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PCR-Based Replicon Typing ofplasmids inKlebsiellapneumoniae producing- ESBL-GenesinUTIspatients in Iraq

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Abstract— This is the first research to identify the characterization of "Klebsiellapneumoniae", a multidrug-resistant plasmid circulating in Iraq. The goal of our work was to examine the distribution in clinical K. pneumoniae of the antibiotic resistance genes of ESBLs from UTI patients as well as identification and classification of K. pneumoniae plasmids associated with virulence and antimicrobial resistance by using Replicon Typing based on PCR (PBRT). Medical isolates of this bacteria in this analysis has been isolated from UTI patients. ESBLs-manufacturing K. Pneumoniae was preliminarily tested and validated by various phenotypic approaches using cephalosporin disks. There was also an antimicrobial susceptibility examination. For identification of genotypics, CTX-M, TEM, and SHV ESBL genes were determined by PCR. PBRT was performed by PBRT 2.0 KIT (Diatheva, Italy, 2020), and provided that ESBLs resistance transfer assay was performed by conjugation.The "ESBLs" were observed in (80.53%) of K. pneumoniae. Overall, bla"CTX-M" was the commonest genotype (95.6) by PCR.The replicons could be determined in ESBL-producing K.pneumoniaestrains; 29 replicons were detected in CTX-M producingK. pneumoniaestrains" HI1; HI2; I1α; I2; X1; X2; X3; X4; L; I1y; N; FIA;FIB; FIC; FII; FIIK; FIB- KN; FIB- KQ; W; Y; P1; I1 A/C; T; K; U; R; B/O; HIB-M and FIB-M". The TEM replicons and SHV were detected 28 replicons. New replicons P1, N2 and X4 were detected in 'ESBLs producing K. pneumoniae'in the current investigation and analysis; as well as FIB-KN and FIB-KQ that were not detected in other previous studies.

Introduction

In the Enterobacteriaceae, 'plasmids' are the main resistance and virulence gene vectors; plasmid typing is a necessary parameter for the study of epidemiology, development, and propagation of anti-bacterial resistance (1, 2). Replicon typing based onpcr (PBRT) system; is a "pcrbased method" mainly ratified and established at the "Italian-National-Instituteof Health: in Rome; Italy" by 'Dr. Alessandra Carattoli, Senior Scientist' and subsequently industrialized via(Diatheva). 'PBRT' is a handy technique for detecting and monitoring the interaction of replicons with unique resistant plasmid-borne genes and for detecting the mobilization potential of resistance genes within different bacterial plasmids (3). Identification of bacterial plasmid is very problematic since the presence of several plasmids in the same cell makes it difficult to establish the plasmid content in a bacterial strain; thus, PBRT has recently been adopted as the tool for plasmid identification and typing based on PCR amplification of replicons (1).

Materials and methods

ClinicalK.pneumoniae isolates

This study included UTI patients attending Al-Anbar Hospitals, Midstream Urine- samples were collected from 715 UTIpatients from (January 2019 to October 2019).Clinical isolates of K.pneumoniaewere isolated from UTI patients. They were identified by conventional morphological methods and biochemical analysis, as well as automated systems represented by API 20E and VITEK 2.

Antimicrobial Susceptibility Test:

Antimicrobial susceptibility test of K.pneumoniaewasCalculated by the Kirby Bauer alteration process of disk diffusion on Mueller Hinton agar against Ampicillin, Pipracillin ,Mecillinam, Imipenem, Meropenem, Gentamicin, Amikacin, Tobramycin, Ciprofloxacin, Levofloxacin, Nalidixic acid, Chloramphenicol, Nitrofurantoin, Trimethoprim-Sulfamethoxazole, Tetracyclin,Doxycycline, Minocycline,Tigecycline, Rifampicin, Fosfomycin, Colistin and Polymyxin B (Oxoid, UK), as well as screening method antibiotics (Oxoid, UK), Recommended by the Clinical and Laboratory Quality Institute adopting standard procedures (CLSI), Wayne, USA (4). Escherichia coli ATCC 25922 was used as a control strain.

