTG CCC GAG GTG AAG	515	[13]
BssS-F BssS-R	GATTCAATTTTGCGATTCCTGC TAATGAAGTCATTCAGACTCATCC	225	[14]
MrKD-F MrKD-R	AT GGA ACC CAC ATC GAC ATT AT GAG TAT TCA ACA TTT CCG	390	[15]

Table 1. Primers Used for Detection Genes.

Gene	Thermocycler conditions				
	Denaturation	Annealing	Extension	Final Extension	cycles
TEM	95°C 3 min.	55 °C 45 sec	72°C 50sec	72°C 7 min	32
SHV	95°C 3 min.	61 °C 45 sec	72°C 50sec	72°C 7 min	32
CTX-M	95°C 3 min.	59 °C 45 sec	72°C 50sec	72°C 7 min	32
BssS	95°C 3 min.	66 °C 45 sec	72°C 50sec	72°C 7 min	32
MrKD	95°C 3 min.	59 °C 45 sec	72°C 50sec	72°C 7 min	32

Table 2. PCR Thermocycler Conditions for Each Gene.

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 2compact 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 [n=34(68%)], then the CTX-M gene [n=33(66%)]. While none of the isolates possessed the TEM gene, as showed in Figure 2.

The results found that the isolates contain MrKD gene at [n=41 (82%)] as shown in the figure A (3). It also, found that [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 [n=3 (6%)] of isolates which are non-ESBL producing carry one or both virulence genes, while [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 abscess. 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 antimicrobial 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 Pishtiwan 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 β -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 Shalib *et al.*, who showed that [n=62 (88.6%)] of *K. pneumoniae*

Antimicrobial Agent	Susceptible	Intermediate	Resistant
	No (%)	No (%)	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)
Doxycycline	3 (6)	0 (0.0)	47 (94)
Tetracycline	2 (4)	0 (0.0)	48 (96)
Doxycycline	3 (6)	0 (0.0)	47 (94)
Ciprofloxacin	36 (72)	1 (2)	13 (26)
Levofloxacin	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)

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

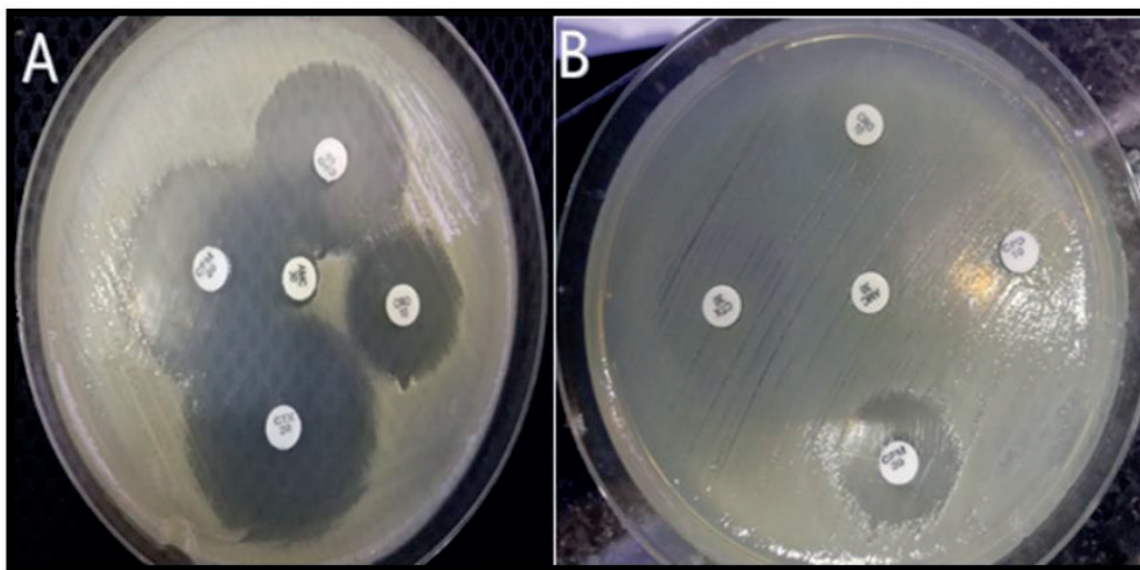


Figure 1. Modified Double Disc Synergy Test (MDDST) (A) shows synergism of ceftazidime, cefpodoxime, ceftriaxone and cefotaxime with amoxicillin-Clavulanate, which indicates ESBL production, while in (B) showed no synergism with amoxicillin-Clavulanate, which indicates none of ESBL production.

