Efficacy of Combination of Meropenem with Gentamicin, and Amikacin against Resistant *E. coli* Isolated from Patients with UTIs: *in vitro* Study[#]

Maysaa A. abdul khaleq*,[1], Abdulkareem H. Abd and Maysaa A. dhahi*****

* Department of pharmacotheraputics,College of Pharmacy, Al- Mustansiryh University,Baghdad,Iraq.
** Department of pharmacology, College of Medicin, Al- Nahrain University, Baghdad, Iraq.
*** Department of Microbiology, College of Medicin, Al-Nahrain University, Baghdad, Iraq.

Abstract

Seventy five *E. coli* isolates were collected from urine of patients with urinary tract infections in AL-Kadhimia and AL-Yarmook teaching hospitals in Baghdad for a period between 22/11/2009 to 15/3/2010, from these samples twenty five isolates were selected according to their pattern of the highest resistance as these showing multi-drug resistances and tested to specify their minimum inhibitory concentration for (meropenem, gentamicin and amikacin), meropenem was found having the lowest MIC comparing with others. This study also includes in vitro effects of various combinations of three types of antimicrobials (meropenem, gentamicin and amikacin) against twenty five *E. coli* isolates.Among combinations the combination of meropenem with the other types of antimicrobials showed high synergistic effect when 1/4+1/4 MIC for each antimicrobial were used. While combinations of amikacin with gentamicin in some isolates showed additive effect when 1/2+1/2 MIC for each antimicrobial were used. The plasmid profile for the twenty five *E. coli* isolates were studied using Pure Yeild ™ plasmid Miniprep system- Cat.# A1220 – Promega- USA. In order to determined the presence of plasmid for antimicrobials resistance.

الخلاصة

جمعت خمسة وسبعون عزلة من الاشيريشيا القولونية من ادرار مرضى المجاري البولية الذين راجعوا مستشفى الكاظمية واليرموك التعليمي في بغداد للفتره من ٢٠٠٩/١١/٢٢ الى ٢٠١٠/٣/١٥ ومنهم تم اختيار خمسة وعشرون عزلة اعتماداً على ما أبدته من مقاومة عالية و متعددة للمضادات الجرثومية ثم حددت التراكيز المثبطة الدنيا (MIC) للمضادات (الميروبنيم، الجنتمايسين والاميكاسين) وقد أظهرت النتائج بان مضاد الميروبنيم هو الأكثر فاعلية و ذلك بتثبيطه نمو البكتريا بأقل تركيز مقارنة بالمضادات الأخرى.تضمنت هذه الدراسة استقصاء تأثير اتحاد المضادات الحيوية ضد خمسة وعشرون عزلة من *E. coli* (*in vitro*) وقد أظهرت النتائج أن اتحاد الميروبنيم مع بقية المضادات الحيوية(الجنتمايسين والاميكاسين) يشير الى تاثير تازري عالي عند استعمال ربع التركيز المثبط الادنى (MIC) لكل مضاد حيوي. بينما اتحاد الاميكاسين مع الجنتامايسين في بعض العزلات يشير الى تاثير اضافي فقط عند استعمال نصف التركيز المثبط الادنى (MIC) لكل مضاد حيوي .شملت ألدراسة ايضاً دراسة ألنمط البلازميدي لخمسة وعشرون عزلة من بكتريا *E. coli* بأستخدام عدة لعزل البلازميد بواسطة نظام Miniprep وقد أظهرت النتائج بأن العزلات (٣٢،٣٧،٥٧،٦) حامله لبلازميد لمقاومة ألمضادات الحيوية.

Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections in humans both in the community and hospital setting [(1)]. Escherichia coli have been documented to be the most important pathogen associated with symptomatic urinary tract infections [(2)].plasmid DNA molecule is separate from, and can replicate independently of, the chromosomal DNA[(3)]. In this study we use combination of meropenem (which is a broad spectrum antimicrobial agent with more activity against gram-negative bacilli and less activity against gram-positive cocci than is imipenem)[(4)], with aminoglycosides which are polar compound with more activity against aerobic gram-negative bacilli and little activity against an aerobic bacteria and use with other antimicrobial agent against gram positive bacteria [(5)].

