RESEARCH ARTICLE

Coexistence of blaOXA-48, blaVIM, and blaSHV genes in *Klebsiella pneumoniae* and *Escherichia coli* isolated from urinary tract infections: Microbiological and epidemiological analysis

Manar Abdallah[1], Soliman Haroun[2], Amin Tahoun[3], Yamen Mohammed Hegazy[4], Ramadan Eldomany[5]

Abstract

Objective: To investigate antimicrobial resistance mechanisms of isolated bacterial strains, and their correlation with virulence profile.

Method: The cross-sectional study was conducted in January 2020 at outpatient health centres in Kafrelsheikh Governorate of Egypt, and comprised urine samples from patients regardless of age and gender. Midstream samples were collected into sterile swaps which were kept in ice-cooled boxes until transported to the laboratory within 5h. Antimicrobial resistance profile of the isolated *Enterobacteriaceae* was done using Kirby–Bauer disk diffusion method and was confirmed with Vitek compact 2. The phenotypic of carbapenemases and extended-spectrum beta lactamase was determined, and polymerase chain reaction was used, as appropriate. Data was analysed using SPSS 20.

Results: Of the 199 patients, 101(50.7%) were females and 98(49.3%) were males. The majority 73(36.6%) were aged 30-50 years. Urinary tract infection was found in 68(34.2%) patients. In 28(41.2%) of these patients, there were 32 isolates of *Enterobacterales*; 21(65.62%) *Klebsiella pneumoniae*, 7(21.87%) *Escherichia coli* and 4(12.5%) *Enterobacter cloacae*. Of the 28(41.2%) patients, 24(85.7%) were infected with a single strain; 17(70.8%) Klebsiella pneumoniae, 4(16.7%) *Escherichia coli* and 3(12.5%) Enterobacter cloacae. In 3(10.7%) cases, there was co-infection with *Escherichia coli* and Klebsiella pneumoniae, and 1(3.6%) sample had mixed infection with *Klebsiella pneumoniae* and *Enterobacter cloacae*. The other 40(58.8%) patients had other causative agents. Housewives, agricultural workers and those aged >50 years had a higher risk of urinary tract infections ($p<0.05$) Among *Klebsiella pneumonia* isolates, 6(28.5%) possessed carbapenemase-related genes and 4(19.1%) extended-spectrum beta lactamase-related genes. The carbapenemase-related genes were bla-Verona integron-encoded metallo beta lactamase 6(100%) bla-New Delhi metallo beta lactamase-1 4(66.6%) and bla-oxacillinase-48 2(33.3%). The 4(19.1%) cases of extended-spectrum beta lactamase-related genes had bla-temoneira gene 3(75%) and bla-sulfhydryl variable gene 4(100%). In *Escherichia coli* isolates, bla-oxacillinase-48 and bla-Cefotaximase genes were observed in 2(28.5%) cases. Virulence genes uridine diphosphate-glucose 4-epimerase, fimbrial adhesion and mannose-resistance adhesin of Klebsiella spp genes in *Klebsiella pneumoniae* isolates were positive in in 16(76.2%), 14(66.7%) and 10(47.6%) cases, respectively. All 21(100%) isolates of *Klebsiella pneumoniae* were negative for mucoviscosity-associated gene A.

Conclusion: There was evidence of the coexistence of bla- oxacillinase-48, bla-Verona integron-encoded metallo beta lactamase and bla-sulfhydryl variable genes in *Klebsiella pneumoniae* and *Escherichia coli* isolates from mixed urinary tract infection samples.

Keywords: Carbapenemase, *Klebsiella pneumoniae*, *Escherichia coli*, Lactams. **DOI: 10.47391/JPMA.EGY-S4-58**

Introduction

Urinary tract infection (UTI) is known as the second most common cause of infections and a major cause of morbidity and mortality[1,2], affecting people of all ages[3-5]. Age, gender and other risk factors, such as pregnancy, diabetes, poor sanitary conditions, poor nutrition and poor socioeconomic status in rural regions, are the key threatening elements implicated in the prevalence of UTIs.[6, 7]

Bacterial UTI can result in both local and systemic disease syndromes, and is alarmingly rising, which could be owing to the development of drug resistance by pathogenic microorganisms as a result of extensive and uncontrolled usage of multiple antibiotics.[8, 9] *Klebsiella (K.) pneumoniae* represents one of most commonly encountered uropathogens in African countries, and its prevalence has steadily grown over the years.[10, 11] In both simple and complex UTI patients, infection with uropathogenic *Escherichia (E.) coli* (UPEC) increases the likelihood of recurrence within 6 months. Enterobacter (E.) cloacae has also been reported as one of the main pathogens causing UTI[12], which is responsible for a variety of infections, especially in UTI patients.[9, 13, 14]

Drug resistance is one of the predominant mechanisms associated with the survival and pathogenicity of bacterial

[1,2]Department of Botany and Microbiology, Kafrelsheikh University, Egypt.
[3,4]Department of Animal Medicine Kafrelsheikh University, Egypt.
[5]Department of Microbiology and Immunology, Kafrelsheikh University, Egypt.
Corrospondience: Amin Tahoun email: amin.abdelhady@vet.kfs.edu.eg

organisms.[15-17] Carbapenemases are beta (β) lactamase enzymes, which were shown to be slow to emerge in Enterobacteriaceae, but are now increasingly causing serious infections leading to mortalities in hospitalised patients.[18-20] The most common carbapenemases are *K. pneumoniae* carbapenemases (KPC), New Delhi metallo-β-lactamase (NDM), imipenem-resistant Pseudomonas (P.)-type carbapenemases (IMP), Verona integron-encoded metallo-β-lactamase (VIM), and oxacillinase (OXA-48-like) types. They are coded by blaKPC, blaNDM, blaIMP, blaVIM, and blaOXA-48 genes, respectively.[21]

