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Phenotypic and Genotypic Characterization of Multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* Isolated from Women with Urinary Tract Infections in Mosul City

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Abstract

This study was established to discover and determine multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* from women suffering urinary tract infections, specifically in Mosul city. A total of 62 *E. coli* and 32 *K. pneumoniae* bacterial isolates were considered for this study. All isolates were characterized using standard bacterial culture methods, including culture on MacConkey agar, Eosin Methylene Blue agar and biochemical tests. Also antibiotic sensitivity test using standard disc method for different antibiotics and also special discs to detect ESBL activity were carried out, in addition to PCR as molecular identification tool. The results showed that most isolated *E. coli* and *K. pneumoniae* demonstrated MDR resistance pattern with highest resistance recorded for *E. coli* to tetracycline 62/62 (100%), trimethoprim/sulfamethoxazole 60/62 (96.8%), gentamycin 51/62 (82.3%) and azithromycin 50/62 (80.7%). While *K. pneumoniae* recorded high resistance to nalidixic acid 32/32 (100%) and tetracycline 26/32 (81.2%). On the other hand, imipenem was the only one that showed ultimate sensitivity for all *E. coli* and *K. pneumoniae* isolates. Furthermore, the current results revealed that 68/94 (72.3%) of the studied isolates have ESBL activity. The results for molecular studies confirmed that *E. coli* and *K. pneumoniae* have resistance genes with dominated CTX-M gene, followed by SHV and finally TEM gene. The study concluded that *E. coli* and *K. pneumoniae* with MDR feature are serious threat to women with UTIs and all necessary measures ought to be performed in order to reduce the antibiotic resistance.

Keywords: *Escherichia coli*, *Klebsiella pneumoniae*, MDR, UTI, ESBL

التوصيف المظهري والجيني للاشريكيا القولونية والكليبسيلا الرئوية ذات المقاومة المتعددة للأدوية والمعزولة من النساء اللاتي تعانين من التهابات الجهاز البولي في مدينة الموصل

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الخلاصة

أُنشئت هذه الدراسة لعزل وتشخيص الاشريكيا القولونية والكليبسيلا الرئوية ذات المقاومة المتعددة للأدوية والمعزولة من النساء اللاتي تعانين من التهابات الجهاز البولي، وخصوصاً في مدينة الموصل. تضمنت الدراسة 62 عزلة من الاشريكيا القولونية و 32 عزلة اخرى من الكليبسيلا الرئوية، وقدتم عزل هذه الجراثيم من النساء

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اللاتي تعانين من التهابات الجهاز البولي. تم توصيف كافة العزلات بإستخدام طرق العزل الجرثومي القياسية والمتضمنة العزل على اكار الماكونكي واكار الايوسين والمثيلين الازرق والاختبارات الكيموحيوية. كما تم اجراء فحص الحساسية للمقاومة للمضادات الحيوية بطريقة الاقراص بالاضافة الى استخدام الاقراص الخاصة للكشف عن فعالية ESBL، فضلا عن اجراء فحص تفاعل البلمرة المتسلسل كطريقة تشخيصيةعلى المستوى الجزيئي. اظهرت النتائج ان اغلب عزلات الاشريكيا القولونية والكليبسيلا الرئوية قد اظهرت صفة المقاومة المتعددة للادوية، حيث سجلت الاشركيا القولونية مقاومة مطلقة للتتراسايكلين 62/62 (100%) ، الترايميثبريم/سلفاميثاكسازول 62/60 (96.8%)، الجنتامايسين 62/51 (82.3%) والازيثرومايسين 62/50 (80.7%). في حين سجلت الكليبسيلا الرئوية مقاومة مطلقة للناليديكسك اسد 32/32 (100%) والتتراسايكلين 32/26 (81.2%). من جانب اخر اظهر الامبنيم وحده فقط حساسية مطلقة لجميع عزلات الاشيريكيا القولونية والكليبسيلا الرئوية. فضلا عن ذلك اظهرت النتائج ان 94/68 (72.3%) من العزلات الخاضعة للدراسة تمتلك فعالية ESBL. واظهرت نتائج الاختبارات الجزيئية ان كل من الاشريكيا القولونية والكليبسيلا الرئوية تمتلك جينات مقاومة للمضادات الحيوية وكان الجين CTX−M هو السائد، يليه SHV واخيرا الجين TEM. استنتجت الدراسة أن الاشريكيا القولونية والكليبسيلا الرئوية التي تمتلك صفة المقاومة المتعددة للأدوية تعتبر خطر كبير على النساء اللاتي تعانين من التهابات الجهاز البولي وانه يجب الاخذ بجميع الاحتياطات الضرورية لتقليل المقاومة للمضادات الحيوية.

