PLASMID PROFILING OF EXTENDED SPECTRUM B-LACTAMASES PRODUCING *ESCHERICHIA COLI* IN SOME HOSPITALS IN BAGHDAD

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ABSTRACT

This study was aimed to determine the prevalence of ESBLs in ***E. coli*** **and their relation to plasmid profiling patterns in a sample of patients in Iraqi hospitals. The total number of *E. coli* isolates 91 (49%) were collected out of 185 clinical samples. The antibiotic sensitivity test for isolates showed high resistance percentage to Nitrofurantion 95.7%, amoxicillin and Ceftriaxone to each of which 91.3% and to Ceftazidime 90.2%, while the highest sensitivity was observed against Imipenem 100%, Meropenem 99% then to Amikacin 93.5%. The Multi drug resistant (MDR) isolates were 53(58.2) % and ESBLs producers were 45 (49.5) %. Plasmid profile analysis showed nineteen different plasmid profile patterns were obtained based on molecular weight and number of plasmids content. Most β-lactamase producers had multiple plasmids, where's single plasmid profile predominant in non-β-lactamase-producing isolates. The presence of common plasmid among the isolates increases the distribution of resistant plasmid in the community. In conclusion, ESBLs producing bacteria can transferred between species by plasmids leading to cross resistance which give a limited options for treatment.**

Keywords: mdr, antibiotics, urinary tract infection and enterobacteriaceae

التشكيل البلازميدي في عينة من العزلات العراقية للاشيريشا القولونية المنتجة للبيتالاكتاميز الواسعة الطيف

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المستخلص:

هذه الدراسة هو تحديد مدى انتشار للبيتالاكتاميز الواسعة الطيف وعلاقتها بالتنميط البلازميدي في عينة من المرضى في المستشفيات العراقية. العدد الإجمالي لعزلات الإشريكية القولونية 91 (49)٪ تم جمعها من 185 عينة سريرية.أظهر اختبار الحساسية للمضادات نسبة مقاومة عالية للنيتروفوارنتيون 95.7٪ وللأموكسيسيلين وسيفاترايكسون لكل منهما 91.3 % و للسيفتازيديم 90.2٪ ،بينما لوحظ أعلى حساسية كانت للاميبينيم 100٪ وللميروبنيم 99 % وللاميكاسين 93.5%, كانت نسبة العزلات ذات المقاومة المتعددة هي 53(58.2) % والمنتجة للبيتالاكتاميز الواسعة هي الطيف 45 (49.5) %. أظهر تحليل تشكيل البلازميد وجود تسعة عشرنمطًا مختلفًا من أنماط البلازميد بناءً على الوزن الجزيي وعدد البلازميدات،اظهرت معظم العزلات المنتجة للبيتا لاكتاميز بلازميدات متعددة بينما اغلب العزلات غير المنتجة للبيتا لاكتاماز كانت من نمط البلازميد المنفرد. وجود البلازميد الشائع بين العزلات يزيد من انتشار البلازميدات المقاومة بالمجتمع. الخلاصة ان البكتريا المنتجة للبيتالاكتميز الواسع الطيف تنتقل بين الانواع بواسطة البلازميد وتؤدي الى مقاومة متقاطعة مما يعطي خيارات محدودة للعلاج

الكلمات المفتاحية: مقاومة الأدوية المتعددة، المضادات الحيوية، عدوى المسالك البولية، البكتريا المعوية