The detection of carbapenemases is difficult; the isolate may be reported as sensitive by phenotypic tests while still harbouring carbapenemases but released in low levels[22, 23], particularly for meropenem (MEM) and imipenem IMP) groups.[24, 25]

Extended-spectrum beta lactamase (ESBL)-producing bacteria represent a public health concern because of the variable prevalence and emergence particularly in *K. pneumoniae*. These enzymes hydrolyze b-lactam antimicrobials, third- and fourth-generation cephalosporins and monobactams, and lead to failure of treatment.[26] ESBLs are grouped into four classes A, B, C and D enzymes. Cefotaximase (CTX-M), temoneira (TEM) and sulfhydryl variable (SHV) are class A ESBLs.[27] Phenotypic detection of ESBLs between Enterobacteriaceae species is important for epidemiological purposes and to limit the spread of resistance mechanisms.[28, 29]

Multiple bacterial determinants have been implicated in the pathogenesis of *K. pneumoniae*. They include capsular serotypes, especially K1 or K2.[30] Examples include mucoviscosity-associated gene A (magA)[31], mannose-resistance adhesin of *Klebsiella* spp (mrkA) gene[32], and uridine diphosphate (UDP)-glucose 4-epimerase (uge).[33] Fimbrial adhesion (fimH) is a major virulence factor implicated in many urinary and systemic pathological consequences, particularly during *K. pneumoniae* colonisation, biofilm formation, and persistence in UTIs.[34, 35]

Although *K. pneumoniae*, *E. coli* and *E. cloacae* are very important causes of UTI, no information reflects these bacteria in the Kafrelsheikh Governorate of northern Egypt. The current study was planned to determine the epidemiological data obtained from UTI patients and to investigate the antimicrobial resistance mechanisms of isolated Enterobacteriaceae strains. Also genotypic characterisation of carbapenemase, metallo-β-lactamase (MBL) and ESBL-producing isolates were planned, and the correlation of profile of *K. pneumoniae* with its virulence profile.

Patients and Methods

The cross-sectional study was conducted in January 2020 at outpatient health centres in Kafrelsheikh Governorate of Egypt (Figure 1). After approval from the institutional ethics review committee, the sample size was calculated using 7% expected prevalence[36] and acceptable margin of error 5%.[37] The sample was inflated by 97%. Urine samples were obtained from patients regardless of age and gender who had clinical evidence suggestive of UTI. Informed consent was obtained from all patients or from parents of patients aged <18 years. Those not willing to participate were excluded. Inadequate urine samples and results showing >3 pathogens, reflecting a contamination more than a real UTI[38], were also excluded.

Demographic data, including age, gender and occupation, was recorded. Urine was collected from patients using the midstream clean catch method. The patients were instructed by the medical staff to void the first portion of the urine stream into the toilet and then collect the midstream into the sterile container.

The samples were kept in ice-cooled boxes until transported to the laboratory within 5h.

All the samples were diluted using 10-fold serial dilution method with the help of peptone water, and the dilution was spread on cysteine lactose electrolyte-deficient (CLED) (Oxoid, UK) agar plates before being incubated at 37°C for 24-48h. A significant colony count was considered on the basis of a pure growth of $\geq 10^5$ CFU/mL.[3, 39-41]

All urine samples were streaked on CLED agar. Each suspected sample was streaked on eosin methylene blue (EMB) agar and MacConkey agar (Oxoid, UK). All plates were incubated at 37°C for 18-48h.[42]

The isolates were identified using classical biochemical methods by standard laboratory techniques.[43]

Multidrug resistence (MDR) was characterised if a strain during in vitro antimicrobial susceptibility test (AST) showed resistance to three or more antimicrobial classes.[41]

The AST test was carried out using the Kirby-Bauer disk diffusion method, as recommended by the Clinical & Laboratory Standards Institute (CLSI)[44-46], which was further confirmed by an automated system (Vitek Compact 2; AST-card, Biomerieux®, France) following the manufacturer's instructions.

A total of 21 antibiotics (Oxoid, UK) commonly used for UTI treatment in the Egyptian drug market were tested. They were ampicillin (AM), ampicillin/sulbactam (SAM), amoxicillin (AMX), amoxicillin/clavulanic acid (AMC),

piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), ertapenem (ERT), imipenem (IMP), meropenem (MEM), amikacin (AN), gentamicin (GM), ciprofloxacin (CIP), norfloxacin (NOR), fosfomycin (FOS), nitrofurantion (FT), trimethoprim/sulfamethoxazoleole (SXT), levofloxacin (LEV), ceftriaxone (CTR) and colistin (CT). The American type culture collection (ATCC) strain *E. coli* (ATCC 25922) was used as the quality-control strain in AST, and *K. pneumoniae* (ATCC 1290) as the control strain.[47, 48]

All carbapenem-resistant strains were subjected to modified Hodge test (MHT) for the detection of carbapenemase-producing *K. pneumoniae* isolates.[49, 50] A 10mg MEM susceptibility disk was placed in the centre of the MHT plate and the tested organism was streaked in a straight line from the edge of the MEM disk to the edge of the plate. It was incubated at 35±2°C for 16-18h. ATCC BAA-1705 and BAA-1706 were used as MHT-positive and MHT-negative controls. Carbapenemase production was detected by the appearance of enhanced ATCC *E. coli* 25922 growth along with the tested organism that revealed a clover leaf-like indentation, indicating a positive test.

The combined disk test (CDT) was used for the phenotypic detection of MBLs in carbapenem-resistant gram-negative bacteria.[51-53] Two 10mg IMP disks were placed on Müller-Hinton agar, and 4µl of 0.5M concentrated ethylenediaminetetraacetic acid (EDTA) solution was added to one of the disks. The inhibition zones of IMP and IMP-EDTA disks were compared after 16-18h incubation at 35°C. CDT compared the increased inhibition zone ≥7mm with the IMP-EDTA disk to the IMP disk alone, which was considered MBL-positive.