1. Introduction

Urinary tract infections (UTI) are a frequent in humans, especially in women [1], and represent about 25% of heath care infections that are mainly unresponsive to treatment with common antibiotics due development of antibiotic resistance [2]. Antibiotic resistance has increased the impact of UTIs treatment, especially ones with multi-drug resistance (MDR) features as a function of increase in the cost and length of treatments, in addition to the burden of the side effects of using several types of antibiotics [2, 3]. Enterobacteriaceae is a principal pathogen of UTI. However, *Escherichia coli* and *Klebsiella pneumoniae* are the main members of Enterobacteriaceae that have been frequently isolated from women suffering urinary tract infections (UTI) [3, 4]. On the other hand, spreading of resistant bacteria has become a global problem nowadays, especially those belonging to Enterobacteriaceae with multidrug-resistance (MDR) properties mainly against aminoglycosides, macrolides, quinolones, and sulphonamides classes [5, 6]. On the other hand, due to their bactericidal properties with low toxicity and their broad spectrum activity, β-Lactams antibiotics are commonly used. Though, it has been shown that the synthesis of β- lactamases by Enterobacteriaceae is the most common form of resistance towards this group of antibiotics [7]. Nevertheless, the emergence of new strains with extended-spectrum ß-lactamase (ESBL) resistance character in addition to MDR add further burden for the treatment of such MDR bacteria [8, 9]. The ESBLs bacteria are able to hydrolyse the antibiotics containing beta lactam such as penicillins and 1st, 2nd, 3rd and 4th generations cephalosporins [1, 10]. The genetic background of ESBL resistance mainly belongs to the presence of CTX-M gene and to a lesser extent SHV and TEM genes [11]. These genes are mediated by plasmids and transmitted to other bacterial species by horizontal transfer [12]. Recent studies on UTI infections have reported an increase in the prevalence of ESBL producer *E. coli* and *K. pneumoniae* with MDR type resistance feature [4, 13]. This combination significantly affects the course of UTI and has left few choices for the physicians to treat such serious infections [8, 9]. Women are generally more vulnerable to UTI infections than men which increases the burden of antibiotic resistance [1, 14]. In Iraq, the antibiotics can be obtained from pharmacies directly by patients without physician advice or standard prescription which leads to misuse of antibiotic doses and treatment period which has further complicated the problem of antibiotic resistance. Few local studies have reported the type and molecular pattern of resistance for bacteria that are responsible for UTIs in women [4, 13, 15]. However,

in Mosul city such information surely contributes the understanding of the genetic background of bacterial resistance. According to the presented facts, the objective of the current work was to isolate and molecularly characterize *E. coli* and *K. pneumoniae* that show multidrug resistance from women suffering UTI.

2. Materials and Methods

2.1 Bacterial Isolates

A total of 62 *E. coli* and 32 *K. pneumoniae* isolates were obtained from Microbiology laboratory, Al-Salam Hospital in Mosul city. These bacteria were isolated from women suffering UTI with ages between 25-60 years who had attended the hospital during the period December 2020 to May 2021. All isolates were further subjected to phenotypic characterization using standard bacteriological methods including culture on MacConkey, Eosin Methylene Blue agars, and biochemical tests to confirm the species of these bacteria [16]. Additionally, bacterial glycerol stocks were made of these isolates and kept at -20°C for further use in determining antibiotic resistance and molecular identification.

2.2 Antibiotic Sensitivity Test (AST)

In this study, AST was conducted according to Kirby-Bauer disc diffusion method as previously described by Clinical and Laboratory Standards Institute (CLSI) [17]. Types of antibiotic discs (Bioanalyse/ Turkey) used are listed in Table 1. Briefly, Mueller-Hinton agar plates (Neogen, USA) were inoculated with 0.5 McFarland bacterial suspension for each isolate. Later, the discs were distributed on the plates and incubated at 37°C for 24 h. The zone of inhibition was measured and the final results were recorded according to CLSI [17].