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INTRODUCTION

E. coli is probably one of the most commonly studied microorganisms and plays a significant role in medicine, biological sciences, and industry (12). The gram-negative bacteria contain genes with a significant role in antimicrobial agent resistance which located on the plasmids (21). Antimicrobial resistance considered as one of the most challenging problems in current empiric infectious diseases therapy. Often for the treatment of inflammatory diseases caused by Gram-negative bacteria, so these infections are usually treated with Cephalosporins, Fluoroquinolones, beta-lactams, as well as beta-lactamase inhibitors. Therefore, medication resistance is a serious problem in underdeveloped nations. Among them, the pathogens that producing via the prevalence of β-lactamases pose a danger to clinical microbiology (13). There is a steadily increasing worldwide in the number of *Enterobacteriaceae* that produce ESBLs on a worldwide scale (6). ESBL species have been discovered in *E. coli* and several Enterobacteriaceae-related Gram-negative bacteria (18). ESBLs are widespread, with much more than 1.5 billion persons infected with ESBL-producing *Enterobacteriaceae* (31). *E. coli* has become the most common type of bacteria to generate these newer lactamases which emerged and spread across the globe as a significant source of nosocomial and community diseases and is now a significant danger (7). The first approach to prevent the spread of these bacteria and avoiding any problems is the early identification of possible ESBL carriers (26). Plasmids serve a significant function in the evolution and dissemination of resistance genes between bacterial pathogens. Although the plasmid confers the evolutionary benefit for antibiotics, acquisition of plasmid tends to create widespread metabolic changes within the bacterial host (27). The majority of ESBLs resistant genes are usually produced through plasmids, especially in *E .coli* isolates (8, 19), Plasmid profiling assists in determining the possibility of propagation of resistance genes. Plasmid profiles are significant and effective in outbreak monitoring as well as antibiotic resistance tracking (29). The purpose of this study was to find out the prevalence of extended-spectrum B lactamase (ESBL) genes among multidrug-resistance *E. coli.* clinical isolates and their correlation with plasmid profiling patterns among patients with *E. coli* infections that were collected from several hospitals in Baghdad.

MATERIALS AND METHODS

Sample's collection

During October 2020 and January 2021, a total of one hundred eighty-five clinical specimens (urine, fluid, sputum, stool, blood, high vaginal swabs, liver abscess, and genital swabs) were collected from patients who were admitted into two hospitals (Al- Imamen Al-kadhimaein medical city and Child Center hospital under controlled conditions. All clinical isolates were identified by using standard biochemical tests.

Identification and distinction of *E. coli* from other lactose-positive organisms

All samples were cultivated in (nutrient broth) at 37°C for 18 to 24 h. After that streaks plate sub-cultured onto MacConkey agar and EMB agar, and identification of these bacteria according to Colony morphology (9, 20), Gram Stain, biochemical test and recognition and detection of bacteria via the BIOMÉRIEUX VITEK 2 program.

Antibiotic susceptibility Test for *E. coli*

According to the Clinical and Laboratory Standards Institute recommendations, the antibiotics susceptibility testing was performed using the Kirby Bauer disc diffusion method on Mueller Hinton agar (MHA) (10). Via utilizing commercially available antimicrobial disks of himedia Manufacturers (Mumbai, India). Imipenem (10µg), Nitrofurantoin (300 µg), levofloxacin (5 µg), Amikacin (30µg), Augmentin (30 µg) Gentamicin (10µg), iperacillin/tazobactam (100/10 µg), Meropenem (10µg), Doxycycline (10µg), Amoxicillin (10µg), Cotrimoxazole (25µg), Cefotaxime (30µg), Cefixime (30µg), Ceftriaxone (30µg), ceftazidime (30µg), Cefoxitin (30µg)} where also tested. Multidrug-resistant *E. coli* was described as *E. coli* that was resistant to three groups or more groups (17). *E. coli* isolates susceptibility to various anti-microbial agents tested by using the single disc diffusion process (4, 1).

Double disc Synergy test (DDST): The synergy test is a phenotypic assay that can be used to assess whether or not an ESBL is present. The hypothesis revolves around the formation of a synergistic relationship between Cephalosporins and Amoxicillin/ Clavulanicacid. Cephalosporins (Ceftazidime, Cefotaxime, and Ceftriaxone) were placed at distances of 30 mm (edge to edge) from the Amoxycillin/Clavulanic acid disc which placed in the middle of the plate. The result was deemed positive if there was an improved zone of inhibition between any of (the Cephalosporin antibiotics and the Amoxycillin/Clavulanic acid disc after a (24-hour) incubation period. This suggested the existence of an ESBL and synergistic interaction with Clavulanic acid, ESBL activity is detected (16).