Double-disk synergy test (DDST) was performed for IMP-EDTA.[51, 54] One blank filter paper disk containing 10mL EDTA was placed at 20mm from the centre of the IMP disk, and the inhibition zones of the IMP and the blank disk with EDTA were compared. Enhancement of the inhibition zone in the area between IMP and the EDTA disk compared to the inhibition zone on the far side of the drug was interpreted as a positive result.

All isolates were screened for ESBL production by CDT following CLSI guidelines[55, 56]. Susceptibility to CTX (30µg), CTX/clavulanate (30/10µg), CAZ (30µg) and CAZ/clavulanate (30/10µg (Oxoid, UK) was determined on Müller-Hinton agar (Oxoid, UK). ESBL-producing strains were identified by at least 5mm increase in zone diameter around CTX/clavulanate and CAZ/clavulanate disks compared to disks without clavulanic acid.[55, 56]

Conventional PCR was performed using specific primers (Metabion, Germany) (Table 1) to detect genes encoding carbapenemases, MBL (blaIMP, blaVIM, blaNDM, blaKPC, and blaOXA-48), and ESBL (blaTEM, blaSHV, and blaCTX-M)[57]. In order to obtain DNA from the isolated strains, a single colony from each plate was taken and inoculated into 5ml of tryptone soya broth (Oxoid, UK) overnight at 37°C. Then 1ml of bacterial culture broth was centrifuged in a micro-centrifuge tube at 13,000rpm for 1min. The supernatant was discarded, and the bacterial pellets were homogenised with nuclease-free water and heated at 95°C for 10min. The boiled lysates were finally centrifuged, and the supernatant was collected as DNA templates, which were kept at -80°C until use.

Primers (Table 2) were used in 25µl uniplex PCR mix, comprising 12.5µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1µl of each primer (20pmol), 5.5µl of water, and 5µl of DNA template[55]. The reaction was performed in a thermal cycler (Applied Biosystems 2720). The cycling condition started with primary denaturation at 94°C for 5min followed by 35 cycles and a final extension at 72°C for 10min. Positive controls were represented by field samples that were previously confirmed to be positive by PCR for the related genes in the reference laboratory for veterinary quality control on poultry production, animal health research institute.[57-60] Sterile water was added to the PCR mix with each primer pair as control negative.

The products were separated by electrophoresis in 1% agarose gel with 1X Tris/borate/EDTA, stained with a safe stain load dye, and visualised under ultraviolet illumination.

PCR for virulence-associated genes led to extraction of bacterial DNA using EmeraldAmp GT PCR Mastermix (Takara, Japan; Code No. RR310A) as was the case with resistance genes.

Data was analysed using SPSS 20. Univariate logistic regression model was built to examine the associations between dependent and independent variables. Variables with $p<0.2$ were subjected to multivariate logistic regression model, in the light of a 2019 study by Abdelwahab et al.[61]. In *K. pneumoniae* and *E. coli* isolates, the relative risk (RR) for resistance to both ERT and IMP was worked out using the formula (D1/N1) * (N2/D2), where D1 was the number of isolates that were phenotypically or genotypically positive for resistance to a particular antibiotic/group of antibiotics, D2 was the number of isolates phenotypically or genotypically positive to both ERT and IMP resistance (baseline group), N1 was the number of isolates resistant to a particular antibiotic/group of antibiotics, and N2 was the number of isolates resistant to both ERT and IMP. Similar forumla was used for *K. pneumoniae* and *E. coli* for CTX and CAZ resistance. The

relative association between the resistance type and the virulence gene of *K. pneumoniae* isolates was calculated using univariable ordinal logistic regression analysis. The resistance type was established as the response variable and the predictor variables were virulent for mrkA (fimbriae), fimH (fimbriae), uge (capsule) and magA (capsule). Odds ratios (ORs) were calculated. Level of significance was set at $p<0.05$.

Results

Of the 199 patients, 101(50.7%) were females and 98(49.3%) were males. The majority 73(36.6%) were aged 30-50 years. Housewives, agricultural workers and those aged >50 years had a higher risk of urinary tract infections (p<0.05). In the multivariate model, gender, occupation and age variables were included; age was a confounder for occupation, which left only the variable of occupation (Table 3**).** UTI was found in 68(34.2%) patients. In 28(41.2%) of these patients, there were 32 isolates of Enterobacterales; 21(65.62%) *K. pneumoniae*, 7(21.87%) *E. coli* and 4(12.5%) E. cloacae. Of the K. pneumonia isolates, 15(71.4%) were from rural areas, and 6(28.6%) from urban areas. Among the *E. coli* isolates, 5(71.4%) were rural and 2(28.6%) urban. Among the E. cloacae isolates, 1(25%) were rural and 3(75%) urban.

Table-1: Primers used for polymerase chain reaction (PCR) to detect genes encoding carbapenemases, Metallo B-Lactamases (MBLs) and extended-spectrum beta lactamases (ESBLs).

Gene		Sequence	Amplified product	Reference
blaIMP	F	CATGGTTTGGTGGTTCTTGT	488 bp	[58]
	R	ATAATTTGGCGGACTTTGGC		
blaVIM	F	AGTGGTGAGTATCCGACA	280 bp	
	R	ATGAAAGTGCGTGGAGAC		
blaNDM-1	F	GGCGGAATGGCTCATCACGA	287 bp	
	R	CGCAACACAGCCTGACTTTC		
blaKPC	F	ATGTCACTGTATCGCCGTCT	892 bp	
	R	TTTTCAGAGCCTTACTGCCC		
blaOXA-48	F	CCATAATCGAAAGCATGTAGC		
	R	TAATCACCGCGATGAAATATTCAGT CTTGCTCATACGTGCCTC	504 bp	[57]
blaTEM	F	ATCAGCAATAAACCAGC	516 bp	[59]
	R	CCCCGAAGAACGTTTTC		
blaSHV	F	AGGATTGACTGCCTTTTTG	392 bp	
	R	ATTTGCTGATTTCGCTCG		
blaCTX-M	F	ATG TGC AGY ACC AGT AAR GTK ATG GC	593 bp	[60]
	R	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		

IMP: Imipenem-resistant Pseudomonas-type carbapenemases, VIM: Verona integron-encoded metallo beta, NDM-1: New Delhi metallo beta lactamase-1, KPC: Klebsiella pneumoniae carbapenemases, OXA-48: Oxacillinase-48, TEM: temoneira gene, SHV: sulfhydryl variable gene, CTX-M: Cefotaximase gene.

