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PCR-Based Replicon Typing of Plasmids in *E.Coli* Producing Esbls in Patients with UTI in Iraq

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Objectives:The aim of this work was to study the distribution of antibiotics resistance genes of ESBLs in clinical *E. coli* in patients with UTI as well as identification and classification of bacterial plasmids associated with virulence and antimicrobial resistance by using PCR-Based Replicon Typing (PBRT).

Materials and methods: ESBLs- producing *E. coli* were preliminary screened by cephalosporin confirmation tests. Antimicrobial susceptibility test was performed of all ESBLs- producing *E. coli*. For genotypic detection, CTX-M, TEM and SHV ESBL genes were determined by PCR. PBRT was performed by PBRT 2.0 KIT (Diatheva, Italy, 2020), as well as ESBLs resistance transfer assay was performed by conjugation method.**Results:** The prevalence of ESBL in this study is high. Overall, bla CTX-M was the commonest genotype (93.6%) in *E. coli* by PCR. 26 replicons were detected in CTX-M producing *E. coli* strains:" HI- 1, HI- 2, I1- α, I- 2, X- 1, X-2,X- 3,X -4, L, I1y, N, FIA, FIB, FIC, FII, W, Y, P1,I1 A/C, T, k, U, R, B/O, HIB-M and FIB-M "; 25 replicons were detected of TEM and SHV by PBRT. After conjugation was performed; transconjugants could be obtained at ratio 100% in *E. coli* producing CTX-M; and 88% in TEM and SHV.

Conclusions: The PBRT scheme exhibited anexcellent specificity and sensitivity, detecting new epidemiologically relevant replicons: P1, N2 and X4 in ESBLs-producing *E. coli*. The PBRT method for the(plasmids analysis) by the(PBRT 2.0) kitretrenches the detection time and provided that simplifies the steps for identification and classification of*E. coli* plasmids.

Introduction

PCR- Based Replicon Typing (PBRT) is a useful technique for detecting and monitoring the interaction of replicons with specific plasmid-borne resistant genes and for detecting the mobilization potential of resistance genes among different bacterial plasmids (1).Bacterial plasmid diagnostic is very difficult because the several plasmids in same bacterial cell make it unmanageable to assess the bacterial plasmid- content of the bacterial - strain. PBRT method based on PCR amplification of replicons has therefore been used as a plasmid identification and typing technique throughout the world in recent years.(2). The (PBRT)method is a PCR- based auscultating initially certified and developed by" Dr. Alessandra Carattoli"(senior scientist) at the Italy ; industrialized by "Diatheva" subsequently.However, in Iraq generally, there are no studies about molecular identification and classification of plasmid conferring drug resistance in these clinical isolates and strain genotypes exacts a distinction to sprawls of antimicrobial resistance

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ESBL genes in patients with UTI.

Materials and methods

Clinical isolates. Midstream urine samples were collected from 715 patients suffering from UTI referred to Al Anbar hospitals during (January 2019 to October 2019).All isolates were diagnosedroutine analysis,and automated diagnosis by API 20E and VITEK 2 compact system.

Antimicrobial Susceptibility Testing

E. coli's antimicrobial susceptibility test was estimated by updated' KirbyBauer disk diffusion method" in the 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, as well as screening method antibiotics (Oxoid, UK) according to CLSI (3). *Escherichia coli ATCC* 25922 were used as a control strain.

Screening methods for ESBLs detection

The CLSI has proposed disc diffusion methods for screening for ESBL production by *E. coli*(3). For ESBL production, these methods can be screened by noting particular zone diameters that suggest a high degree of suspicion for ESBL production. Ceftazidime, Cefotaxime, Ceftriaxone, Cefpodoxime, Cefepime and Aztreonam were used. Even then, using more than one of these screening agents increases detection sensitivity. If any ESBL diameter indicates suspicion, the condition should be checked using phenotypic confirmatory tests.