Table 1: Types of antibiotic discs used for AST

No.	Type of Antibiotic	Disc Conc. µg
1.	Imipenem (IP)	10
2.	Tobramycin (TOB)	10
3.	Gentamycin (CN)	10
4.	Streptomycin (S)	25
5.	Ciprofloxacin (CIP)	10
6.	Levofloxacin (LEV)	5
7.	Nalidixic acid (NA)	30
8.	Tetracycline (TE)	10
9.	Nitrofurantoin (F)	100
10.	Trimethoprim/ sulfamethoxazole (SXT)	25
11.	Chloramphenicol (C)	10
12.	Azithromycin (AZM)	15

2.3 Detection of ESBL Producing *E. coli* and *K. pneumoniae*

All isolates were primary screened for ESBL activity by culture on selective MacConkey agar (SMC^+) with cefotaxime (Primocef 500 mg, Julphar, UAE) according to the procedure described by Jacob *et al.* [18]. Positive suspected colonies were selected for further phenotypic confirmation to detect the ESBL using specific discs D68C MASTDISCS® set (Mast Group, Germany). According to manufacturer instruction, these discs are formulated of four types of discs (A, B, C, and D). The screening agent on disc A is 10 µg of cefpodoxime, the ESBL inhibitor on disc B is 10 µg of cefpodoxime and clavulanate, the AmpC inhibitor on disc C is 10 µg of cefpodoxime and cloxacillin, and disc D has 10 µg of cefpodoxime in combination

with both clavulanate and cloxacillin. After that, Kirby-Bauer disc diffusion method was conducted as an antibiotic sensitivity test, as previously described [17]. The zone of inhibition for each type was estimated according to the instructions of the manufacture.

2.4 Molecular Characterization

Extraction of DNA

Positive isolates of *E. coli* 43/62 and *K. pneumoniae* 25/32 that confirmed their ability to produce ESBL by phenotypic methods, were selected for molecular characterization. DNA extraction was done by using AddPrep Bacterial Genomic DNA Extraction Kit (AddBio, Korea) following the instructions of manufacture. The extracted DNA was stored at -20°C for further use.

Polymerase Chain Reaction (PCR)

Before phenotypic studies, *E. coli* and *K. pneumoniae* isolates were confirmed using specific primers (*E. coli*: EC-F and EC-R) and (*K. pneumoniae*: KP-F and KP-R) respectively. In addition, CTX-M, SHV and TEM genes were detected using specific primers (Table 2). The primers were obtained from Macrogen Co, Korea. The PCR reaction mixture was prepared using HS Prime Taq Premix (2X) (AddBio, Korea). Briefly, the mixture was set at a total volume of 20 µl in 0.2 ml PCR tube containing final concentration of 1X HS Prime Taq Premix, 1 µM of each F and R primers, 2 µl of extracted DNA (at 2 ng/µl) and finally 6 µl of the PCR water. The PCR was carried out using a thermal cycler (T100 BioRad, USA). Following a single cycle of initial denaturation at 95°C for 10 min, there were 35 cycles with denaturation at that temperature, primer annealing at (EC=55ºC, KP=60ºC, CTX-M=54ºC, SHV=57ºC and TEM=45ºC) and extension at 72ºC. Then 1 cycle of final extension at 72°C for 5 min before finally cooling at 4ºC. The obtained PCR products were separated by using 1.2% agarose gel (Bio-Rad, USA). The conditions of the electrophoresis were set at 80 V, 350 mA for 60 min by use of gel electrophoresis system (Bio-Rad, USA) loaded with 1X TBE buffer (Bio-Rad, USA).

Table 2: Primers are used in PCR.

No.	Primer	Sequence 5' – 3'	Tm °C	Amplified size (bp)	Ref.
1	EC-F	ATCAACCGAGATTCCCCCAGT	55	232	[19]
2	EC-R	TCACTATCGGTCAGTCAGGAG			
3	KP-F	GGAACTGAGACACGGTCCAG	60	700	[20]
4	KP-R	CCAGGTAAGGTTCTTCGCGT			
5	CTX-M-F	CGCTTTGCGATGTGCAG	54	550	[21]
6	CTX-M-R	ACCGCGATATCGTTGGT			
7	SHV-F	ATGCGTTATATTCGCCTGTG	57	753	[15]
8	SHV-R	TGCTTTGTTATTCGGGCCAA			
9	TEM-F	AAACGCTGGTGAAAGTA	45	822	[15]
10	TEM-R	AGCGATCTGTCTAT			

2.5 Statistical Analysis

Chi-square was used in order to compute *P* values at level *P*>0.05 (MedCalc V.19.4).