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[1] Corresponding author E- mail : maysaa_ali82a@yahoo.com

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Material and Methods

The *E. coli* identification depended on morphological, biochemical testes in addition to API 20E system. Susceptibility of isolates to seventeenth antimicrobials was tested using disk diffusion assay according to modified Kirby–Bauer method [6]. Meropenem, nitrfurantoin , amikacin and imepenem were to be the most effective antimicrobials, while the other antimicrobials were less effective. Minimum inhibitory concentration (MIC) was determined using tubes dilution method [7]. The combination of antimicrobials weather it's synergistic, additives, antagonistic, or indifference depending on the fractional inhibitory concentration (FIC) was determine as follow: (≤0.5) synergism, (0.5–<1) additive, (1–<4) indifference,(≥4) antagonism, and calculated using the following equation [8].

$$\text{FIC} = \frac{\text{MIC for antibiotic in combination}}{\text{MIC for antibiotic alone}}$$

Plasmid DNA isolated using Pure Yeild ™ plasmid Miniprep system, according to the manufacture manual. Then the extracted plasmid DNA was loaded in 0.8% agarose gel stained with ethidium bromide and electrophoresis for 60 minutes at 2V/Cm using 1X TBE buffer. Then agarose gel was visualized using UV-transluminator.

Result and Discussion

Colonies of *E. coli* had marked as a flat smooth and pink in color as a result of lactose fermentation in the media on MacConky agar, while on blood agar it gave small pink convex colonies surrounded by zone of β- haemolysis. In Microscopic Examination it showed as small single bacilli non spore forming with red color (gram –negative bacteria), it occurred separately and singly, but often they are accumulated in groups. The result of biochemical tests for most of *E. coli* showed its ability to catalase production and lactose fermentation while it gave a negative result in Oxidase, Urease and Simmon Citrate tests. Further identification of the isolates was done by using Api 20E system, as in Figure (1).

***E. coli* (4)**

Figure 1: Identification of *E. coli* by Api20E system

Antimicrobial Sensitivity Test

1-Qualitative Method (Disc Diffusion Test)

In this study we found that antimicrobials sensitivity among *E. coli* isolates varied according to the nature of antimicrobials. The percentage of resistant isolates to each antimicrobial is shown in Figure (2).Standard disc diffusion assay was used to detect the sensitivity of pathogenic bacteria and results obtained were compared with those of Clinical and laboratory standard institute [9].The results of the current study (Figure 2) revealed that most of *E. coli* isolates resist the β- lactam antimicrobials (like ampicillin and amoxicillin) [10].noted the high resistance rates of gram positive and gram negative species to penicillins and some of cephalosporins. Increasing of bacterial resistance rates to this group of antimicrobials may be a result of either production of β-lactamase enzyme that had the ability to destroy the β- lactam ring in these antimicrobials [11, 12]. Also it may be due to minimizing the interaction of antimicrobials with target site (Penicillin Binding Proteins) [13].Augamentin (amoxicillin + clavulanic acid) had more activity than other penicillin due to its presence of clavulanic acid, which inhibit β- lactamase enzyme, and increase the spectrum of amoxicillin against gram- positive

and gram- negative bacteria [14]. Many research illustrated the higher activity of imipenem and meropenem (related to carbapenems group) against gram- positive and gram- negative bacteria [15].Regarding aminoglycoside group, amikacin was more active than gentamicin on the current *E. coli* isolates, many researches showed that the increasing resistance against aminoglycoside group was due to production of the modified enzymes and losing outer membrane pores, which are responsible of permeability of surface cell layer to antimicrobials [16]. The current results (Figure 2) was in agreement with that of Shevelev *et al.* (2002) [17] who found in a study that the resistance percentage of the isolates to amikacin was (0%) , while the resistant rate to gentamicin was (48.6%). The results also was in agreement with Bashir *et al.*(2008) [18] who found in a study in Pakistan that the resistance percentage of the isolates to gentamicin was (49%) . Resistant to tobramycin was (40.7%) and this result was near that found by Pape *et al.* (2004) [19] who found that the resistant percentage of *E. coli* to tobramycin was (30%).Many studies were illustrated the activity of naldixic acid, and most of quinolones antimicrobials against wide range of bacteria that were in a good agreement with the currently result. For example the resistant rate to ciprofloxacin was (40.7%) this result was comparable to the result of Shamm *et al.*(2001) [20] found in a study that the resistant percentage of *E. coli* to ciprofloxacin was (39%). Resistance to pipracillin was (85.5%), this result was in agreement with that of Bujdakova *et al.*(1998) [21] who found that (86%) of *E. coli* isolates resistant to pipracillin , and this may be due to the ability of *E. coli* to develop resistance to these antimicrobials through the production of β-lactamase enzyme which break the β-lactam ring of pipracillin. Resistance to nitrofurantoin was (2.6%), this result was in agreement with Akyar (2008) [22] who found that the resistant rate of *E. coli* against nitrofurantoin was (3%).Resistance to trimethoprim/ sulfamethoxazole (SXT) was (43.4%), this result may be attributed to the wide use of (SXT) as empirical therapy for urinary tract infection, however this result was in agreement with Gupta; Hooton and Stamm (2001) [23] who found that the resistance to (SXT) among *E. coli* isolates from patient with UTIs has increased, with a prevalence of resistance which is reported 30 to 50 percent .