Confirmatory Tests for ESBLsdetection: Phenotypic Tests

The creation of *E. coli* isolates toESBLswas tested to at least onecephalosporinindicator resistant. (4); Double Disk Synergy (DDST),Broth Microdilution, CombinedDisk Test (CDT), ESBL E Test,*inhibitor-potentiated- disk- diffusion test*(*IPDD*)Test, Culture on (ChromeID- ESBLs and Brilliance ESBLs agar) andESBL NDP Test (5). As ESBL positive or negative controls, *Klebsiella pneumonia ATCC 700603* and *Escherichia coli ATCC* 25922 were used for all phenotypic methods ofdetection of*E.coli*.

Molecular Detection of ESBLs Genes

For ESBL production, molecular analysis has been conducted for all phonotypicalESBL *E. coli* isolates,for detection of molecular *E. coli*; the traditional Polymerase chain reaction was performed by the*E. coli* harboring ESBL genes (PCR).Total bacterial genomic DNA was isolated according to the Promega kit (USA).

Conventional PCRTechnique:

For detecting of ESBLs"Ambler: class A" of *E.coli*"CTX-M, TEM and SHV" encoding, standard PCR as a prototype with full bacterial genomic DNA. For PCRs with these primers, thermal cycling conditions (shv genes) consisted: 30 cycles at"94°C for 30 S", "68°C for 60S" and "72 for 60 S", and a final extension of" 72°C for 7min". A PCR with primers and conditions defined by

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genes (bla CTX-M) was performed (8). Amplicon is an intragenic (544)bp fragment. In 1-hour W/V agarose gel, 10 µl of the pcr product was electrophorised. 140V with an intelligent DNA ladder.

Table 1: sequences of SHV and TEM

Gene	Primers' Sequences (5'→3')	P. s. (bp)	Refernce
SHV	F: CGC CGG GTT ATT CTT ATT TGT CGC	**1016**	**6**
	R:TCT TTC CGA TGC CGCCGCCAGTCA		
TEM	F:ATA AAA TTC TTG AAG ACG AAA	**1080**	**7**
	R:GAC AGT TAC CAA TGC TTA ATC A		
CTX-M	F: TTTGCGATGTGCAGTACCAGTAA R:CGATATCGTTGGTGGTGCCATA	**544**	**8**

* F: Forward sequences, R: Reverse sequences.

PBRT method

The "resistance plasmids" in the ESBLs- *E.coli* isolates wereidentified by using "PBRT-kit": (Diatheva, Italy, 2020). Complete bacterial DNAwas prepared according to Promega kit (USA) and used as a pcr prototype according to" the PBRT 2.0 kit protocol". The following 8 multiplex PCR tests (groups of incompatibility) with "positive –controls" were used for amplifying "30 replicons":- (HI-1, HI -2, I1 -α, I-2, X-1, X-2, X-3,X-4, L, I1y, N, FIA, FIB,FIC, FII,FIIS, FIIK, FIB KN, FIB KQ, W, Y, P1, A/C, T, K, U, R, B/O, HIB-M and FIB-M) in this kit representing the main plasmidIncompatibility- groups and plasmid genes in the "Enterobactericeae" set (2).

Transfer of ESBLs resistance assays (conjugation)

The plasmid that confers cephalosporin resistance in all ESBL-producing *E. coli* strains were transferred by plasmid-free, rifampicin-resistant *E. coli* strain K12 J53R (positive lac) was used as acceptor (9). 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) (10). When resistance plasmid was transferred in mating experiments: DNA was extracted using Promega kit (USA) and plasmid replicates were determined using PCR-based replication typing (2).

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Statistical Analysis:

All the data was analyzed with descriptive statistical analysis using SAS , 2012 software, .In order to compare the different classes, we used chi-squared testfor descriptive parameters.