Of the 28(41.2%) patients, 24(85.7%) were infected with a single strain; 17(70.8%) *K. pneumoniae*, 4(16.7%) *E. coli* and 3(12.5%) *E. cloacae*. In 3(10.7%) cases there was co-infection with *E. coli* and *K. pneumoniae*, and 1(3.6%) sample had mixed infection with *K. pneumoniae* and *E. cloacae*. The other 40(58.8%) patients had other causative agents.

AST with 21 antibiotics led to 441 *K. pneumoniae*, 147 *E. coli* and 84 *E. cloacae* isolates. *K. pneumoniae* had the highest overall resistance to the tested antibiotics 256(58.04%), followed by *E. coli* 84(57.14%), and *E. cloacae* 29(34.52%). For all the 32 bacterial isolates analysed, 31(96.87%) (95% confidence interval [CI]: 98.7-100%).

All the 32(100%) isolates were resistant to AM and AMX, except 1(14.28%) *E. coli* isolates, while 13(61.9%) *K. pneumoniae*, 5(71.4%) *E. coli* and 3(75%) E. cloacae isolates were resistant to AMC. The association of the isolates with each of the 21 antibiotics was noted in detail (Table 4; Figures 2-3).

Table-2: Primers used for polymerase chain reaction (PCR) of virulence associated genes.

Gene		Sequence	Amplified product	Reference
MagA	F	GGTGCTCTTTACATCATTGC	1282 bp	[74]
	R	GCAATGGCCATTTGCGTTAG		
Uge	F	TCTTCACGCCTTCCTTCACT	534 bp	[75]
	R	GATCATCCGGTCTCCCTGTA		
MrkA	F	CGGTAAAGTTACCGACGTATCTTGTACTG	475 bp	[76]
	R	GCTGTTAACCACACCGGTGGTAAC		
FimH	F	TGCAGAACGGATAAGCCGTGG	508 bp	[77]
	R	GCAGTCACCTGCCCTCCGGTA		

MagA: Mucoviscosity-associated gene A, Uge: Uridine diphosphate-glucose 4-epimerase gene, FimH: Fimbrial adhesion gene, mrkA: mannose-resistance adhesin of Klebsiella spp gene.

Table-3: Univariate logistic regression analysis for identification of risk factors for uropathogens of bacterial urinary tract infection (UTI).

Variable	Number tested	Number positive	*p-value*	OR	95% CI
Occupation					
Students	37	2	-	-	-
Teachers*	59	5	0.58	1.6	0.30 – 8.82
Nurses**	3	0	0.99	0.0	0.000
Agricultural workers	28	6	0.07	4.77	0.88 – 25.78
Housewives	72	15	0.005	4.61	0.99 – 21.36
Age					
0- 15 years	30	2	-	-	-
> 15 - 30 years	43	1	0.38	0.33	0.03 – 3.85
> 30 - 50 years	73	11	0.26	2.48	0.52 – 11.96
>50 years	53	14	0.04	5.03	1.06 - 23.90
Gender					
Females	101	15	-	-	-
Males	98	13	0.75	0.88	0.39 – 1.95

*Including any employed men (engineers, salesmen, lawyers, and accountants).
**Including any employed woman.
OR: Odds ratio, CI: Confidence interval.

Table-4: Table 4: The 32 strains along with their origin, antibiotic resistance profile as well as phenotypic and genotypic tests.