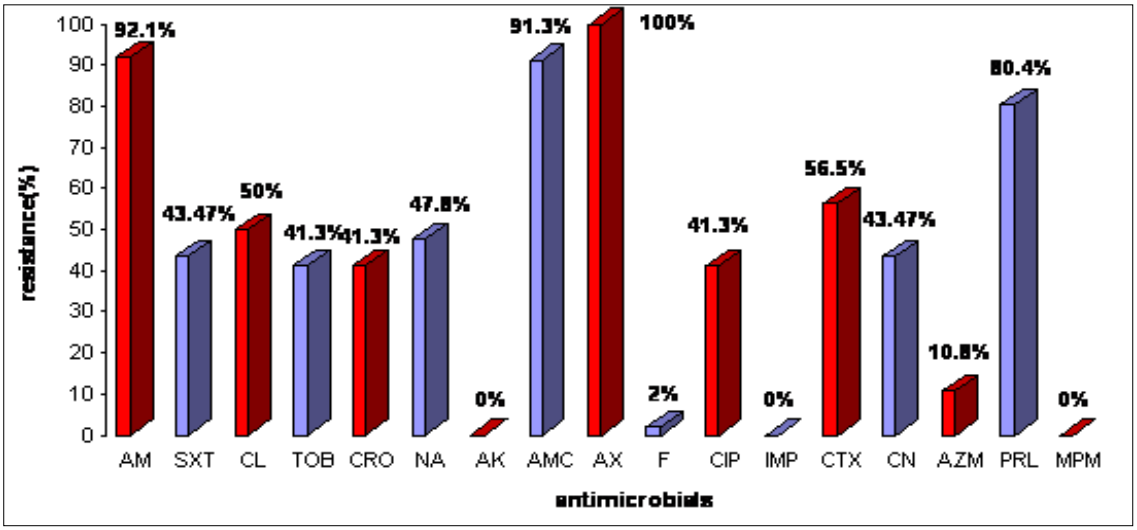

Figure 2: Percentage of resistant *E. coli* isolates to antimicrobial
ToB: Tobramycin; CN: Gentamicin; Sxt: Triomethoprime and sulfamethoxazole; Cip: Ciprofloxacin; Na: Naldixic acid; Ctx: Cefotaxime; Ipm: Imipenim;Am: Ampicillin; CL: Cephalexin; CRO:Ceftriaxone;AMC:Amoxicillin and Clavulonicacid; F:Nitrofurantoin; AZM:Azithromycin;PRL:Pipracillin;MPM: Meropenem; AX:Amoxicillin AK:Amikacin

2- Quantitative Method (Minimum Inhibitory Concentration) (MIC)

Table 1 showed that MIC of meropenem ranged from (0.003-12.5µg/ml) this result was in agreement with Marie *et al.* [24] who found in his study that *E. coli* was moderately susceptible to meropenem at MIC (8µg/ml) .The results of this study also showed that the MIC of gentamicin ranged from (12.5 to 480 µg/ml), this result was in agreement with Jakobsem *et al.* [25] who found in his study that the MIC of gentamicin distributed from (8-› 512 µg/ml).·On the other hand MIC of

amikacin ranged from (0.3-2.5µg/ml), this result was in agreement with Shrivastava and Chaudhary [26] whose found that the MIC of amikacin in *E. coli* was (2µg/ml).while Celine *et al*. [27] who found in his study that the MIC of amikacin in *E. coli* ranged from (1 to16 µg/ml).