Plasmid DNA extraction: For plasmid DNA extraction the miniprep plasmid DNA extraction kit (Qiagen\USA) were used according the manufacturer's instructions. For later use, the purified DNA plasmids were aliquoted and stored at -20 °C (2).

Agarose gel electrophoresis: Agarose gel was prepared in 1 % concentration in TBE buffer (1x). 5 µl (10 mg/ml) of ethidium bromide dye was used for staining. The gel electric current has been transmitted via the gel for 60 minutes at 120 v. A 48.5 Kb DNA ladder (Bio-lab\USA) was added with each run to detect the size of the plasmids (30). The positive control (*E. coli*\ BI21) and the Negative control (without template), both have been run within the samples during DNA extraction and gel electrophoresis.

RESULTS AND DISCUSSION

Sample Collection: A total number of 185 clinical samples were collected over Seven months (from October 2020 until the end of March 2021). The samples were selected randomly from several patients of different ages under sterile conditions., who were admitted to Imam Kadhimin Medical City and Child Center Hospital, which consisting of 105 (57.%) urine samples, 30 (16%) stool samples, 25 (14%) blood samples, 15(8.7%) wound swabs, 10 (5.4%) vaginal swabs, 9 (4.9%) Fluid samples, 5 (2.2%) sputum samples and 1 (0.5%) liver abscess swabs. The distribution of female to male among 185 samples (Figure 1) represented that the urine samples in female was 65 (35.2%) more than that in male 40 (21.7 %), this result in agreement with a study by Ani and Mgbechi (3), they showed that the UTI in women are more than males due to the genital tract structure (urethra is much shorter and closer to the anus than in males). The urine samples more than other clinical samples due to that the UTI infection is more frequent than other cases which may developed when bacteria pass easily from the anus can into the urinary tract.

Identification of *E. coli* : Identification of *E. coli* isolates were characterized according to Bergey's Manual of Systematic Bacteriology (5). Culture using MacConkey agar and EMB, morphology and biochemical features in addition to VITC system identification kit, were used for identification of all isolates (24).

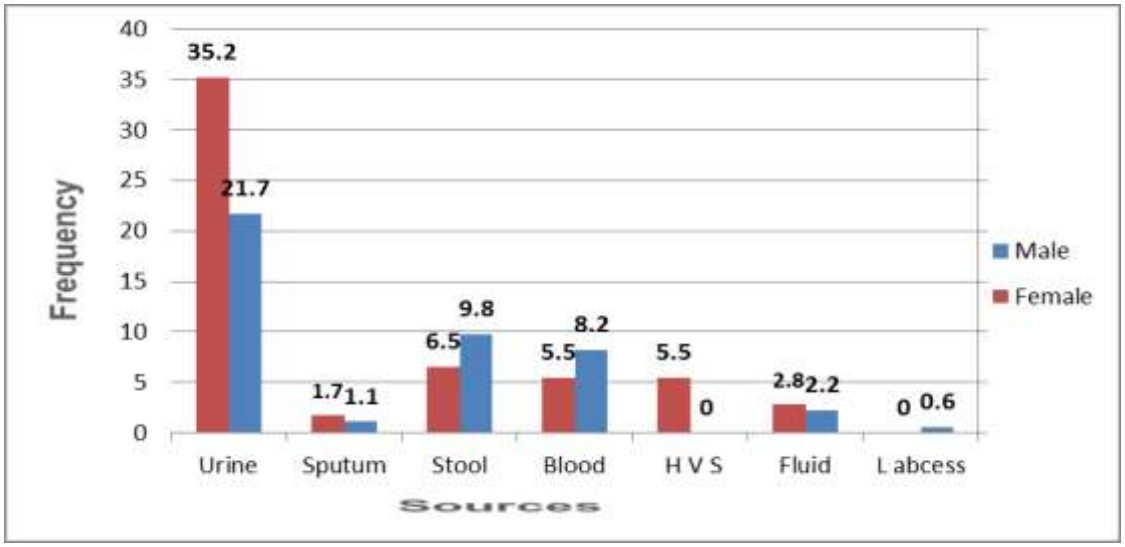


Figure 1. Percentage frequencies of sex distribution among 185 collected samples. HVS: high vascular swabs; L. abscess: liver.