3. Results

The majority of the *E. coli* and *K. pneumoniae* isolates investigated in this study had an MDR resistance pattern to the antibiotics used. However, high resistance was recorded for *E. coli* to tetracycline 62/62 (100%), trimethoprim/sulfamethoxazole 60/62 (96.8%), gentamycin 51/62 (82.3%) and azithromycin 50/62 (80.7%). While *K. pneumoniae* recorded high resistance to nalidixic acid 32/32(100%) and tetracycline 26/32 (81.2%). Nevertheless, different resistance patterns were recorded for the rest of the antibiotics for both *E. coli* and *K. pneumoniae*. Imipenem, however, was the only one that showed absolute sensitivity for all *E. coli* and *K. pneumoniae* isolates (Table 3 and 4). Our results showed that MDR was clearly present in most studied isolates and recorded the highest number 8/12 of antibiotic resistance with total 33/94 (35.1%) which represents 21/62 (33.9%) for *E. coli* and 12/32 (37.5%) for *K. pneumoniae* isolates with no significant differences $P>0.05$ (Table 5 and Figure 1). Using specific D68C Mastdiscs, the results showed that 68/94 (72.3%) of the studied isolates had ESBL activity with highest value 25/32 (78.1%) recorded for *K. pneumoniae* and 43/62 (69.4%) for *E. coli* isolates with no significant differences $P>0.05$ between them (Table 6 and Figure 2). The results for molecular studies confirmed *E. coli* and *K. pneumoniae* isolates by using PCR with product size 232 bp and 700 bp, respectively (Figure 3 and 4). Furthermore, PCR screening for resistance genes of the confirmed *E. coli* and *K. pneumoniae* ESBL producer isolates revealed that CTX-M gene with product size 550 bp dominated and recorded 59/68 (86.8%) with significant differences $P\leq 0.05$, followed by SHV gene with product size 753 bp 8/68 (11.8%) and TEM gene with product size 822 bp 4/68 (5.9%) with no significant differences $P>0.05$ for them (Table 7 and Figures 5, 6 and 7).

Table 3: Antimicrobial sensitivity profiles of *E. coli* isolated from UTI, (n=62).

Type of Antibiotic	Resistance n (%)	Intermediate n (%)	Sensitive n (%)
Imipenem (IP)	0	0	62 (100)
Tobramycin (TOB)	21 (33.9)	2 (3.2)	39 (62.9)
Gentamycin (CN)	51 (82.3)	3 (4.8)	8 (12.9)
Streptomycin (S)	17 (27.4)	0	45 (72.6)
Ciprofloxacin (CIP)	27 (43.6)	2 (3.2)	33 (53.2)
Levofloxacin (LEV)	33 (53.2)	4 (6.5)	25 (40.3)
Nalidixic acid (NA)	45 (72.6)	5 (8.1)	12 (19.3)
Tetracycline (TE)	62 (100)	0	0
Nitrofurantoin (F)	42 (67.8)	3 (4.8)	17 (27.4)
Trimethoprim/ sulfamethoxazole (SXT)	60 (96.8)	0	2 (3.2)
Chloramphenicol (C)	38 (61.3)	4 (6.5)	20 (32.2)
Azithromycin (AZM)	50 (80.7)	2 (3.2)	10 (16.1)

Table 4: Antimicrobial sensitivity profiles of *K. pneumoniae* isolated from UTI, (n=32).

Type of Antibiotic	Resistance n (%)	Intermediate n (%)	Sensitive n (%)
Imipenem (IP)	0	0	32 (100)
Tobramycin (TOB)	5 (15.6)	3 (9.4)	24 (75)
Gentamycin (CN)	3 (9.4)	2 (6.2)	27 (84)
Streptomycin (S)	4 (12.5)	0	28 (87.5)
Ciprofloxacin (CIP)	8 (25)	3 (9.4)	21 (65.6)
Levofloxacin (LEV)	9 (28.1)	4 (12.5)	19 (59.4)
Nalidixic acid (NA)	32 (100)	0	0
Tetracycline (TE)	26 (81.2)	3 (9.4)	3 (9.4)
Nitrofurantoin (F)	4 (12.5)	3 (9.4)	25 (78.1)
Trimethoprim/ sulfamethoxazole (SXT)	8 (25)	5 (15.6)	19 (59.4)
Chloramphenicol (C)	7 (21.9)	3 (9.4)	22 (68.7)
Azithromycin (AZM)	12 (37.5)	3 (9.4)	17 (53.1)