Screening methods for ESBL Production:
The CLSI has proposed disc diffusion methods for screening of ESBLs production byK. pneumoniae(4).For ESBL production, these approaches may be screened by noting particular zone diameters that suggest a high degree of suspicion for ESBL production. ceftriaxone, Ceftazidim, cefotaxime, cefotaxime, cefpodoxime , cefepime, and aztreonam were used.

Confirmatory Tests for ESBL Production(Phenotypic methods)
K.pneumoniae isolates resistant to at least one of the Cephalosporin markers were tested for the development of ESBLs (5); Broth microdilution, Combination Disc Test (CDT), Double Disc Synergy Test (DDST), ESBL E. Strip test (E test)," Inhibitor- Potentiated- Disk Diffusion Test (IPDD)", Agar Culture (Chrom ID ESBL and Brilliance ESBL) and NDP Test ESBL. EUCAST (6). K. Pneumonia ATCC: 700603 and Escherichia coli ATCC: 25922 have been used as 'positive and negative-controls' ESBL tests for all phenotypic methods, respectively.

Molecular-Detection of ESBL- Genes
The whole phenotypical ESBL K. Pneumoniae isolates have been subjected to molecular analysis to validate the development of ESBL. Detection of K. Pneumoniaeby Conventional polymerase chain reaction was conducted by harboring ESBL genes (bla-CTX-M, bla-TEM, and bla-SHV) (PCR). According to the Promega package, complete bacterial genomic DNA was insulated (USA).

Polymerase Chain Reaction
For the identification of(CTX-M, TEM and SHV) ESBLs whichencoding:Ambler"class A", from K.pneumoniae; A regular PCR was performed , with complete bacterial genomic DNA as a template.Forward sequences:- 5'-CGC CGG GTT ATT CTT ATT TGT CGC-3 'and Reverse sequences:- 5'-TCT TTC CGA TGC CGC CGC CAG TCA-3' were used to amplify ESBL genes encoding SHV expanded range β-lactamases (bla SHV) primers (7). For these primers,theconditions of thermal- cycling that used for the PCR in this process; involved 30 cycles at 94 °C for 30 S, 68 °C for 60 S, and 72 for 60 S, respectively.For 7min with a final extension of 72 °C. The whole ORF was found in the (1016) bp PCR product. ESBLs (bla TEM), primers (TEM-F) 5'- ATA AAA TTC TTG AAG ACG AAA-3 'and (TEM-R) 5'-GAC AGT TAC CAA TGC TTA ATC A-3' were used for gene encoding amplification (TEM); with a thermal cycling conditions as defined by the thermal cycling conditions described by (8). From 214 bp upstream of the original codon, the (1080) bpamplicon entered the stop codon. A PCR with primers (CTX-M) F: TTTGCGATGTGCAGTACCAGTAA and R: CGA TAT CGT TGGTGGTGCCATA and conditions identified by the bla CTX-M genes was performed to detect (bla CTX-M) genes (9); The amplicon is an intragenic fragment of (544) bp . In a (1%) W/V agarose gel, 10 µl of pcr product was electrophoresed for 1 h. Together with a smart ladder DNA, at 140 V.

PCR-Based RepliconTypingTechnique
A PBRT package was used to classify the resistance plasmids of the K.pneumoniae isolates by PBRT-Kit :Diatheva; Italy, 2020. The complete bacterial DNA was prepared in compliance with the manufacturer's protocol of the "PBRT 2.0 kit" by the Promega kit (USA) and also used as a PCR template. (30) Replicons "Incompatibility groups" were amplified by eight multiplex PCR assays: (HI-1, HI-2, I-1 alpha, I-2, X-1, X-2, X-3, X-4, L, I1y,N, FI-A, FI-B, FI-C, FII, FII-S, FII-K, FIB KN, FIB KQ, W, Y, P1, A/C, T, K, U, R, B/O, HIBM and FIBM) in PBRT- kit.,The primary plasmid that is indicative ofIncompatibility groups and replicase genes for resistance plasmids circulating amongst Enterobacteriaceae (1) are included in this package, as well as positive controls for all the respective replicons.