The principle of this reaction is due to the presence of methylene blue dye and eosin Y, act as pH indicators which at acid pH combine to form a green-metallic precipitate and act as an inhibitors of gram-positive bacteria. Also, to isolate *E. coli*, the MacConkey agar was employed. It is like EMB agar, all too many G^{+ve} bacteria are inhibited from growing. *E. coli* differentiated from other gram-negative bacteria by producing pink colonies (Lactose-fermenting organisms) addition to colony morphology (11). The total number of *E. coli* isolates was 91 obtained from different ages and sources. The *E. coli* percentage among the total number was 49% which is in agreement with the study by Ngonzi and his colleagues (23), who got the same percentage among the collected samples. The identification was characterized as gram-negative which grown on selective media, while the mixed culture of the samples were excluded from this study. The colonies grown on MacConkey agar were confirmed by biochemical tests i.e. (IMViC), gram-negative isolates gave positive results for the indole, methyl-red test and gave a negative result for Voges-Proskauer and Citrate Utilization Test which confirms that the tested organism was *E coli.* As shown in Table (1), the *E. coli* isolates were more frequent in females 33 (36.3%) and more than that in male 20 (22%), the same results reported by Naqid team (22). There is significant differences between the total number of clinical isolates in female 49 (53.8%) and in male 42 (46.2%). The isolates from blood and stool samples showed the same ratio in male to female which is approximately 2:1 which represented as 9 (9.9%) isolates from male blood samples to 5 (5.5%) isolates from female blood samples, while the stool samples represented as 10 (11%) from male and 5 (5.5 %) from female. A study by Shams and her colleagues showed that the percentage of *E. coli* isolated from male stool samples was (54.2), while the percentage (45.8%) of *E. coli* isolated from female stool samples (28).

Table 1. Frequency and Percentage of 91 *E. coli* isolates among Gender and Source of samples

Sample source	Female		Male		Total No.	Total %
	No.	%	No.	%		
Urine	33	36.3	20	22	53	58.3
High vaginal swab	3	3.3	0	0	3	3.3
Blood	5	5.5	9	9.9	14	15.4
Stool	5	5.5	10	11	15	16.5
Fluid	2	2.2	1	1.1	3	3.3
Liver abscess	0	0	1	1.1	1	1.1
Sputum	1	1.1	1	1.1	2	2.2
Total	49	53.8	42	46.2	91	100

Regarding Figure (2), the distribution of 91 *E. coli* isolates among age group and gender showed that the female total isolates number was 50 (55%) and the male total isolates number. was 41 (45%). The age group (11-20), (21-30), (31-40) had the highest frequency in female 10 (11%) each of which, while the highest frequency in male at age group (31-40) was 9 (9.9%) then (41-50) was 8 (8.8%).