Table 1: MIC value for three antimicrobials (µg/ml) tested against *E. coli* isolates

E. coli isolates	Meropenem µg/ml		Gentamicin µg/ml		Amikacin µg/ml	
	MIC	MBC	MIC	MBC	MIC	MBC
A1	0.12	0.125	300	300	2.5	5
A2	1.25	12.5	200	300	1.25	2.5
A3	0.12	1.25	300	300	1.25	2.5
A4	1.25	1.25	300	480	0.6	1.25
A6	0.12	1.25	480	480	0.3	0.6
A7	1.25	1.25	300	300	2.5	5
A10	12.5	12.5	480	480	0.6	1.25
A11	0.003	0.003	12.5	12.5	1.25	2.5
A13	0.12	0.12	300	300	2.5	5
A24	0.12	1.2	300	300	1.25	2.5
A28	0.03	0.03	200	200	1.25	2.5
A32	12.5	12.5	480	480	0.6	1.25
A35	1.25	1.25	200	300	0.6	1.25
A37	0.12	1.25	480	480	0.3	0.6
A41	12.5	12.5	100	200	1.25	2.5
A42	12.5	12.5	100	200	1.25	2.5
A43	1.25	1.25	200	300	0.6	1.25
A44	0.06	0.06	200	300	2.5	5
A45	1.25	12.5	200	300	1.25	2.5
A47	0.12	1.25	480	480	0.3	0.6
A51	12.5	12.5	100	200	1.25	2.5
A55	0.06	0.03	200	300	2.5	5
A57	0.12	1.25	480	480	0.3	0.6
A58	0.12	0.12	300	300	2.5	5
A67	0.12	0.125	300	300	2.5	5
LSD value	4.945 *	5.418 *	137.95 *	118.38 *	0.830 *	1.651 *

* ($P<0.05$), LSD: Least significant difference

3- Antimicrobials Combination

The result in Table2 shows that the synergistic effect noticed from combination of meropenem with gentamicin on isolate No. (1, 2, 3, 4, 6, 7, 10, 13, 24, 28, 35, 37, 41, 42, 43, 44, 45, 47, 51, 55, 57, 58, 67), this result similar to that shown by Richared *et al.* [28] found that aminoglycoside synergized with β-lactams antimicrobials against *E. coli* isolates, because of the latter action on cell wall synthesis, which enhance diffusion of the aminoglycoside into the bacterium. While isolate No.(32) show the additive effect with combination of meropenem with gentamicin, and that may be due to their resistance to gentamicin (MIC 480) and to meropenem (MIC 12.5). table3 shows Another synergistic effect resulted from combination of meropenem with amikacin when its effect tested on isolate No. (1, 2, 3, 4, 7, 10, 13, 24, 28, 37, 41,42,43, 44,45,47, 51, 55, 57, 58, 67) this result was in agreement with and Piroska *et al*. [29] whose found that there is synergistic effect result from combination of meropenem with amikacin against *E. coli* isolates . While isolates No. (6, 32, 35) showed no effect toward combination of meropenem with amikacin.On the other hand combination of amikacin with gentamicin (table 4 showed additive effect when tested on isolates No. (1, 2) but other isolates show no effect.

Table2: Results of combination of meropenem with gentamicin (1/4+1/4MIC)