Klebsiella pneumonia	Case number	Gender	Occupation	District	Age(y)	AM	SAM	AMX	AMC	TZP	CAZ	CTX	FEP	ERT	IMP	MEM	AN	CIP	GM	FOS	NOR	SXT	FT	CTX	LEV	CT	MHT	D.D.S.T	C.D.T	ESBL-C.D.T	*bla*TEM	*bla*SHV	*bla*OXA48	*bla*KPC	*bla*MP	*bla*VIM	*bla*TEM	*bla*SHV	*bla*CTX-M
ID1	2	male	Student	urban	11	R	R	R	R	R	R	R	R	R	R	S	S	R	S	S	R	S	R	R	R	S	-	-	-	(+)	-	-	-	-	-	(+)	(+)	-
ID2	7	male	Agricultural worker	urban	50	R	I	R	R	R	R	R	R	R	R	S	S	I	S	S	R	S	R	R	I	S	-	-	-	-	-	-	-	-	-	-	-	-	-
ID3	9	female	housewife	rural	53	R	R	R	R	R	R	R	R	R	R	S	S	R	S	S	R	S	R	R	R	S	-	-	-	-	-	-	-	-	-	(+)	-	-	-
ID4	22	male	Student	rural	14	R	R	R	S	S	R	R	R	S	S	S	S	S	S	R	S	R	I	R	S	S	-	-	-	(+)	-	-	-	-	-	(+)	-	-	-
ID5	30	male	Lawyer	rural	45	R	R	R	R	R	R	R	R	R	R	S	S	R	S	S	R	R	S	R	R	I	-	-	-	-	-	-	-	-	-	-	-	-	-
ID6	36	female	Housewife	rural	36	R	R	R	S	S	R	R	R	S	S	S	S	S	S	R	S	R	I	R	S	I	-	-	-	(+)	-	-	-	-	-	-	(+)	-
ID7	37	female	Housewife	rural	93	R	R	R	R	S	R	R	R	R	S	S	S	S	S	R	S	R	I	R	S	S	-	-	-	-	-	-	-	-	-	-	-	-
ID8	38	female	Housewife	rural	57	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	(+)	-	(+)	-	(+)	(+)	-	-	(+)	-	-	-
ID9	40	female	Housewife	rural	55	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	I	-	-	(+)	-	(+)	(+)	-	-	-	-	-	-
ID10	43	male	Accountant	urban	33	R	S	R	S	S	R	R	R	R	S	S	S	R	S	S	S	R	I	R	S	I	-	-	-	-	-	-	-	-	-	-	-	-
ID11	46	female	Housewife	urban	40	R	R	R	R	R	R	R	R	S	S	S	S	R	S	S	R	R	I	R	R	S	-	-	-	-	-	-	-	-	-	-	-	-
ID12	47	female	Housewife	rural	43	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	S	I	-	-	-	-	-	-	-	-	-	-	-	-	-
ID13	48	female	Housewife	rural	65	R	R	R	S	R	S	S	I	I	S	S	S	I	S	S	S	R	I	R	S	I	-	-	-	-	-	-	-	-	-	-	-	-	-
ID14	50	female	Housewife	urban	60	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	S	R	R	R	S	-	(+)	(+)	-	(+)	(+)	-	-	-	-	-	-	-
ID15	55	male	teacher	urban	50	R	R	R	R	R	R	R	R	R	R	S	S	S	R	S	R	I	R	S	S	S	(+)	(+)	(+)	-	(+)	(+)	-	-	-	-	-	-	-
ID16	56	female	Housewife	rural	70	R	R	R	R	R	R	R	S	S	S	S	R	R	R	R	R	R	R	R	R	I	-	-	-	(+)	-	-	-	-	-	-	(+)	-
ID17	57	female	housewife	rural	39	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	(+)	-	-	-	(+)	-	-	(+)	-	-	-	-	-
ID18	61	male	Agricultural worker	rural	51	R	S	R	S	S	R	R	R	S	S	S	S	S	S	R	S	R	I	R	S	S	-	(+)	-	(+)	(+)	-	-	-	-	-	(+)	-	-
ID19	71	male	Accountant	rural	32	R	S	R	S	S	R	R	S	R	S	S	S	S	S	R	S	R	I	R	S	S	-	-	-	-	-	-		-	-	-	-	-	-
ID20	79	female	Housewife	rural	26	R	S	R	S	S	R	R	R	S	S	S	S	I	S	R	S	R	R	R	I	S	-	-	-	-	-	-	-	-	-	-	-	-	-
ID21	86	female	Housewife	rural	69	R	R	R	R	S	S	S	R	R	S	S	S	S	S	S	R	R	I	R	S	S	-	-	-	-	-	-	-	-	-	-	-	-	-
E-coli																																							
ID22	6	male	Agricultural worker	urban	60	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S	R	R	S	R	R	I	-	(+)	-	-	-	-	-	-	-	-	-	-	-
ID23	32	male	Agricultural worker	rural	63	R	R	R	R	R	R	R	R	S	S	S	S	R	S	S	R	R	S	R	R	S	-	-	-	-	-	-	-	-	-	-	-	-	-
ID24	38	female	Housewife	rural	75	R	R	R	R	R	R	R	R	R	S	S	R	S	S	S	R	R	S	R	R	I	(+)	-	-	-	(+)	(+)	-	-	(+)	-	-	-	-
ID25	44	female	Housewife	urban	39	R	R	R	R	R	R	R	R	S	S	S	S	R	S	S	R	R	S	R	R	S	-	-	-	-	-	-	-	-	-	-	-	-	-
ID26	45	male	Sales	rural	61	R	R	R	I	S	R	R	R	S	S	S	S	R	R	S	R	R	S	R	R	I	-	-	-	(+)	-	-	-	-	-	(+)	-	(+)
ID27	47	female	Housewife	rural	43	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-	-	-	-
ID28	56	female	Housewife	rural	70	R	R	R	R	R	R	R	R	S	R	R	S	R	S	S	R	R	I	R	R	S	(+)	(+)	(+)	(+)	-	-	-	-	(+)	-	(+)	(+)	(+)
Enterobacter cloacae																																							
ID29	7	male	Agricultural worker	urban	50	R	R	R	I	S	R	S	I	I	S	S	S	R	S	S	I	R	S	I	I	I	-	-	-	-	-	-	-	-	-	-	-	-	-
ID30	25	male	Agricultural worker	urban	65	R	R	R	R	S	R	S	S	S	S	S	S	R	S	S	I	R	S	I	R	S	-	-	-	-	-	-	-	-	-	-	-	-	-
ID31	35	female	Housewife	rural	62	R	R	R	R	S	R	S	I	I	S	S	S	R	S	S	I	R	S	I	R	S	-	-	-	-	-	-	-	-	-	-	-	-	-
ID32	53	male	Agricultural worker	urban	49	R	R	R	R	S	R	S	S	S	S	S	S	R	S	S	I	R	S	I	I	I	-	-	-	-	-	-	-	-	-	-	-	-	-

AM: Ampicillin, SAM: Ampicillin/sulbactam, AMX: Amoxicillin, AMC: Amoxicillin/clavulanic acid, TZP: Piperacillin/tazobactam, CTX: Cefotaxime, FEP: Cefepime, ERT: Ertapenem, MEM: Meropenem, CIP: Ciprofloxacin, GM: Gentamicin, FOS: Fosfomycin, NOR: Norfloxacin, SXT: Trimethoprim, Sulf: Sulfamethoxazoleole, FT: Nitrofurantion, CTR: Ceftriaxone, LEV: Levofloxacin, MHT: Modified Hodge test, DDST: Double-disk synergy test, CDT: Combined disk test, ESBL: Extended-spectrum beta lactamase, IMP: Imipenem-resistant Pseudomonas-type carbapenemases gene, VIM: Verona integron-encoded metallo beta gene, NDM-1: New Delhi metallo beta lactamase-1 gene, KPC: Klebsiella pneumoniae carbapenemases gene, OXA-48: Oxacillinase-48 gene, TEM: temoneira gene, SHV: sulfhydryl variable gene, CTX-M: Cefotaximase gene.