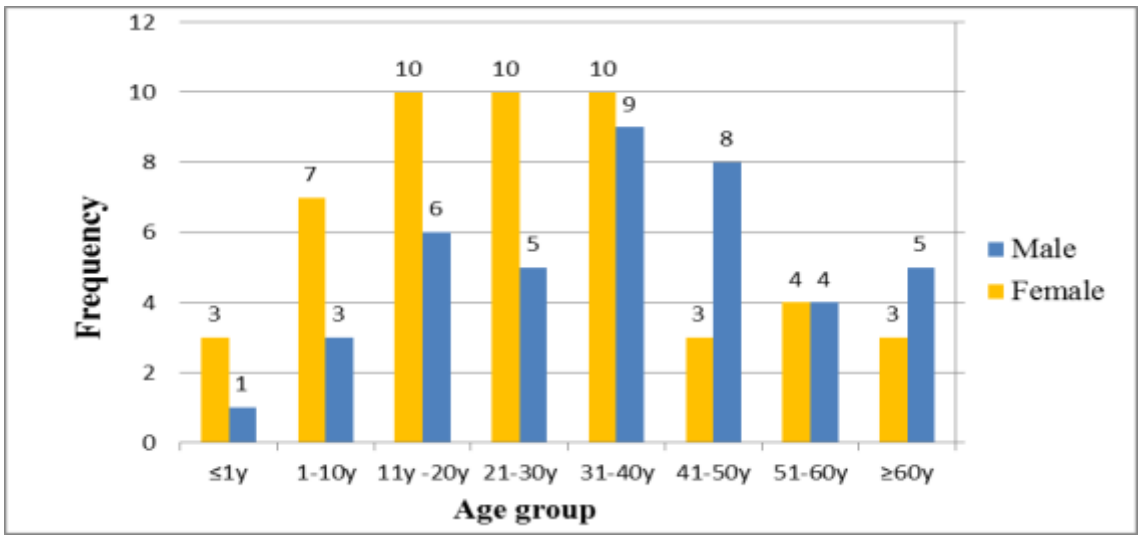


Figure 2. Age group distribution of 91 *E. coli* isolates among males and females

Antimicrobial susceptibility of *E. coli*

Single disk diffusion: The susceptibility of 91 isolates of *E. coli* was tested towards sixteen antimicrobial disks (Figure 3). The *E. coli* from different sources showed antibiotic sensitivity patterns with high resistance to commonly used antibiotics and displayed high resistance rates to Nitrofurantion (95.7) %, amoxicillin and Ceftriaxone to each of which (91.3) %, Ceftazidime (90.2) %, and to Cefixim (89.1) %, while the highest sensitivity was observed against Imipenem (100) %, Meropenem (99) % then to Amikacin (93.5) % which proposed to be selected by physicians as the most effective antibiotics. Naqid and his team showed that the most effective antibiotics against *E. coli* isolates were Ertapenem, Imipenem, and Nitrofurantoin (22). The MDR isolates Were 53 (58.2%). In comparing with a study by Odonkor and Addo showed that the prevalence of multidrug resistant *Escherichia coli* was 49.48% from drinking water (25). *E. coli* isolates showed the highest resistance towards Amoxicillin, Amoxicillin and Clavulanic Acid, Piperacilin, Doxycycline, Cotrimoxazole, and Levofloxacin, in addition to different types of cephalosporin, which results disagree with the study by Fatima and her colleagues, who represented that the MDR E. coli specimens showed the pattern with the greatest resistance against Imipenem which put it in a hazardous condition in Pakistan (14).