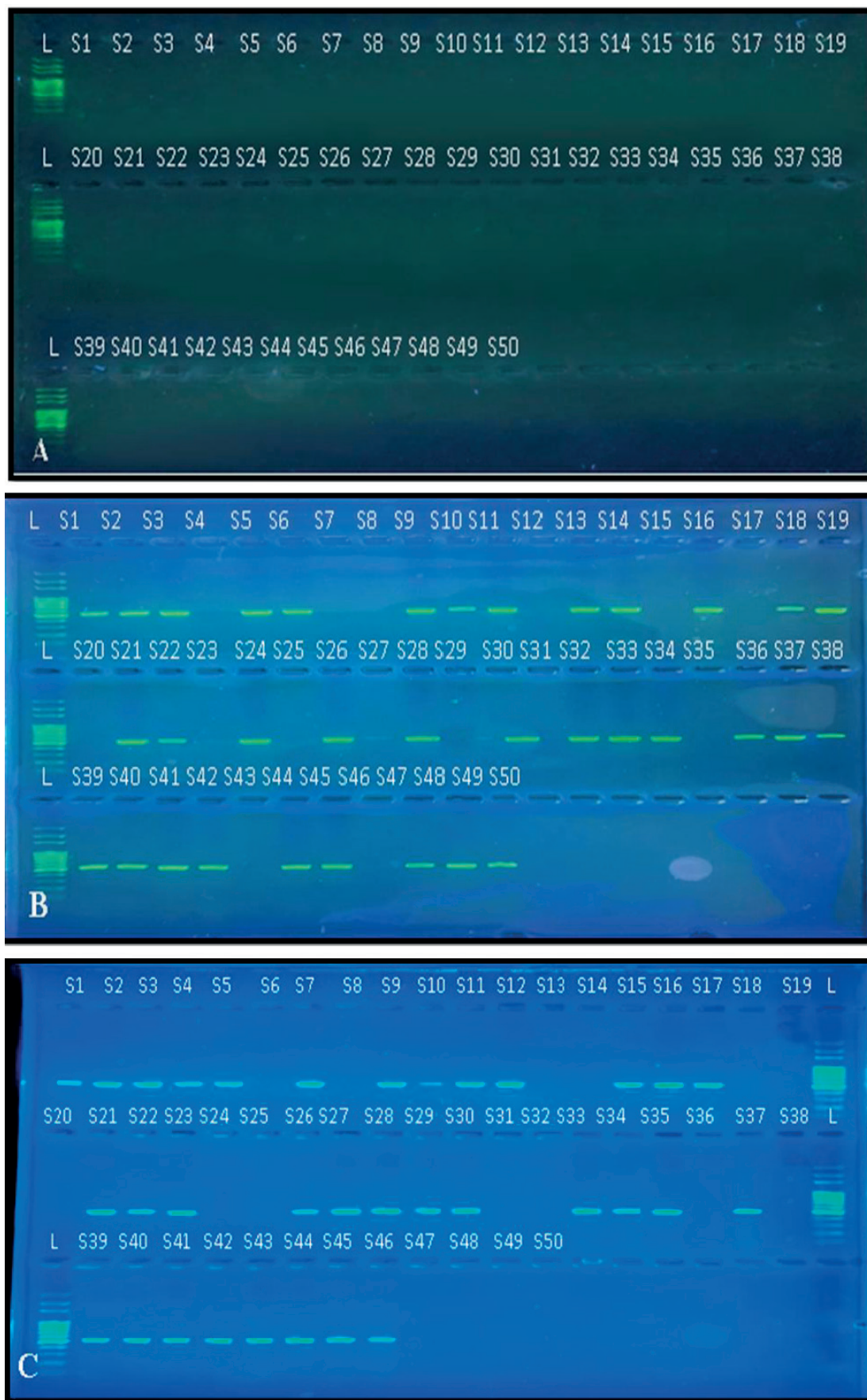


Figure 2. Agarose gel electrophoresis of PCR products of (A) TEM gene, (B) SHV gene and (C) CTX-M gene in *K. pneumoniae* isolates visualized under UV after staining by RedSafe™ nucleic acid staining solution for 2% agarose gel at 100 volts, 70 amps for 60 min. L: ladder DNA (100pb), S: Sample.