E. Coli isolates	MIC of meropenem before combination (ug/ml)	MIC of meropenem after combination (ug/ml)	MIC of gentamicin before combination (ug/ml)	MIC of gentamicin after combination (ug/ml)	FIC	Result
A1	0.12	0.03	300	75	0.5	Syn
A2	1.25	0.31	200	50	0.5	Syn
A3	0.12	0.03	300	75	0.5	Syn
A4	1.25	0.31	300	75	0.5	Syn
A6	0.12	0.03	480	120	0.5	Syn
A7	1.25	0.31	300	75	0.5	Syn
A10	12.5	3.125	480	120	0.5	Syn
A13	0.12	0.03	300	75	0.5	Syn
A28	0.03	0.007	200	50	0.5	Syn
A24	0.12	0.03	300	75	0.5	Syn
A35	1.25	0.31	200	50	0.5	Syn
A37	0.12	0.03	480	120	0.5	Syn
A41	12.5	3.12	100	25	0.5	Syn
A42	12.5	3.12	100	25	0.5	Syn
A43	1.25	0.31	200	50	0.5	Syn
A44	0.06	0.015	200	50	0.5	Syn
A45	1.25	0.31	200	50	0.5	Syn
A47	0.12	0.03	480	120	0.5	Syn
A51	12.5	3.12	100	25	0.5	Syn
A55	0.06	0.01	200	50	0.5	Syn
A57	0.12	0.03	480	120	0.5	Syn
A58	0.12	0.03	300	75	0.5	Syn
A67	0.12	0.03	300	75	0.5	Syn
LSD value	5.030 *	4.234 *	213.56 *	122.23 *	--	--

* (P<0.05); LSD: Least significant difference; Syn: Synergism; FIC: Fractional Inhibitory Concentration

Table 3: Results of combination of meropenem with amikacin (1/4+1/4 MIC):

E. Coli isolates	MIC of meropenem before combination (ug/ml)	MIC of meropenem after combination (ug/ml)	MIC of amikacin before combination (ug/ml)	MIC of amikacin after combination (ug/ml)	FIC	Result
A1	0.12	0.03	2.5	0.62	0.5	Syn
A2	1.25	0.31	1.25	0.31	0.5	Syn
A3	0.12	0.03	1.25	0.31	0.5	Syn
A4	1.25	0.31	0.6	0.15	0.5	Syn
A7	1.25	0.31	2.5	0.62	0.5	Syn
A10	12.5	3.12	0.6	0.15	0.5	Syn
A13	0.12	0.03	2.5	0.62	0.5	Syn
A24	0.12	0.03	1.25	0.31	0.5	Syn
A28	0.03	0.007	1.25	0.31	0.5	Syn
A37	0.12	0.03	0.3	0.07	0.5	Syn
A41	12.5	3.12	1.25	0.31	0.5	Syn
A42	12.5	3.12	1.25	0.31	0.5	Syn
A43	1.25	0.31	0.6	0.15	0.5	Syn
A44	0.06	0.01	2.5	0.62	0.5	Syn
A45	1.25	0.31	1.25	0.31	0.5	Syn
A47	0.12	0.03	0.3	0.07	0.5	Syn
A51	12.5	3.12	1.25	0.31	0.5	Syn
A55	0.06	0.01	2.5	0.62	0.5	Syn
A57	0.12	0.03	0.3	0.07	0.5	Syn
A58	0.12	0.03	2.5	0.62	0.5	Syn
A67	0.12	0.03	2.5	0.62	0.5	Syn
LSD value	5.030 *	4.234 *	213.56 *	122.23 *	--	--

* (P<0.05); LSD: Least significant difference; Syn: Synergism; FIC: Fractional Inhibitory Concentrations

Table 4: Antimicrobials combination (1/2+1/2 MIC for each antimicrobials)

E. coli isolates	Antimicrobials combination	MIC of first antimicroal alone (µg/ml)	MIC of first antimicrobial in combination (µg/ml)	MIC of second antimicrobial alone (µg/ml)	MIC of second antimicrobial in combination (µg/ml)	FIC	Results
A32	MPM+CN	12. 5	6. 25	480	240	1	Add
A1	AK+CN	2. 5	1. 25	300	150	1	Add
A2	AK+CN	1.25	0. 625	200	100	1	Add

Add: Addition; FIC: Fractional Inhibitory Concentration MPM: meropenem; CN: gentamicin; AK: amikacin.