Table-5: Relative association between the resistance of Klebsiella (K.) pneumoniae isolates to MEM, ERT and IMP antibiotics and its carbapenemase phenotypic and genotypic resistance profiles.

Resistance	Total	Phenotypic resistance	RR	*p- value*	Genotypic resistance	RR	*p- value*
Susceptible	7	1	1.9	0.7	1	1.9	0.7
Resistant to ERT	5	0	0.9	0.9	0	0.9	0.9
Resistant to ERT and IMP (base group)	4	0	1	-	0	1	-
Resistant to MEM and either ERT or IMP or both	5	5	9.2	0.1	5	9.2	0.1
Total	21	6	-	-	6	-	-

RR: Risk ratio or relative risk, MEM: Meropenem, ERT: Ertapenem, IMP: Imipenem.

Table-6: Relative association between the resistance of Klebsiella (K.) pneumoniae isolates to antibiotics CTX and CAZ and its ESBL phenotypic and genotypic resistance profiles.

Resistance	Total	Phenotypic resistance	RR	*p- value*	Genotypic resistance	RR	*p- value*
Susceptible (base group)	3	0	1	-	0	1	-
Resistant to CTX and CAZ	18	5	2.3	0.7	6	2.7	0.6
Total	21	5	-	-	6	-	-

RR: Risk ratio or relative risk, CTX, Cefotaxime, CAZ: Ceftazidime, ESBL: Extended-spectrum beta lactamase.

Table-7: Relative association between Escherichia (E.) coli to antibiotics MEM, ERT and IMP and its carbapenemase phenotypic and genotypic resistance profiles.

Resistance	Total	Phenotypic resistance	RR	*p- value*	Genotypic resistance	RR	*p- value*
Susceptible (base group)	4	0	1	-	0	1	-
Resistant to IMP and either MEM or IMP or both	3	3	8.9	0.1	2	8.3	0.17
Total	7	3	-	-	2	-	-

RR: Risk ratio or relative risk, MEM: Meropenem, ERT: Ertapenem, IMP: Imipenem

Table-8: Relative association between the resistance of Escherichia (E.) coli to antibiotics CTX and CAZ and its ESBL phenotypic and genotypic resistance profiles.

Resistance	Total	Phenotypic resistance	RR	*p- value*	Genotypic resistance	RR	*p- value*
Susceptible (base group)	1	0	1	-	0	1	-
Resistant to CTX and CAZ	6	2	1.4	0.8	2	1.4	0.8
Total	6	2	-	-	2	-	-

RR: Risk ratio or relative risk, CTX, Cefotaxime, CAZ: Ceftazidime, ESBL: Extended-spectrum beta lactamase.

Table-9: Distribution of the virulence genes among Klebsiella (K.) pneumoniae isolates.

Sample ID	Case number	mrkA(fimbriae)	fimH(fimbriae)	magA(capsule)	Uge(capsule)
Sample ID	Case number	mrkA(fimbriae)	fimH(fimbriae)	magA(capsule)	Uge(capsule)
1	57	+ve	-	-	+ve
2	38	+ve	+ve	-	+ve
3	40	-	+ve	-	+ve
4	50	+ve	+ve	-	+ve
5	2	+ve	+ve	-	+ve
6	55	+ve	+ve	-	+ve
7	22	+ve	+ve	-	+ve
8	46	-	-	-	+ve
9	30	-	-	-	-
10	36	+ve	+ve	-	+ve
11	61	-	+ve	-	-
12	56	-	+ve	-	+ve
13	9	+ve	+ve	-	+ve
14	71	-	+ve	-	
15	7	-	-	-	+ve
16	48	-	+ve	-	-
17	86	-	-	-	+ve
18	43	+ve	+ve	-	+ve
19	79		-	-	+ve
20	47	+ve	-	-	+ve
21	37	-	+ve	-	-

RR: Risk ratio or relative risk, CTX, Cefotaxime, CAZ: Ceftazidime, ESBL: Extended-spectrum beta lactamase.

Table-10: Association between resistant and virulent genes of Klebsiella (K.) pneumoniae isolates.

Variable	Total	*p- value*	OR	95% CI
mrkA (fimbriae)	Yes			
	No	0.17-	3.2-	0.61 – 17.19-
fimH (fimbriae)	Yes			
	No	0.61-	1.6-	0.28 – 8.90-
Uge (capsule)	Yes			
	No	0.29-	2.7-	0.43 – 16.99

Uge: Uridine diphosphate-glucose 4-epimerase gene, FimH: Fimbrial adhesion gene, mrkA: mannose-resistance adhesin of Klebsiella spp gene OR: Odds ratio, CI: Confidence interval.

Figure 1: The map of Egypt (left) and that of Kafrelsheikh Governorate (right) showing the distribution of different bacterial isolates across urban and rural areas.