Results and Discussion

In our study, *E. coli* was the most predominant species(45.1% 166 out 368 positive urine samples).High-ratio explanation for these bacteria is found in the urine, thereby triggering autoinfection. Additionally, after joining the human bladder;E. coli can bind to the bladder wall and form a "biofilm" that resists body immune response (11).Antibiotic susceptibility test showed full resistance of ESBLs- producing *E. coli* to Ampicillin, Pipracillin, Azetronam, third generation Cephalosporin and to Cefepime, but 100% sensitive to Carbapenems & Mecillinam. Pathogenic bacteria typically resist several antibiotics in hospitals due to increased antibiotic selection pressure.Weobserved that no resistant in the pattern to Carbapenems and Mecillinam, because they may be not used asa choice for the management of multidrug-resistant *E. coli*.
Our study found that *E. coli* producing ESBLs were 100% resistant to (Ceftazidime, Cefotaxime, Ceftriaxone, Cefpodoxime), Cefepime and Aztreonam. Due to the large amount consuming of thirdgeneration cephalosporins, cefepime and Aztreonam. Furthermore, resistance of ESBLs isolates to non-Beta-lactam antibiotics were threshed; Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Nalidixic acid, Chloramphenicol, Trimethoprim-Sulfamethoxazole, Tetracycline, Doxycycline and Rifampicin with resistant rates of respectively 44%, 50.4%, 71.2%, 70.4%, 88.8%, 33.6%, 83.2%, 76%, 55.2% and 64% against *E .coli.*Multi-drug resistance is a big concern in treating uropathogens ESBLs. This (MDR) may be attributable to plasmids with multiple resistance genes transferred from one uropathogenic bacterium to another. ESBL is usually encoded with resistant genes (aminoglycosides, chloramphenicol, sulfonamides, trimethoprene and other antigens) that significantly restrict alternative therapies (including the use of multiple resistant plasmids). Amikacin, Nitrofurantoin, Minocyclin, Tigecycline, Fosfomycin,Colstin and polymyxin- B. Showed best performance(high sensitivity ratio) percent against *E.coli*indicating these medications can still be used to treat UTIs. The antibiotic resistance of ESBL-producing strains to Beta-lactam and non-Beta lactam antibiotics which are usually recommended for initial treatment of UTI; was significantly higher ($p < 0.01$) than that of ESBL-non-producing strains in our study. Similarobservations have been published by other studies (12).
In the present study, we found 125(75.30%) ESBLs producers' from166 *E. coli* isolates based on MIC ESBLs screening breakpoints and confirmatory tests bydifferent phenotypic methods; high Sensitivites and Specificities of thesetestsforidentification of ESBL-production *E. coli*. The overall high sensitivities (100%) of the *E. coli* producing ESBL by broth micro dilution method, IPDD and the sensitivity of the combined disk method, with and without clavulanic acid as screening substrates for clavulanic acid synergy.
Among all ESBLs phenotypic detection methods, (DDS)test was the least sensitive phenotypic ones. The Combined disc test tends to have a higher Sensitivity 100% with ESBLs-positive isolates (11 of *E.coli*) that were missed by DDST. As a phenotypic method, CDM was shown to be more sensitive than the DDS test; thus, CDM is better than DDST for detection of ESBLs (13), as well as we found that The IPDD test seemed to be a better method than the DDST, showing 100

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percent sensitivity and specificity in confirming ESBL production. DDST failed to detect 11 isolates of*E.coli* therefore; 91.2 percent sensitivity *E.coli* ESBL producers by this test. The sensitivity of the DDST as a phénotypic tool depends on the exact location of the disks (14). In our research, the E-strip sensitivity is 100% in *E. coli* with 100 percent specificity; thus, the (E-strip) phenotypic testing method is responsive, accurate and comfortable. In addition, our analysis showed that cefpodoxime and ceftazide have a larger zone (increase) than cefotaxime; and that even the IPDD testing is more sensitive when ceftazidime disk is used than when the cefotaxime disk is used, as has the cephalosporins used in the IPDD test (14)

The advantage of the IPDD test is that isolates produced in ESBLs may be differentiated from non-produced ESBL isolates by (almost) mm cut-off for area increase and three Oxyimino cephalosporins may also be measured on one agar plate. Ceftazidime is an excellent platform for most ESBL enzymes. The new Brilliance ESBLs, by comparison, proved worthwhile and worked just as well as the chrom ID ESBLs to detect ESBLs. Having an excellent Negative Predictive Value (NPV) of (24) h,*E.coli* was a promising phenotype that could easily remove patients not transporting producers of ESBL. The ESBL NDP Test was recently developed for rapid identification of ESBLs-Enterobactericeae. This test is simple, sensitive and precise, based on hydrolysis detection in the beta-lactam ring of Cefotaxime (15).