Table 5: MDR *E. coli* and *K. pneumoniae* that exhibit resistance to different numbers of antibiotics

Bacteria	No. of Isolates	No. of Antibiotics					
		3/12	5/12	7/12	8/12	9/12	10/12
E. coli	62	0 (0%)	11 (17.7%)	7 (11.3%)	21 (33.9%)	10 (16.1%)	13 (21%)
K. pneumoniae	32	2 (6.3%)	6 (18.7%)	4 (12.5%)	12 (37.5%)	3 (9.4%)	5 (15.6%)
Total	94	2 (2.1%)	17 (18.1%)	11 (11.7%)	33 (35.1%)	13 (13.8%)	18 (19.2%)
***P* value**		0.046	0.900	0.865	0.730	0.375	0.611

P value ≤ 0.05 is statistically significant.

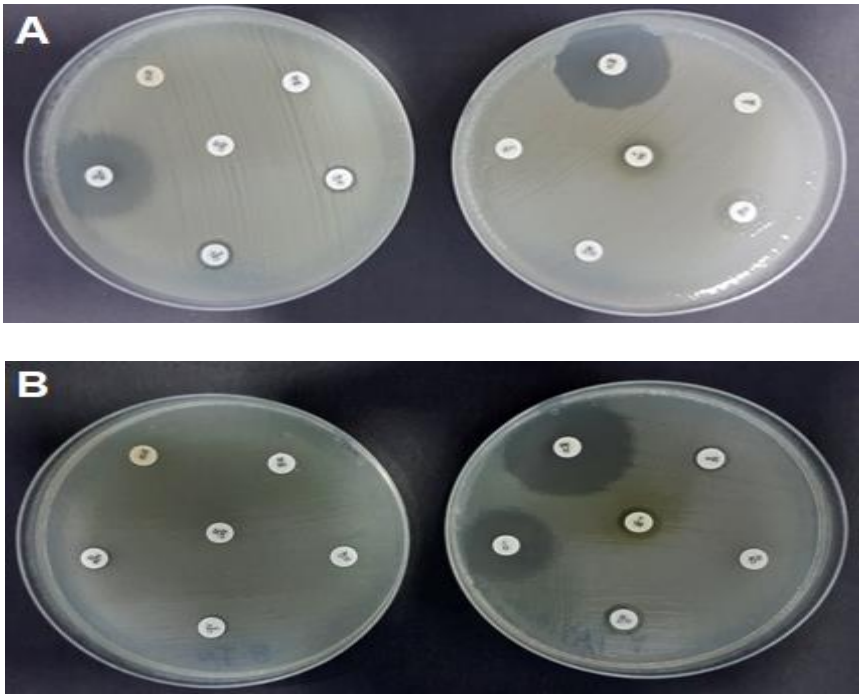

Figure 1: A. *Escherichia coli*; B. *K. pneumoniae* showed MDR with complete resistance to 10 types of antibiotics.

Table 6: Recovery of *E. coli* and *K. pneumoniae* using D68C Mastdiscs

Bacteria	No. of Isolates	No. of ESBL Isolates	Recovery (%)
E. coli	62	43	69.4
K. pneumoniae	32	25	78.1
Total	94	68	72.3
***P* value**			0.374

P value ≤ 0.05 is statistically significant.

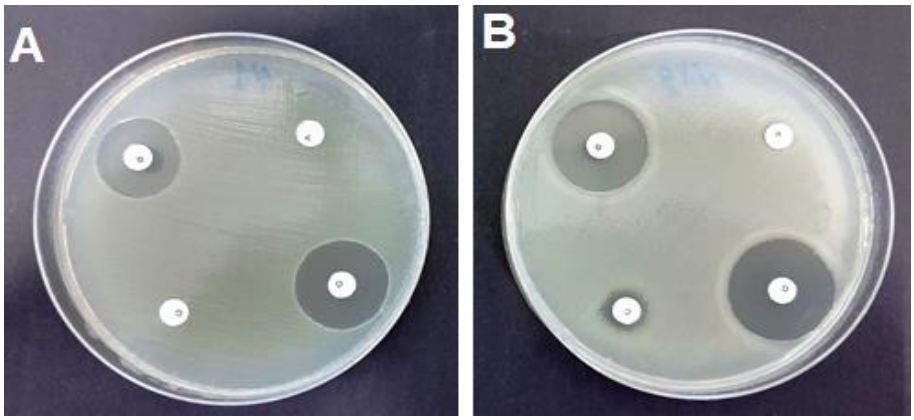

Figure 2: A and B represent positive ESBL *E. coli* and *K. pneumoniae* respectively, using specific ESBL detection discs D68C having zones of resistance (discs A and C), whereas discs B and D represent sensitive zones.