Transfer ofESBLs resistance assays (Conjugation)
The plasmid that confers resistance to "third-generation cephalosporin" in all ESBL-producing K. Pneumoniae strains were transferred by mating with plasmid-free:E.coliwhich is resistant to rifampicin. The E. coli strain was used as an acceptor (K12 J53R Lac positive) (10). The Luria Bertani (LB) boron crop was mixed with the respective donor strain at 1:2 and the mixture was "incubated at 37°C for 18 h".A mixture was spread (100) µl on rifampicin-containing 250 mg/l and the Ceftazidime 2 mg/l (CAZ) rifampicin-containing MacConkey agar plates for (TEM) or (SHV) donors strains styling overnight (10).For CTX-M:Producing-Donors; they were selected on MacConkey agar- plates containing rifampicin 250 mg/l as well as 2.5 mg/l of Cefotaxime (CTX) (11). The plasmid was transferred after resistance in the Conjugation- experiment, the DNA was extracted using (Promega kit, USA) and the plasmid replicon was determined using the PBRT method(1).

Statistical Analysis
To detect the effect of different factors on study parameters, the Statistical Analysis System- SAS (2012) program was used. The Chi-square test was used in this study to compare the percentage (0.05 and 0.01 probability) significantly.

Discussion of Results
Of a total of (715) urine samples, K.pneumoniae was identified in 113 (36.10 percent) specimens. K. Pneumoniae was the prevalent pathogen isolated from UTI patients (12, 13). The results of a test for antimicrobial susceptibility to K. Pneumoniae have shown that all isolates producing ESBL are resistant to β -lactam Antibiotics, but 100% sensitive to Mecillinam and Carbapenem,because this group of antimicrobial agents are among the mostantimicrobial agents commonly used for treating UTIs- infections caused by K-producing ESBLs (14). Besides, in the present study, ESBLs that produce Kpneumoniae, display co- resistance to many non- β -lactam groups of antibiotics; Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Nalidixic acid, Chloramphenicol, Trimethoprim-Sulfamethoxazole, Tetracycline, Doxycycline and Rifampicin with resistant rates of respectively 47.3%, 56%,67%, 52.7%, 86.8%, 50.5%, 76.9%, 60.4%, 73.6% and 65.9% against K. pneumoniae, resulting in limitation of UTI treatment alternative; whereas susceptibility rates of Amikacin, NitrofurantoinMinocyclin, Tigecycline, Fosfomycin, Colstin and Polymyxin B. in K. pneumoniaewere respectively 96.7%, 86.8%, 100%, 95.6%, 93.4%, 100%, 87.9% ;therefore in the present study, Carbapenems and Mecillinamarethe β-lactam drugs of choice appear to be used for the treatment of UTIs caused by ESBL-producing K. Pneumoniae, taking into account their rational use (15). Under like circumstances, UTI treatment options of non- β -lactam antibiotics are Amikacin, Nitrofurantoin, Minocyclin, Tigecycline, Fosfomycin, Colstin, and Polymyxin

B. Bamford et al. demonstrated a significant decline in sensitivity to beta-lactam antibiotics and Fluoroquinolones, but the sensitivity to Amikacin remained significantly high (16).While Carbapenems represent the first option of beta-lactam when UTI s are caused by ESBLs producers (17).
Our investigation discovered thatK.pneumoniae withESBLswere entirely resistant to Third-generation cephalosporins (Ceftazidime, Cefotaxime, Ceftriaxone, Cefpodoxime), Cefepime and Aztreonam. With the exception of Carbapenems or Cephamycins,K. pneumonia developing ESBLs confers tolerance to all generations of penicillins, cephalosporins and aztreonam; these bacteria effectively pyrolyzeoxyiminocephalosporins, intentional resistance to Monobactams and Cephalosporins of the 3rd generation. However, the development of ESBLs in Enterobacteriaceae is an important mechanism of resistance to β-lactam; particulate K. Pneumoniae, and E.coli(18).

However, the resistance to antibiotics of ESBL-producingK.pneumoniae was significantly higherthan($p < 0.01$)that of ESBL-non-producing isolates.