Results of the PCR showed that the most common ESBL chanced gene in *E.coli* urinary isolates was CTX-M. The gene CTX-M was discovered in 93.6 percent *E. coli*. In this analysis, the frequency of the TEM-type and SHV-type enzymes were 90.4% and 60% respectively. Devi et al. have found rising prevalence of *E.coli* in recent studies. CTX-M-type ESBL development of*E. coli* and *Klebsiella pneumoniae* (16). High prevalence of ESBL-induced UTI in many hospitals worldwide has been identified (17; 18).The

Replicons could be determined in ESBLs-producing E. coli strains strains in (Table -1), PCR-based Replicon Type 2.0 Kit; 26 repliconswere detected in CTX-M producing*E.coli*. HI-1, HI-2, I1-α, I-2, X-1, X-2,X-3,X-4, L, I1y, N, FIA, FIB, FIC, FII, W, Y, P1,I1 A/C, T, K, U, R, B/ O, HIB-M, FIB-M Strengths: (fig. 1)

The TEM replicas in ESBL-produced *E. coli* strains (Table-1); 25 replica: HI-1, I1-α, I-2, X-2, X-3, X-4, L, I1y, N, FIA, FIB, FIC FII, W, Y, P1, A/C, T, K, U, R, B/O, HIB-M and FIB-M were detected (fig.3). 25 replicas were found on SHV generating E in the same table (Table-1). HI-1, HI-2, I1-α, I-2, X-1, X-2,X-3,X-4,I1y, N, FIA, FIB, FIC, FII, W, Y, P1, A/C, T, K, U, R, B/O, HIB-M and FIB-M .(fig. 5).

PBRT in *E. coli* producing CTX-M, TEM and SHV after conjugation was performed (Table-1); transconjugants could be obtained at ratio 100% in *E. coli* producing CTX-M (Fig. 2). A mating experiment (conjugation) was successfulinour studywithCTX as a selective agent. In vitro,large number of the strains capable of transferring their resistance was CTX-M producers, which involved transconjugation in gene dissemination (10). The replicons rep B/O, FIB and FII which were found in the *E. coli* donors producing TEM (Fig.3) as well as rep I-2, B/O and FII in the *E. coli* donors producing SHV (Fig 5) were absent in the transconjugants of *E .coli* donors producing SHV (Fig 5); therefore the ratio is 88% in *E. coli* producing TEM and SHV (Fig. 4 & 6). Inc I plasmids were more likely to be transferred by conjugation than Inc F plasmids (19).

In the present study the replicon FIIS was not recognized by PBRT, because this replicon PCR recognizes a divergent FII replicon identified on *Salmonella* virulence plasmids; as well as in our

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results the replicons FIIK, FIB KN and FIB KQ was not recognized by this method. The FIIK PCR recognizes a divergent FII replicon identified on plasmids from *Klebsiella spp* but not in *E. coli* (20). In this study X Replicons (X1, X2, X3 and new replicon X4) were associated with the three ESBL types studied CTX-M,TEM and SHV(Fig. 1,3,5) of *E. coli* strains by PBRT 2.0 kit; but Incx family: (X1, X2, X3) types of IncX plasmids are detected by PBRT, as defined the previous studies. Typing plasmid is an important parameter for evolution research, epidemiology and antibacterial resistance spread (2; 21). The replicate content well-defined by the "PBRT" method which used for"Microbiological analyzing" of the outbreaks bacterial clones and for monitoringspreading of particular groups of epidemiologically related resistant determinants; but genetically unassociated bacterial isolates (22). In 2017, Carloni et al. concluded that "PBRT-kit" detected replicates in" 100% "of the analyzed strains; pretended to increase commercial sensitivity and specificity (2). It has been surmised that larger than "10,000 strain" of Enterobacteriaceae tested by using this technique to classify epidemic plasmids that have spread away species confines in very remote geographer locations in Enterobacteriaceae (22;23).