Table 7: PCR screening of CTX-M, SHV, and TEM genes in ESBL *E. coli* and *K. pneumoniae* isolates.

Bacteria	No. of Isolates	CTX-M n (%)	SHV n (%)	TEM n (%)
E. coli	43	37 (86.1)	5 (11.6)	4 (9.3)
K. pneumoniae	25	22 (88)	3 (12)	0
Total	68	59 (86.8)	8 (11.8)	4 (5.9)
***P* value**		0.049	0.960	0.118

P value ≤ 0.05 is statistically significant.

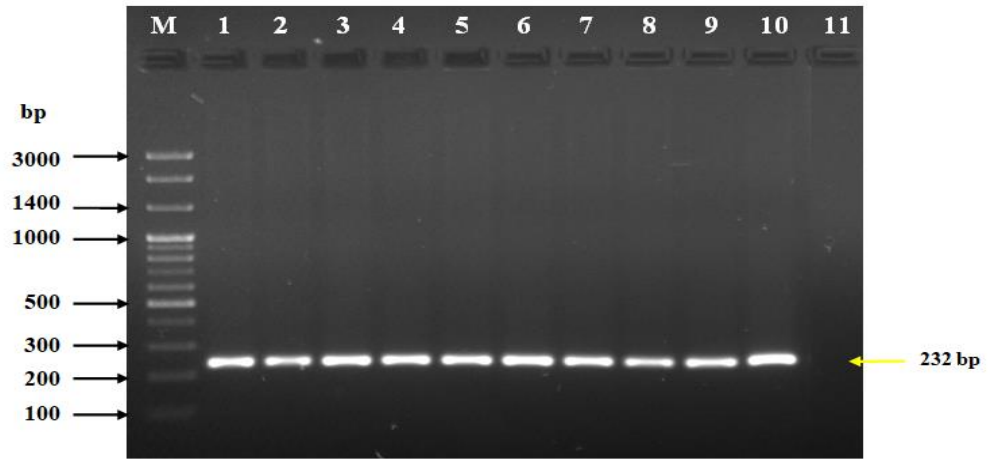

Figure 3: Products of PCR for *E. coli*. Lane M, DNA ladder; lanes 1-10 positive samples; lane 11, control negative

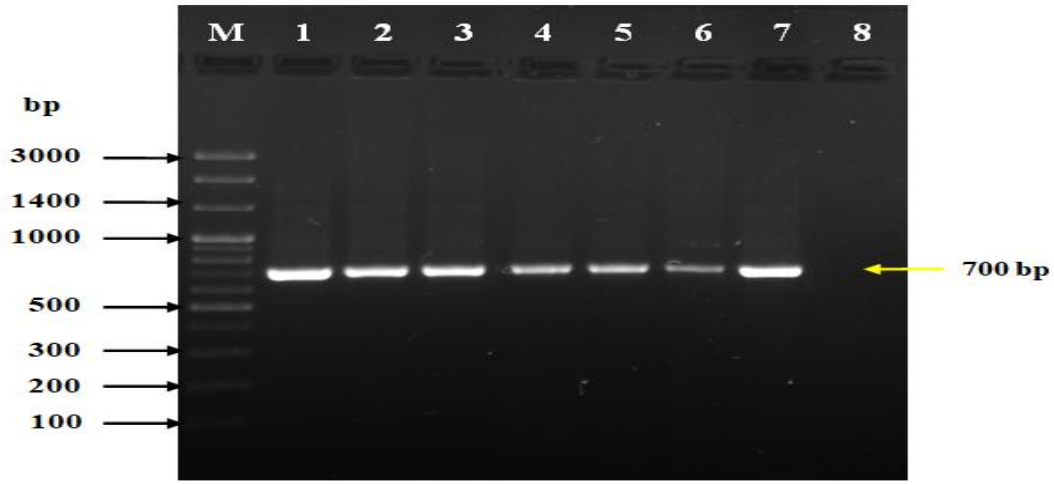

Figure 4: Products of PCR for *K. pneumoniae*. Lane M, DNA ladder; lanes 1-7 positive samples; lane 8, negative control