In the present study, we found 91(82.3%) ESBLs producers' from113K. pneumoniaeisolates based on MIC, ESBLs screening breakpoints, different phenotypic methods and Polymerase Chain Reaction (PCR). There are no phenotypic and molecular data regarding ESBLs detection from UTI patients in our hospitals. However, our results showed high Sensitivities and Specificities of phenotypic methods for detection of ESBLs production inK. pneumoniae.The phenotypic methods: Broth-Micro dilution, IPDD, combined disk method and E. test in the present studyshowed100% sensitivity in K. pneumoniae strains.

The Combined disc test manages to have a higher sensitivity 100% with Seven ESBLs-positive isolates of K.pneumoniaethat were missed by DDS test. Where CD test was shown to be more sensitive than the DDS test as a phenotypic method (19); thus, CD test is better than DDST for detection of ESBLs, as well asIPDDmethod proved to be a better test for verifying the development of ESBLs than DDST, demonstrating 100% sensitivity and specificity. Although the DDST failed to identify 7 ESBL isolates, producerK.pneumoniae, with a sensitivity of 92.3%.Therefore, among all ESBLs phenotypic detection methods, DDStest was the least sensitive one (20).As it is similarly sensitive as the E test, the IPDD test can be favored. The procedure, although less costly, can be used to test more than one cephalosporin as well as a single plate; hence, this test was used as the gold standard in our research to classify ESBLs developing K.pneumoniae as a phenotypic method.In this study,both (Brilliance ESBL and Chrom ID ESBL)agar obtained sensitivity and specificity of (>90%). Brilliance-ESBL agar containsCpd(Cefpodoxime)for inhibition non-ESBL Enterobacteriaceae and for suppressingthe development of most AmpC and other non-ESBL flora species in conjunction with supplementary anti-bacterial agents (20). This new medium"Brilliance- ESBLs- agar" proved useful and showed output as high as that of Chrom ID ESBL agar for the phenotypic identification of K.pneumoniae-producing ESBLs; yielded 96.7 percent high sensitivity with excellent (NPV) at 24 hours; and appeared to be a promising phenotypic method; which could allow for rapid elimination of non-ESBL-producing patients (21).For the rapid identification of ESBLs producers; The ESBL NDP test has been developed in recent years (21). This phenotypic test, based on the detection of Cefotaxime (an extended-spectrum cephalosporin) ring hydrolysis "β-lactam", is fast, sensitive, and precise. Results revealed that this test used directly for contaminated urine samples may be a helpful guide for selecting a high (98.9 percent sensitivity & 100 percent specificity) first-line antibiotic therapy in K. Pneumoniasis. Dortet et al. (2014) stated that there was a sensitivity of 98% and a specificity of 100% when this test was conducted directly on cultured bacteria (22).

The prevalence of ESBL- K. pneumoniae in this study is high. CTX-M was the main genotype (95.6), followed by SHVgenotype (92.3) and TEM genotype (65.9) by PCR. Furthermore, most ESBL-producing K. pneumoniae produced more than one type of ESBLs. The CTX-M type of ESBLs has been recognized to be the cause of outbreaks, as stated elsewhere, as an exceedingly important public health issue worldwide. (18).Howevere, excessive and inaccurate use of Cefotaxime in the treatment of UTI patients maybe lead to a high prevalence of CTX-M genotype. -¬¬

The replicons could be determined in ESBL-producing K. pneumoniae strains by PCR-based Replicon Typing 2.0 kit; 29 replicons were detected in CTX-M producing K. pneumoniaestrains: HI-1, HI-2, I1α, I-2, X-1, X-2,X-3,X-4, L, I1y, N, FI-A, FI-B, FI-C, FII, FII-K, FIB -KN, FIB- KQ, W, Y, P-1,I1 A/C, T, K, U, R, B/O, HIBM and FIBM (Fig.1). The replicons of TEM in ESBL-producing K. pneumoniaestrains were detected (Table-1); 28 replicons: HI-1, HI-2, I1α, I-2, X-1, X-2,X-3,X-4, L, I1y, FI-A, FI-B, FI-C, FII, FII-K, FIB- KN, FIB- KQ, W, Y, P1,I1 A/C, T, K, U, R, B/O, HIBM and FIBM (Fig.3). In the same table (Table-1); 28 replicons were detected in SHV producing K. pneumoniaestrains: HI-1, HI-2, I1α, I-2, X-1, X-2, X-3, X-4, I1y, N, FI-A, FI-B, FI-C, FII, FII-K, FIB- KN, FIB- KQ, W, Y, P1, I1 A/C, T, K, U, R, B/O, HIBM and FIBM (Fig.5).