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

ESBL – genes of *E. coli*					
CTX-M		**TEM**		**SHV**	
Presence of replicon types before conjugation	**Replicons transfer after conjugation**	**Presence of replicon types before conjugation**	**Replicons transfer after conjugation**	**Presence of replicon types before conjugation**	**Replicons transfer after conjugation**
HI1	+	**HI1**	+	**HI1**	+
HI2	+	**I1-α**	+	**HI2**	+
I-1α	+	**M**	+	**I-1α**	+
M	+	**N**	+	**M**	+
N	+	**I2**	+	**N**	+
I-2	+	**B\O**	**-**	**I2**	**-**
B\O	+	**FIB**	**-**	**B\O**	**-**
FIB	+	**FIA**	+	**FIB**	+
B1	+	**P1**	+	**FIA**	+
w	+	**w**	+	**P1**	+

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L	+	**L**	+	**W**	+
X- 3	+	**X3**	+	**X3**	+
I1Y	+	**I1Y**	+	**I1Y**	+
T	+	**T**	+	**T**	+
A\C	+	**A\C**	+	**A\C**	+
N2	+	**N2**	+	**N2**	+
U	+	**U**	+	**U**	+
X -1	+	**X1**	+	**X-1**	+
R	+	**R**	+	**R**	+
X-2	+	**X -2**	+	**X-2**	+
K	+	**K**	+	**K**	+
HIB-M	+	**HIB - M**	+	**HIB-M**	+
FIB-M	+	**FIB – M**	+	**FIB – M**	+
F-I I	+	**F-II**	**-**	**F-II**	**-**
X4	+	**X4**	+	**X4**	+
26 Replicon	**26 Replicon**	**25 Replicon**	**22 Replicon**	**25 Replicon**	**22 Replicon**

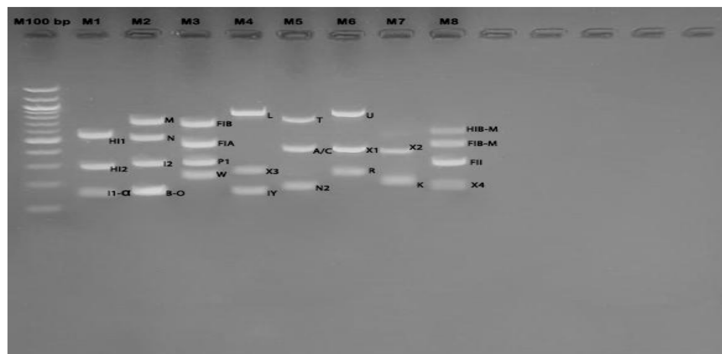

Fig. 1.Agarose gel electrophoresis analysis of multiple amplicons in CTX-M of *E.coli* obtained via amplification of positive controls using all PCR mixes (M1 to M8).

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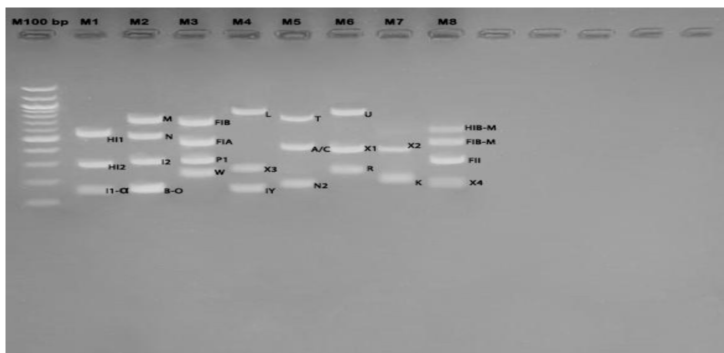

Fig. 2. Agarose gel electrophoresis analysis of multiple amplicons in CTX-M of *E.coli* after conjugation obtained via amplification of positive controls using all PCR mixes (M1 to M8).