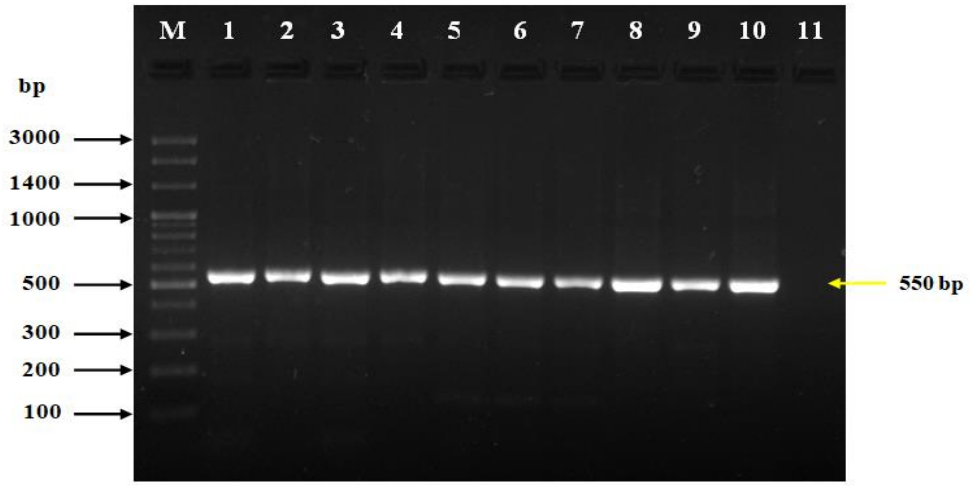

Figure 5: Products of PCR for CTX-M. Lane M, DNA ladder; lanes 1-7 are *E. coli* CTX-M positive samples; lanes 8-10 are *K. pneumoniae* CTX-M positive samples; lane 11, negative control

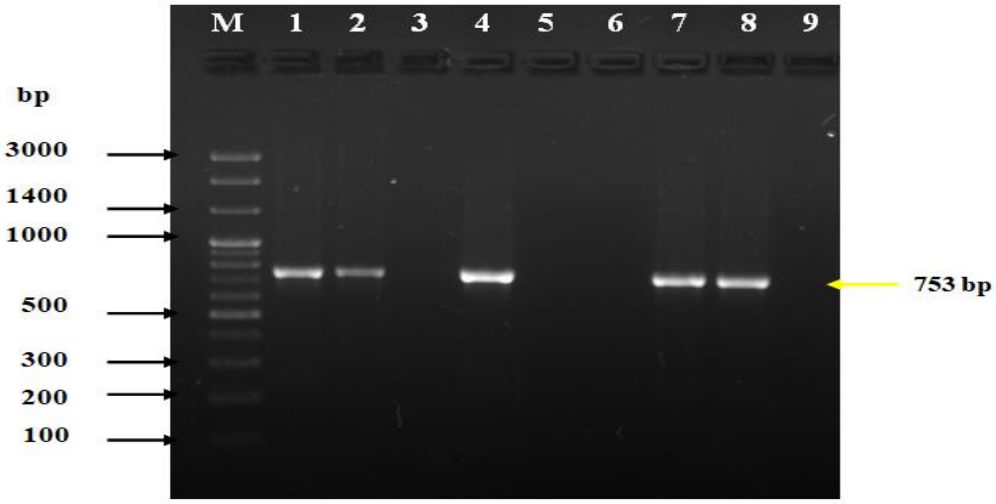

Figure 6: Products of PCR for SHV. Lane M, DNA ladder; lanes 1,2,4 *E. coli* SHV positive samples; lane 7 and 8 *K. pneumoniae* SHV positive sample; lane 9, negative control.