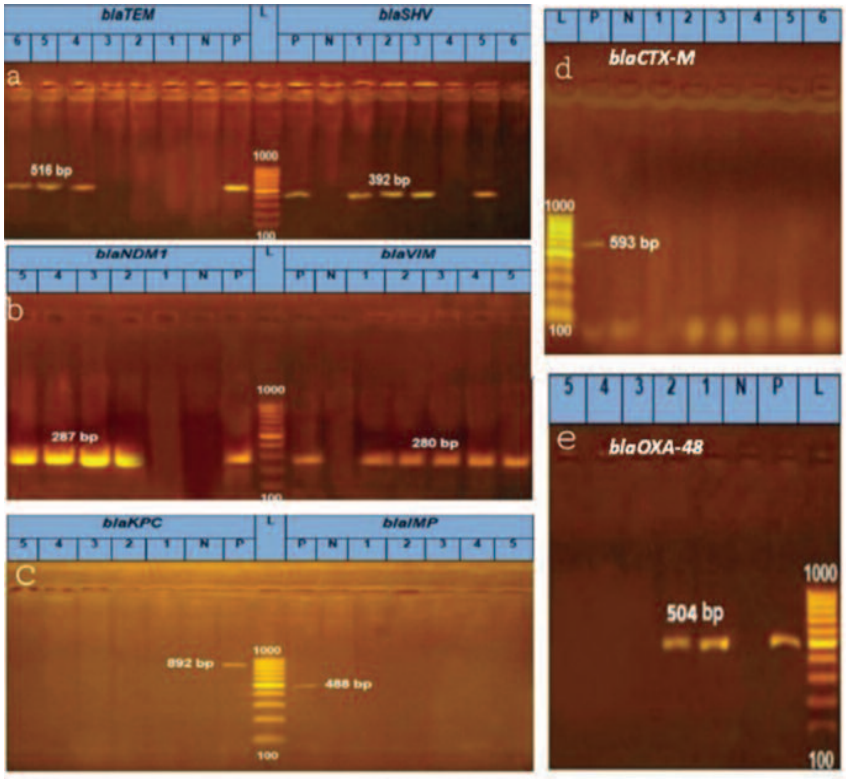

Figure 2: Representative electrophoresis of metallo B-lactamases MBL and extended-spectrum beta lactamases (ESBL) associated with B lactam resistance in Klebsiella (K.) pneumonia isolated from urinary tract infections (UTIs). (a) blaTEM, blaSHV, (b) blaNDM1, blaVIM (c) blaKPC, blaIMP (d) blaCTX-M and (e) blaOXA-48. Lane L is a 100bp deoxyribonucleic acid (DNA) ladder.
IMP: Imipenem-resistant Pseudomonas-type carbapenemases gene, VIM: Verona integron-encoded metallo beta gene, NDM-1: New Delhi metallo beta lactamase-1 gene, KPC: Klebsiella pneumoniae carbapenemases gene, OXA-48: Oxacillinase-48 gene, TEM: temoneira gene, SHV: sulfhydryl variable gene, CTX-M: Cefotaximase gene.

The resistance of *K. pneumoniae* isolates to MEM was associated 9.2 times other than any antibiotic or susceptible isolates with positive results for carbapenemases phenotypic and genotypic resistance (Table 5). The *K. pneumoniae* isolates resistant to CTX and CAZ were 2.3 and 2.7 times, respectively, associated with the presence of ESBLs phenotypic and genotypic resistance profiles than the susceptible isolates (Table 6). For *E. coli*, isolates resistant to IMP were 8.9 times associated with the susceptible isolates with the presence of carbapenemases phenotypic and genotypic resistance ((Table 7; Figure 3). On the other hand, *E. coli* isolates resistant to CTX and CAZ were 1.4 times associated with the presence of ESBLs phenotypic and genotypic resistance profiles than susceptible isolates (Table 8; Figure 3).

Prevalence of virulence genes among *K. pneumoniae* isolates, mrkA, fimH and uge genes were investigated in *K. pneumoniae* isolates, with 10(47.6%), 14(66.7%) and 16(76.2%), respectively, being positive. Conversely, all 21(100%) isolates of *K. pneumoniae* were negative for the magA gene (Table 9; Figure 4). The association between the resistance and any of the virulence genes in *K. pneumoniae* isolates was not significant, but it showed a tendency for an increase in resistance with the presence of virulence genes (Table 10).

Discussion

To the best of our knowledge, the current study is the first evidence of the coexistence of blaOXA-48, blaVIM and blaSHV genes in *K. pneumoniae* and *E. coli* isolated from mixed UTI in Egypt. The study indicated that *K. pneumoniae* was the most frequent pathogen isolated from UTIs, which was consistent with earlier findings.[62] In addition, UTI prevalence was significantly higher in patients aged >50 years than the younger groups. A similar trend was observed by Medina et al.[63] in Italy.

Rural-urban differences in using appropriate antibiotic durations may be related to patient and provider-level factors, such as distance to healthcare facility.[64] In the current study, rural patients were more likely to experience longer treatment durations, which is in agreement with literature.[65, 66]

This study found that most of the strains were MDR as they were resistant to most antimicrobial agents tested.[67] The