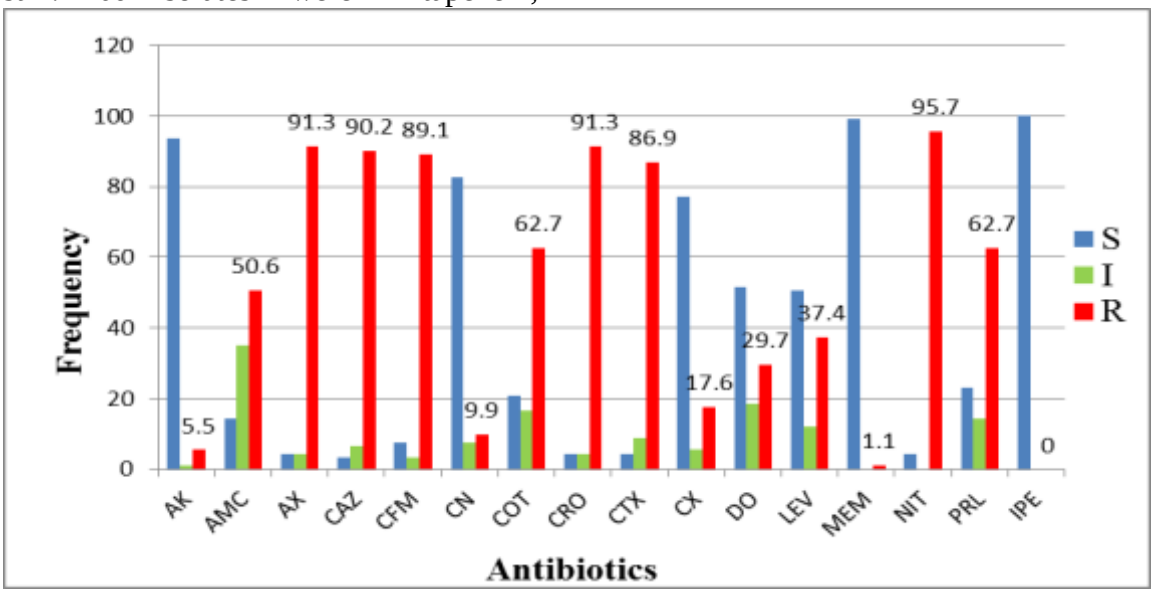


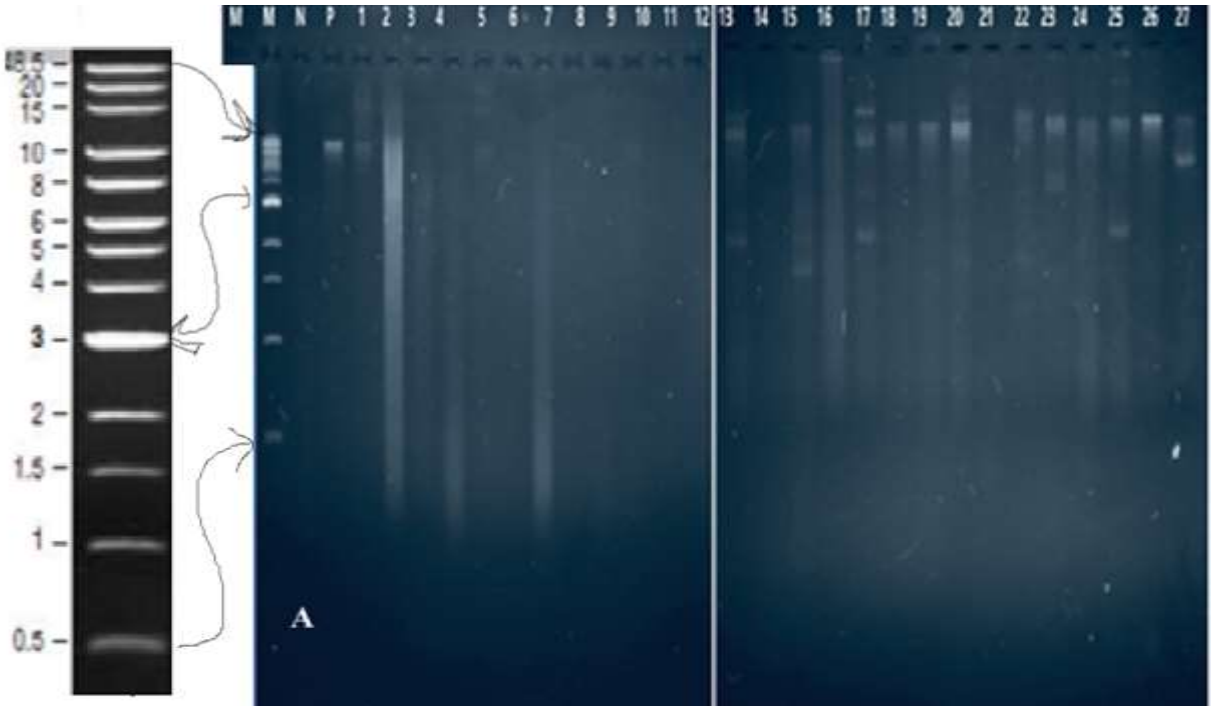
Figure 3. Antibiotic resistance was found in 91 *E. coli* specimens. MEM: Meropenem/ AX: Amoxicilin/ LEV: Levofloxacin/ CN: Gentamicin/ COT: Cotrimoxazole/ NIT: Nitrofurantion/ AMC: Amoxicilin and Clavulanic Acid/ CRO: Ceftriaxone/ CAZ: Ceftazidime/ IPE: Imipenem/ CX: Cefoxitin/ PRL: Piperacilin/ CFM: Cefixime/ CTX: Cephotaxime/ DO: Doxycycline/ AK: Amikacin. S: sensitive/ R: resist/ I: intermediate