The only replicon that was not recognized PBRTin K.pneumoniae strains in the present study is FIIS,because this A divergent FII replicon replicated only on Salmonella virulence plasmids is recognized by replicon PCR. In our findings, on the other hand, replications of FII-K, FIB- KN, and FIB- KQ were recognized in strains of K.pneumoniae. FII-K recognizes a divergent FII replicon found on Klebsiellaspp plasmids(23). The new replicons (FIB KN and FIB KQ) were detected in our study are useful to identify and distinguish the two most common IncFIIK plasmids in K. pneumoniae.FIB- KN recognizes the PKPN3 like plasmids; FIB-KQ recognizes the PKPQILlike plasmids; therefore, PBRT 2.0 Kit – PCR based replicon typing (Diatheva, Italy, 2020) in our study was detected new replicons P1, N2, and X4; as well as FIB-KNand FIB-KQ in ESBL-producing K. pneumoniae that not detected in other previous studies.Generally IncF plasmid is one of the most common forms of plasmids associated with the dissemination of determinants of antimicrobial resistance in Enterobacteriaceae (23). In Klebsiellaspp, FIIK is a popular plasmid replicon (24). (FIIK) plasmids with several genes for antibiotic resistance, including CTX-M, have recently been described in K. Pneumoniae(25). PBRT in K. pneumoniae producing CTX-M, TEM, and SHV after conjugation was performed (Table 1); transconjugants couldbe obtained ata ratio100% in K. pneumoniae producing CTX-M (Fig. 2). A mating experiment wassuccessful with Cefotaxime; because this is a good selective agent (10). However, resistance transfer after conjugation was also successful with Ceftazidime as aselective agent at ratio 93.1% in K.pneumoniae producing TEM; that only tworeplicons (R&K) were not transferred and at ratio 96.5% inK. pneumoniae producingSHV that only 1 replicon (P1) were not transferred (Fig. 4& 6).In 100 percent of the tested K.pneumoniae strains, this kit classified replicons, indicating improved sensitivity and specificity of the commercial test (1).

Table (1). Number of Replicons according to :CTX-M, TEM and SHV identified in K.pneumoniae before and after conjugation by PCR-based Replicon typing.

ESBL-genes of *K. pneumoniae*					
CTX-M		**TEM**		**SHV**	
Presences of Replicon types before conjugation	**Replicons transfer after conjugation**	**Presences of Replicon types before conjugation**	**Replicons transfer after conjugation**	**Presences of Replicon types before conjugation**	**Replicons transfer after conjugation**
HI-1	+	**HI-1**	+	**HI-1**	+
HI-2	+	**HI-2**	+	**HI-2**	+
I1-a	+	**I1-a**	+	**I1-a**	+
M	+	**M**	+	**M**	+
N	+	**I2**	+	**N**	+
I-2	+	**B-O**	+	**I2**	+
B\ O	+	**FI-B**	+	**B\O**	+
FI-B	+	**FI-A**	+	**FI-B**	+
FI-A	+	**P-1**	+	**FI-A**	+
B1	+	**W**	+	**P-1**	-
W	+	**L**	+	**W**	+
L	+	**X-3**	+	**X-3**	+
X3	+	**I1-Y**	+	**I1-Y**	+
I1-Y	+	**T**	+	**T**	+
T	+	**A-C**	+	**A\C**	+
A\C	+	**N-2**	+	**N2**	+
N2	+	**U**	+	**U**	+
Y	+	**X-1**	+	**X-1**	+
X1	+	**R**	-	**R**	+
R	+	**FII-K**	+	**FII-K**	+
FII-K	+	**FIB-KN**	+	**FIB-KN**	+
FIB-KN	+	**X2**	+	**X2**	+
X2	+	**FIB-KQ**	+	**FIB-KQ**	+
FIB-KQ	+	**K**	-	**K**	+
K	+	**HIB M**	+	**HIBM**	+
HIBM	+	**FIBM**	+	**FIBM**	+
FIBM	+	**FII**	+	**FII**	+
FII	+	**X-4**	+	**X-4**	+
X-4	+				