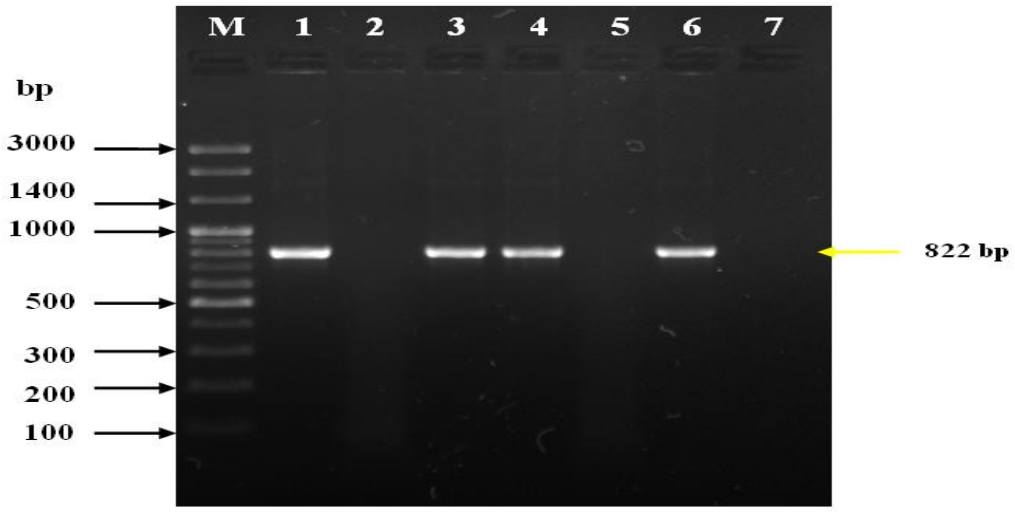

Figure 7: Products of PCR for TEM. Lane M, DNA ladder; lanes 1,3,4 and 6 *E. coli* TEM positive samples; lane 7, negative control.

4. Discussion

Recently, the emergence of MDR *E. coli* and *K. pneumonia* has been reported worldwide as serious pathogens among women with UTI infection in both hospitals and community [5, 8, 22]. Therefore, in this study we only focused on these bacteria as common multidrug-resistant pathogens involved in UTI in women. Our results revealed that MDR was present in total of 33/94 (35.1%) of *E. coli* and *K. pneumoniae* isolates. Other recent studies also showed high rates of MDR resistance among UTI patients due to *E. coli* [9, 22-24] and *K. pneumonia* [3, 25, 26] which indicates the increased concerns about repeated treatment failure of UTIs. The arbitrary administration and misuse of different types of antibiotics has attributed significantly to develop such high rates of bacterial resistance. Another reason might be due to the limited facilities for detection of resistance bacteria in most available diagnostic laboratories in our city. Our results also revealed that tetracycline, trimethoprim/sulfamethoxazole, gentamycin, azithromycin and nalidixic acid had the greatest resistance rate than other types of antibiotics which might be due to the repeated use of such antibiotics or transfer of such resistance from animal sources [27, 28]. Additionally, 68/94 (72.3%) of the tested isolates were ESBL producer using Mastdiscs (D68C) with majority of the isolates belonging to *K. pneumoniae* rather than *E. coli*. This is in contrast with previous studies that usually reported *E. coli* as the main reservoir for ESBL in humans [3, 15, 29, 30]. However, the direct prescription of antibiotics without performing antibiotic sensitivity test also has its direct impact for increasing such type of resistance for most involved cases of UTI in women. And the repeated use of new antibiotic generations for mild UTI cases has left no choice to be taken for the patients, especially in the advance stages where most antibiotics exhibit resistance. This fact indicates that screening ESBL would be a useful tool before establishing any antibiotic treatment. On the other hand, confirmation of ESBL production at the molecular level revealed that CTX-M gene was the dominant type that is responsible for ESBL resistance and recorded 59 (86.8%), followed by SHV 8 (11.8%) and TEM 4 (5.9%) genes. Many studies have also reported domination of CTX-M gene as the main mechanism of resistance [4, 11, 15]. A recent study in Duhok, Iraq by Michael and Saadi [11] has also reported large predominance of CTX-M gene in *E. coli* isolates in patients suffering from recurrent UTI. Although several resistance patterns were recorded in the current study, imipenem was the only drug that showed ultimate sensitivity for all *E. coli* and *K. pneumoniae* isolates [5, 31-33]. Nevertheless, this must not be taken as the only drug that can be used for treatment of UTI unless other choices have been considered to minimize the rapid development of antibiotic resistance for the few last choices lifted.

5. Conclusion

It can be concluded that MDR *E. coli* and *K. pneumoniae* increased significantly among women with UTI with majority of ESBL features which might represent an additional threat for treatment and control of such important infection.

6. Disclosure and Conflict of Interest

The authors declare that they have no conflicts of interest.

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