Plasmid profile patterns

The DNA of plasmids which extracted from *E. coli* was separated on agarose gel stained with ethidium bromide according to molecular weight (Figure 4). In respect to molecular weight and number, various pattern plasmid profiles were observed. The total number was nineteen plasmids, distributed among seventy four isolates (Table 2). The number of plasmid profile was five in β-lactamase producers and only two plasmids related to not producing ESBLs isolates. Among plasmid profiling of isolates, only one was greater than 48.5 kb size and found in one isolate which related to β-lactamase producers. The rang of plasmid's sizes among all isolates was (0.5 kb to >48.5 kb) and plasmids number one to four and seven. Plasmid profile group have varied number of isolates ranged from 1 to 10. The Number of isolates with one plasmid was 26 (55.3%), two plasmids 10 (21.3%), followed by three plasmids 6 (12.8%), four plasmid 4 (8.5%) and Eight plasmids 1 (2.2%). Molecular weight pattern of 15kb was most frequently observed among 18 (38.3%) isolates, then (19.1%) isolates possessing one plasmid of 9 kb. Producing beta-lactamases isolates were most frequently associated with multiple plasmids, while non-producing βeta-lactamases isolates were predominantly harbor one plasmid. The ESBLs producers contain variable number of plasmids with a common one plasmid size (15kb). The molecular weight (0.5 kb) was the lowest plasmid found among all examined isolates, which present within the eight plasmid profile in β-lactamase-producing isolates. Plasmid profiling of *E. coli* isolates represent one plasmid >48.5 kb in (1) ESBLs producing isolate as a single plasmid and with another plasmids profile in eleven ESBLs producing isolates, which disagree with the result by Thapa Shrestha and his colleagues (30), who found that most isolates contain plasmid profile >33.5 kb. Most isolates containing plasmid number 2, 3, 4 and 8 in ESBLs producing isolate except the plasmid profile 7K, 6K, 1.5k which found in non ESBLs producing isolate. The molecular weight (0.5 kb to >48.5 kb) of plasmids were isolated from all ESBLs producing *E. coli*, except one plasmid with molecular weight 7kb possessed in non ESBLs producing isolate. In the same host cell, many profiles of plasmids were co-exist. The plasmid sizes in previous report ranged (0.12 kb up to 65 kb), for example 11.8 kb to 33.5 kb in south-western Nigeria and from 0.12 kb to 23 kb in west Nigeria (30). In conclusion, the plasmid with the same molecular size was present commonly in many isolates with the same β-lactamase production. The presence of this type of plasmid (resistant plasmids) among the isolates increases the distribution of resistance in community (30). The most predominant mechanism of β-lactams resistance among *E. coli* is the β-lactamases production due to continuous using many of β-lactams (15). The production of β-lactamase mediated by transferable genes (on chromosome or plasmid) which expressed by induction (external stimuli enhances gene transcription). The role of plasmid in resistancy is crucial and more important because, plasmids containing beta-lactamase genes are usually encod resistancy to other antibiotics like: Amino-glycosides, Sulfon-amides, Fluoro-quinolones, Tetra-cyclines and other antibiotics. ESBLs producing bacteria can transferred between species by plasmids leading to cross resistance which give a limited options for treatment. AmpCs, ESBLs and carbapenemases producer bacteria can be transferred between species through plasmids.