29Replicon	29Replicon	28Replicon	26Replicon	28Replicon	27Replicon

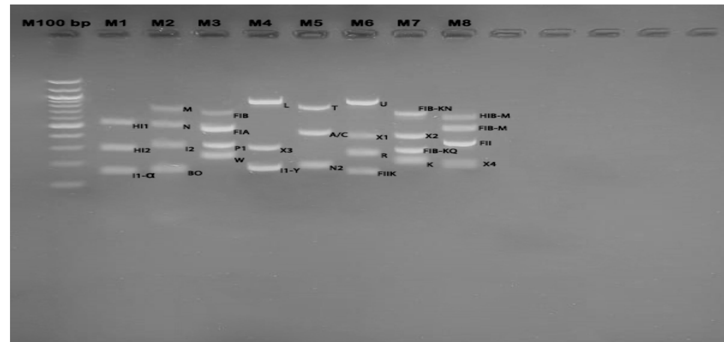

Fig.1.Agarose gel electrophoresis analysis of multiple Amplicons of CTX-M ofK. pneumoniaeobtained via amplification of positive controls using all PCR mixes M1 to M8.

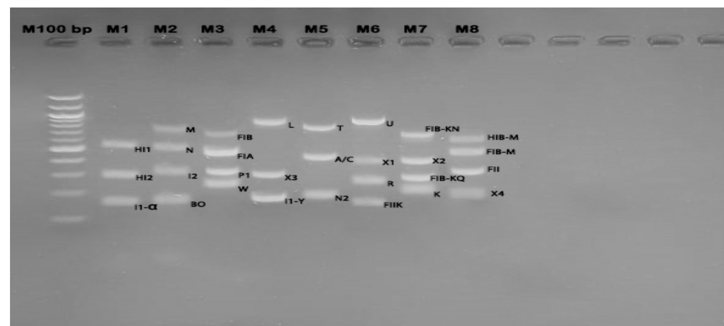

Fig.2. Agarose gel electrophoresis analysis of multiple Amplicons of CTX-M of K.pneumoniaeafter conjugation obtained via amplification of positive controls using all PCR mixes M1 to M8.

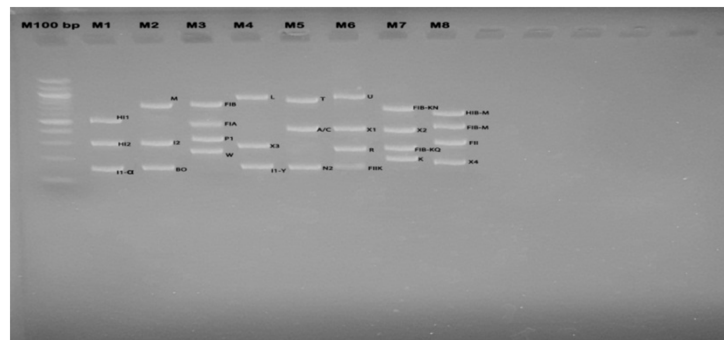

Fig. 3.Agarose gel electrophoresis analysis of multiple Amplicons of TEM of K. pneumoniae obtained via amplification of positive controls using all pcr mixes M1 to M8.