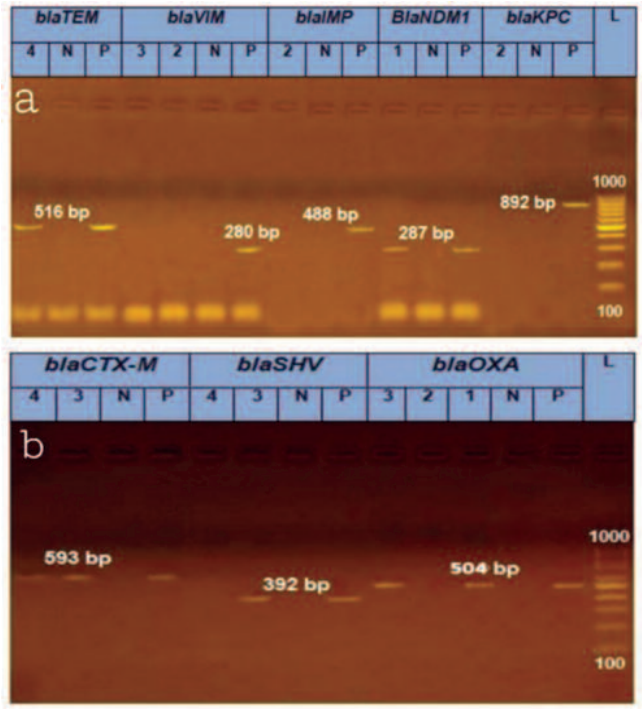

Figure 3: Representative polymerase chain reaction (PCR) amplification of metallo B-lactamase (MBL), and extended-spectrum beta lactamases (ESBL) related genes from different Escherichia (E.) coli isolated from urinary tract infections (UTIs). Lane L is a 100bp deoxyribonucleic acid (DNA) ladder. (a) MBL-related genes (blaIMP, blaVIM, blaNDM, blaKPC and blaOXA-48) (b) extended-spectrum beta lactamases (ESBL)-related genes (blaTEM, blaSHV and blaCTX-M). N = negative control; P: Positive control.
IMP: Imipenem-resistant Pseudomonas-type carbapenemases gene, VIM: Verona integron-encoded metallo beta gene, NDM-1: New Delhi metallo beta lactamase-1 gene, KPC: Klebsiella pneumoniae carbapenemases, OXA-48: Oxacillinase-48 gene, TEM: temoneira gene, SHV: sulfhydryl variable gene, CTX-M: Cefotaximase gene.

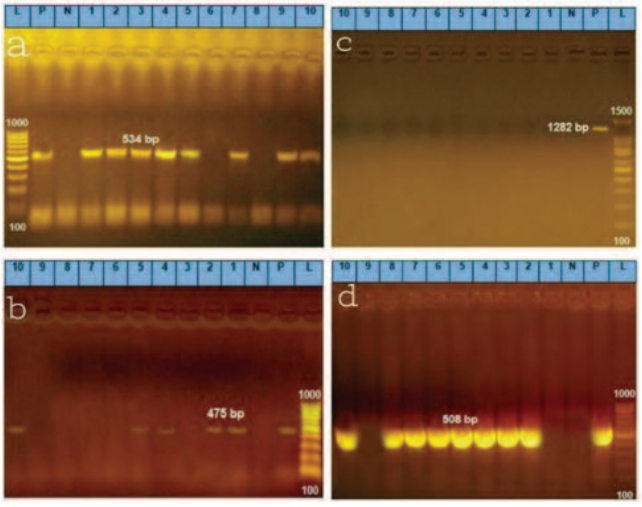

Figure 4: RRepresentative polymerase chain reaction (PCR) for pneumonia virulence-associated genes. PCR amplification of (a) uge, (b) mrkA (c) magA (d) fimH. Lane L is a 100bp deoxyribonucleic acid (DNA) ladder. P: Positive control. N: Negative control.
Uge: Uridine diphosphate-glucose 4-epimerase gene, FimH: Fimbrial adhesion gene, magA: mucoviscosity-associated gene A, mrkA: mannose-resistance adhesin of Klebsiella spp gene

frequency of antibiotic use was more for AM, AMX and AMC, which are the most commonly prescribed in hospitals even before urine analysis results arrive.

A decline in CIP activity against *E. cloacae, E. coli* and K. *pneumoniae* strains would be especially problematic given the ability of gram-negative bacilli to gain resistance to all other classes of antimicrobials. These findings agree with those reported earlier.[15, 68]

FT had low resistance rates for *K. pneumoniae, E. coli* and *E. cloacae* and this was supported by a 2017 study.[69]

K. pneumoniae showed a resistance rate of 52.7% against NOR, which was consistent with a study done in Italy.[26] There was a decline in STX activity, with resistance ranging from 76.8% and 85.7% to 100% for *K. pneumoniae, E. coli* and *E. cloacae*, respectively. This was in line with literature.[15]

Compared to carbapenem sensitivity of >90 reported from Mexico[38], the current study showed a reduction in sensitivities ranging from 33.3% to 76.2%.

In this study, aminoglycosides, including GM and AN, showed high efficacy against tested UTI pathogens *E. coli* and *E. cloacae*. The *K. pneumoniae* isolates also showed a low resistance rate against GM and AN. This may be due to the low and irregular treatment regimens for recorded aminoglycosides.[39]

This study found that the most carbapenemase-related genes detected in *K. pneumoniae* tested isolates were blaVIM and blaNDM, followed by blaOXA-48. No blaNDM-1 gene was detected in *E. coli* strains. The blaIMP and blaKPC genes were not found in any of the bacterial isolates. This was in kine with a study in Tehran.[70]

In this study, two mixed or co-infected samples containing *K. pneumoniae* and *E. coli* strains carried the blaSHV gene. In addition, the two bacterial strains carried the blaVIM and blaOXA-48 genes in the other mixed or co-infected sample. This may show gene transfer among different bacterial UTI strains.[71]

One *K. pneumoniae* isolate simultaneously carried one ESBL gene with one carbapenemase gene. The coexistence of two ESBL-related genes and one carbapenemase-related gene was observed in one *E. coli* isolate of UTI.

Overall, uge was the most commonly detected putative virulence gene, followed by fimH gene. This result agrees with Asani A et al.[72] Also, this analysis found that 11 isolates harboured one virulence gene at least with one of the carbapenemase-related genes and/or ESBL-related genes. Four isolates harboured genes with at least one ESBL-related gene. Three isolates harboured genes with at least

two carbapenemase-related genes. This may indicate the relationship between virulence and drug resistance. Similar trends were reported from Iraq and India.[31, 73]

Conclusions

There was evidence of the coexistence of blaOXA-48, blaVIM and blaSHV genes in *Klebsiella pneumoniae* and *Escherichia coli* isolates from mixed UTI samples. Data on antibiotic resistance and virulence mechanisms could be further developed and used in a national antibiotic sales and antimicrobial resistance monitoring programme.

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Conflicts of Interest: None.

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