Extraction of Plasmid DNA

The result of Figures (3and 4) indicate that each of the isolates (A6 , A37)containing two bands of plasmid DNA with approximate molecular weight (2000 and 1900) bp comparing with molecular weight marker. Also, isolates No.(A32, A57) containing one plasmid DNA with approximate molecular weight (2000) bp when comparing with molecular weight marker.There are many studies referred to the isolation of antimicrobial resistance plasmid from *E. coli*. Joseph *et al.* (2001) [30] found in their study that *E. coli* isolates contain plasmid coding for resistance of aminoglycoside antimicrobials, including gentamicin and tobramycin. Also, March Galimand *et al*(2003) [31] found in their study that *E. coli* isolated from patient suffering from urinary tract infection contain plasmid coding high level of resistance to aminoglycoside. Piddock (1999) [32] found in his study that *E. coli* contain plasmid coding for resistance of flouroquinolone .Sisson *et al.* (2002) [33] found in their study that resistance to nitrofurantoin may be chromosomal or plasmid mediated. Minch chau phuc Nguyen *et al.* [34] found in their study that the plasmid gene that confers resistance to azithromycin had recently emerged in non multidrug resistant *E. coli*; Philippon; Arlet and Jacoby (2002) [35] found in their study that *E. coli* contains plasmid coding for resistance of ampicillin. In the other hand, other *E. coli* isolates that show no plasmid may be due to carrying plasmids with low copy number.

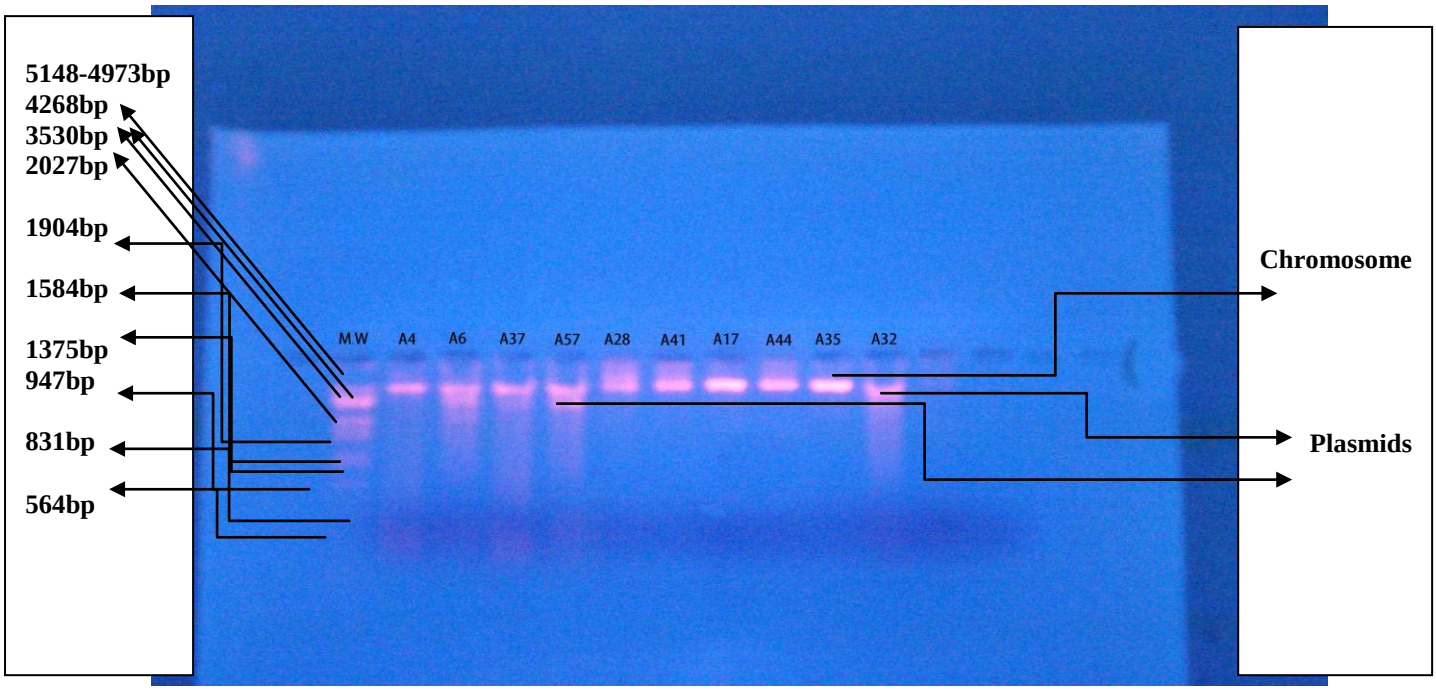

Figure 3: plasmid profile of *E. coli* strains Lane (A6, A37, A57, A32): Plasmid DNA extracted from *E. coli* strains; M.W: Molecular weight marker of lambda DNA digested with EcoRI+HindIII . Electrophoresis was carried in 0.8% agarose gel at (2V/Cm) for 30 min.