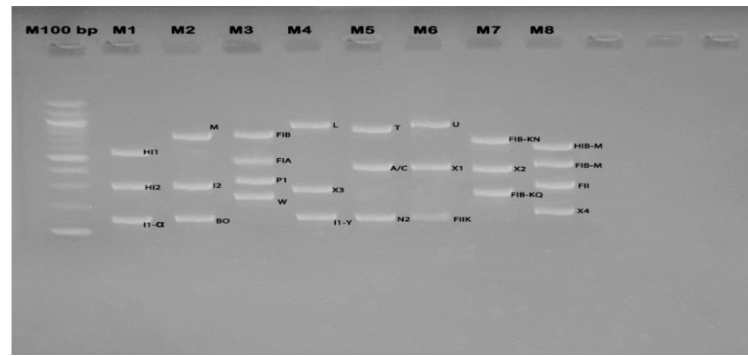

Fig. 4. agarose gel electrophoresis analysis of multiple Amplicons of TEM ofK. pneumoniae after conjugation obtained via amplification of positive controls using all PCRmixes M1 to M8.

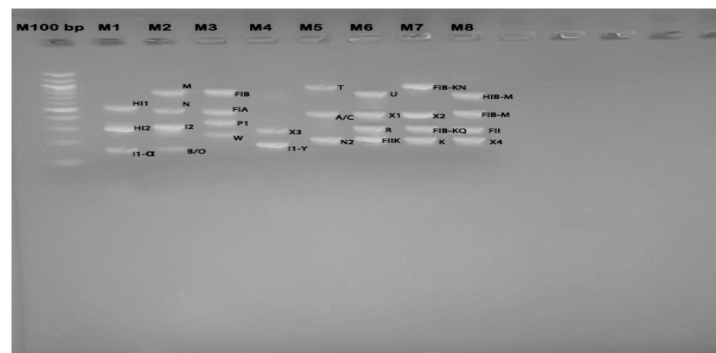

Fig. 5. agarose gel electrophoresis analysis of multiple Amplicons of SHV of K.pneumoniae obtained via amplification of positive controls using all PCR mixes M1 to M8.

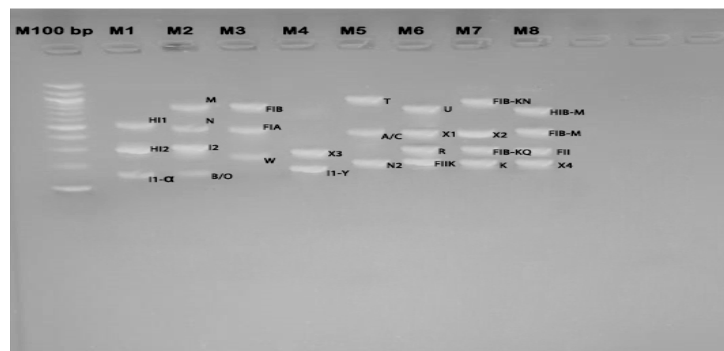

Fig. 6. agarose gel electrophoresis analysis of multiple Amplicons of SHV of K. pneumoniaeafter conjugation obtained through the amplification of positive controls using all PCR mixes M1 to M8.

In conclusion, our study demonstrates the rapid increase of CTX-M-producing urinaryK. pneumoniaeisolates in Al-Anbar hospitals; high ESBLs incidence with SHV genotype as the emerging strain was observed warranting sake need of surveillance for effective management of these strains and the decline of TEM-type, mediated mainly with the highly conjugative and adapted (IncF) plasmids carrying CTX-M genotype. The findings highlight the evolution of "IncFII-K" plasmids into new variants

that contain new elements of antibiotic resistance and their important role in the spread of K-producing ESBLs. among UTI patients hospitalized.The PBRT method, detecting new epidemiologically important replicas: (P1, N2 and X4); showed excellent specificity and sensitivity as well as (FIB-KNandFIB-KQ) in ESBLs-producing K. pneumoniaethat not detected in other previous studies. Characterization of these ESBLs encoding conjugative plasmids extends source understanding on these resistance markers in Multi Drug Resistant (MDR) K. pneumoniae isolated from AL-Anbar patients with UTI. The PBRT method for the plasmids analysis by the PBRT 2.0 kitdepreciates the detection time and trivializes the steps for identification and classification of multiple plasmids within the same K.pneumoniae strains.

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