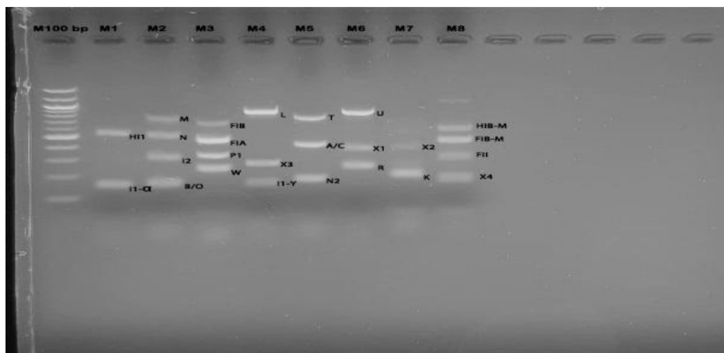

Fig. 3. Agarose gel electrophoresis analysis of multiple amplicons in TEM of *E.coli* obtained (via amplification of positive controls using all PCR mixes M1 to M8).

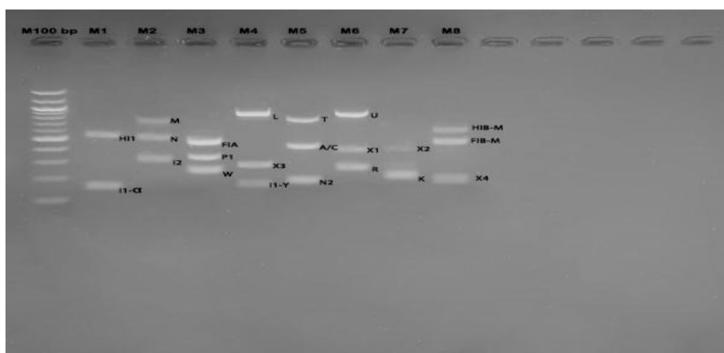

Fig. 4. Agarose gel electrophoresis analysis of multiple amplicons in TEM of *E. coli* after conjugation obtained via amplification of positive controls using all PCR mixes (M1 to M8).

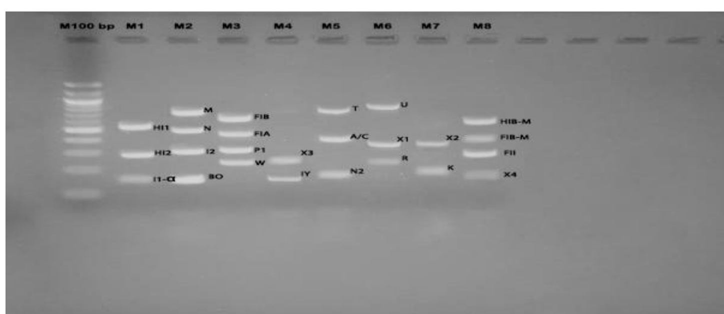

Fig. 5. Agarose gel electrophoresis analysis of multiple amplicons in SHV of *E.coli* obtained

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(via amplification of positive controls using all PCR mixes (M1 to M8).

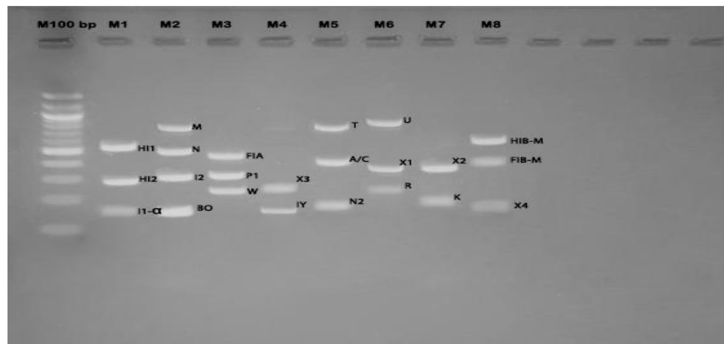

Fig. 6. Agarose gel electrophoresis analysis of multiple amplicons in SHV of *E.coli* after conjugation obtained via amplifications of positive- controls using all pcr mixes (M-1 to M-8).

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