Table 2. Plasmid profiling distribution of *E. coli* Isolates in relation to β-Lactamase production

Plasmid No.	Molecular weight	No. of isolates	ESBLs	Non ESBLs
	9K	9	3	6
1	15K	10	4	6
	30K	6	4	2
	> 48.5 K	1	1	0
	15K, 20K	1	1	0
	15K, 3 K	3	3	0
2	>48.5, 15K	3	3	0
	15K, 4K	1	1	0
	>48.5, 20K	2	2	0
	20K, 15K, 3K	1	1	0
3	>48.5, 20K, 2.5k	2	2	0
	> 48.5, 48.5K, 2k	1	1	0
	15K, 6K,1.5k	1	1	0
	7K, 6K, 1.5k	1	0	1
	20K, 3K, 2.5K, 1K	1	1	0
	>48.5,10K, 3.2K, 2K	1	1	0
4	>48.5, 15K, 5K, 2K	1	1	0
	15K, 4K, 3K, 2K	1	1	0
8	>48.5 15K, 5.5k, 3.5K,	1	1	0
	2.3K, 0.5k, 1.2k, 1.5k			



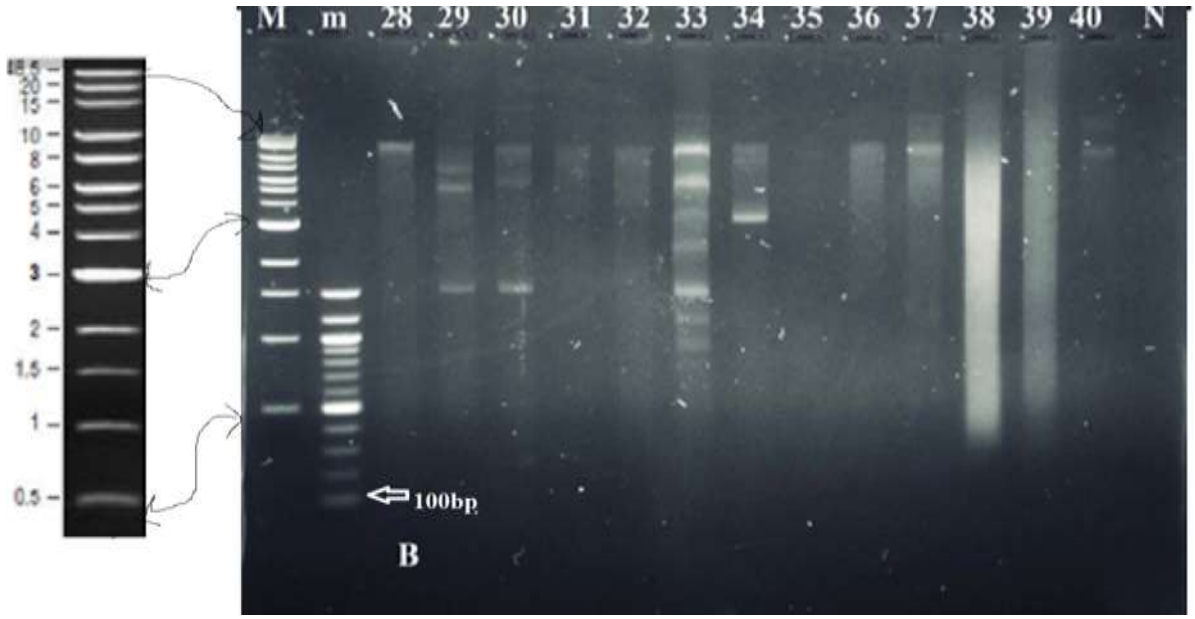


Figure 4 A, B. Separation of plasmid DNA molecular weight on agarose gel (1%) stained with ethidium bromide. The gel electric current has been transmitted via the gel for 60 minutes at 120 v. Llanes (1–40): plasmid DNA of clinical isolated *E. coli*; Lane (M) marker DNA 48.5 kb; Lane (m): DNA marker 1.5kb; P: positive control and N: negative control.

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