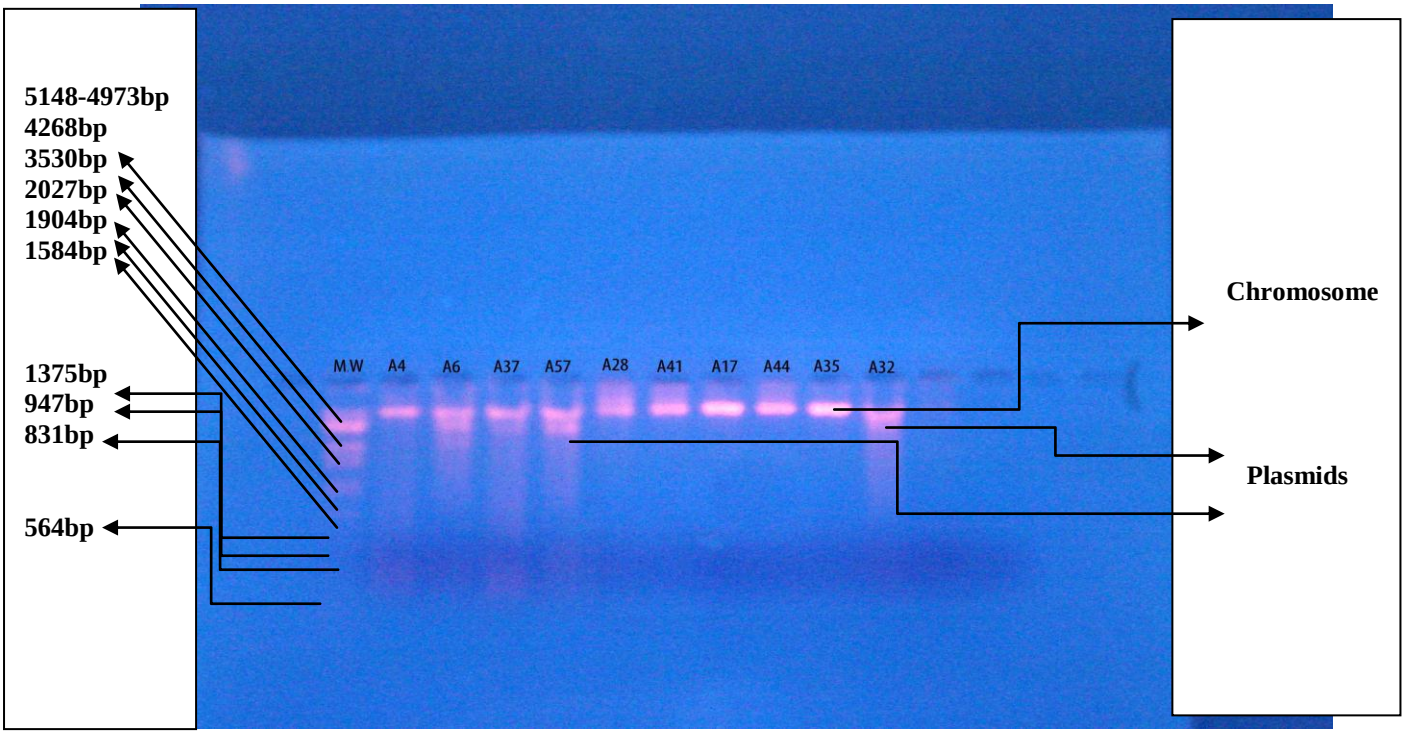

Figure 4: plasmid profile of E. coli strains isolated from UTIs patients Lane (A6, A37, A57, A32): Plasmid DNA extracted from E. coli strains; M.W: Molecular weight marker of lambda DNA digested with EcoRI+HindIII . Electrophoresis was carried in 0.8% agarose gel at (2V/Cm) for 60 min.

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