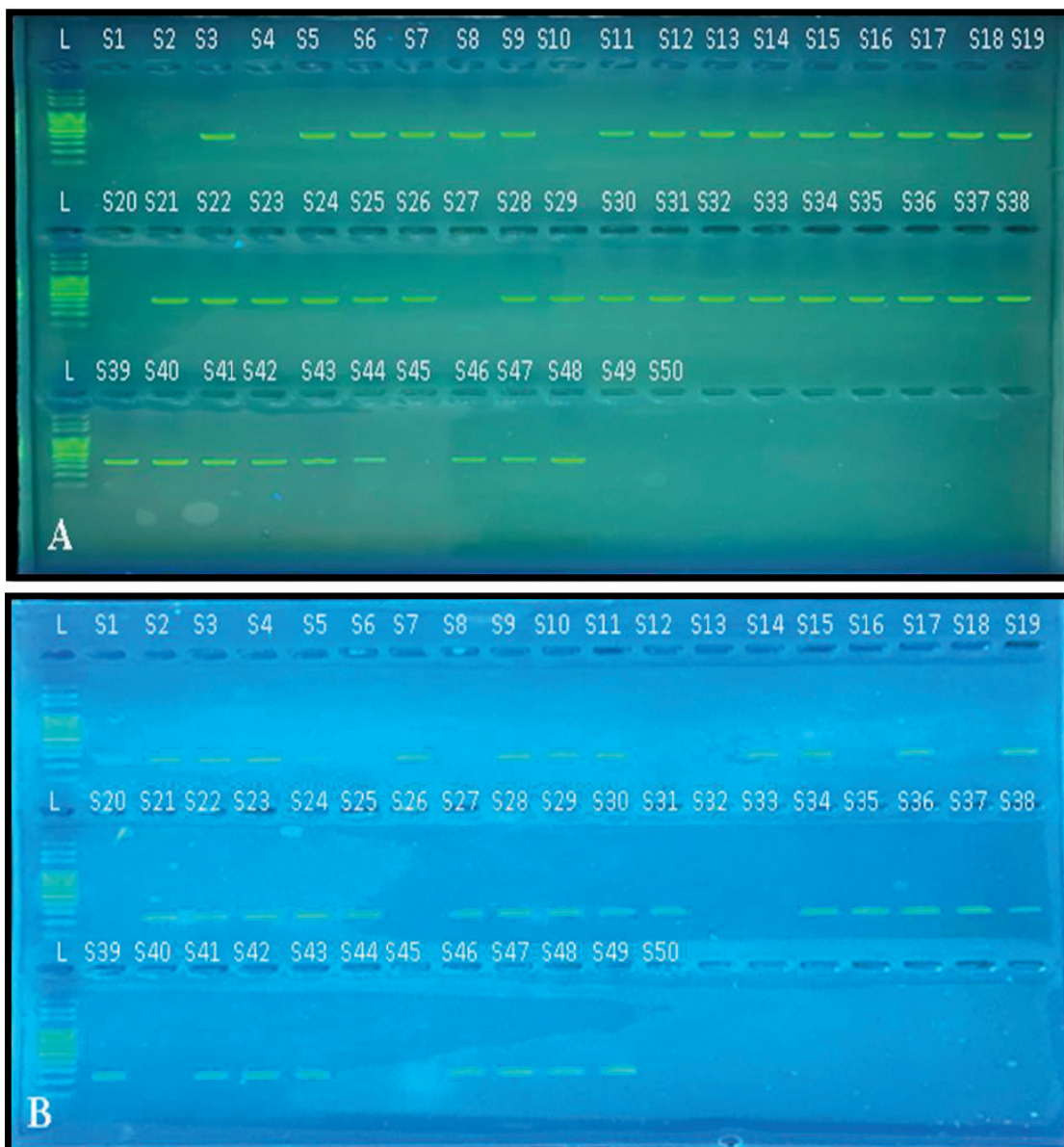


Figure 3. Agarose gel electrophoresis of PCR products of (A) MrkD gene and (B) BssS gene in *K. pneumoniae* isolates visualized under UV after staining by RedSafe™ nucleic acid staining solution for 2% agarose gel at 100 volts, 70 amps for 60 min. L: ladder DNA (100pb), S: Sample.

isolates were ESBL-producing²⁹. Pishtiwan and Khadija *et al.*'s research revealed that [n=17(85%)] of *K. pneumoniae* isolates were positive for ESBL production²³. The prevalence of ESBL-producing clinical isolates is related to risk factors such as current antibiotic use and hospitalization²⁹.

The ability of *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 β -lactamase gene was SHV, followed by TEM and CTX-M³⁰. Also, Pishtiwan and Khadija *et al.* reveal that the TEM gene has the highest prevalence in *K. pneumoniae* isolates at 64.7%, followed by the CTX-M gene at 41.1% and the SHV gene at 35.2%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 genes³¹. 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 animals³². International travel can facilitate the transfer of resistant bacteria and their genes from their endemic regions to other locations³³.

Conclusions

The capacity of *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 *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

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, BssS	+
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	+

Table 4. Distribution Virulence genes within ESBLs and non-ESBLs producing *K. pneumoniae* isolates.

some virulence factors³⁴. In conclusion, the prevalence of ESBL-producing *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 *K. pneumoniae*.

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