



Antibiotic Resistance Study and Detection of Virulence

Gene among Uropathogenic *E.coli*

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Received date: 4 / 1 / 2015

Accepted date: 8 / 3 / 2015

ABSTRACT

A total of two hundred and fourteen outpatient and inpatient children their ages ranged from 1day to 14 years, whether symptomatic or asymptomatic were studied for urinary tract infections (UTIs) during the period (February to May 2012) at Rapareen Teaching Hospital for Children in Erbil City. Urine samples were collected and examined using microscopic, dipstick test for detection of leukocyte esterase and culture techniques. Isolated organisms were identified using microscopical, morphological and biochemical tests (including recent Vitek 2 system) and the results showed that positive urine cultures were detected in 134 (62.6 %) children, among females were 98 (73 %), while among males were 36 (27 %). Microorganisms that had been isolated from urine culture were mainly Escherichia coli 70 (52.2 %) followed by others. The isolates appeared to be varied in their resistance to antibiotics, and the highest resistance were for ampicillin 67 (95.7 %), while the most effective antibiotic used was imipenem .Total E.coli isolates also tested for their ability to produce hemolysin and the results showed that 27 (38.5%) were α -hemolysin, 9 (12.8%) β -hemolysin and 34(48.5%) were γ -hemolysin producers.. Out of 70 Escherichia coli isolates, 41 (58.5%) were found to be ESBLs producers, while 29 (41.4%) were non ESBLs producers. The plasmid profile of studied E.coli isolates revealed that plasmids were found in 64 (91.6%) of isolates with different molecular weights ranged from 1- more than10 kbp. Curing of plasmid DNA content were conducted using sodium dodecyl sulfate (SDS) and elevated temperature at 46C°. The results revealed that the curing percentage for isolate E3 was (50%) while for isolate E25

was (41.6%) for antibiotics under study. On the other hand these two isolates when incubated at 46 C° the curing percentage were (66.6%) and (58.3%) respectively. Also the cured colonies tested for their ability to produce ESBLs and the results demonstrated that the cured colonies cannot produce ESBL indicating that the genes responsible for this trait are plasmid situated. Among 70 UPEC isolates, 61(87.1%) UPEC were positive for the existence of aerobactin virulence gene using PCR.

Keywords: *Uropathogenic E.coli , antibiotic resistance, , and virulence gene.*

دراسة مقاومة المضادات الحيوية والكشف عن الجين الضاري في بكتيريا اشيرشيا

كولاي للمجاري البولية

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تاريخ قبول البحث: 8 / 3 / 2015

تاريخ استلام البحث: 4 / 1 / 2015

الملخص

شملت الدراسة جمع 214 عينة ادرار من الاطفال المرضى الرافقين في المستشفى رايرين والمرضى الوافدين الى العيادة الاستشارية سواء لديهم اعراض المرض او بدون اعراض والتي بلغت اعمارهم من يوم واحد الى أربع عشرة سنة من حيث اصابتهم بالتهاب المجاري البولية في مستشفى رايرين للأطفال في مدينة اربيل في الفترة ما بين شباط ومايس 2012 .

جمعت عينات البول وفحصت بالعين المجردة، باستعمال المجهر؛ اختبار **dipstick** للكشف عن انزيم **leukocyte esterase** والزرع المختبري. تم تشخيص البكتيريا المعزولة بوساطة الصفات المظهرية، المزرعية، الاختبارات البايوكيميائية واختبار نظام **Vitek 2**. تم الحصول على نتيجة زرعية موجبة (زرع الادrar) في 134 (62.6%) طفل و طفلة، 98 (

(73%) حالة للإناث و 36 (27%) حالة للذكور، حيث أظهرت الدراسة أن الأصابة بالتهاب المجاري البولية لدى الإناث أعلى نسبة مقارنة بالذكور. مثلت بكتيريا *Escherichia coli* (52.2%) 70 أعلى نسبة يليها بقية الأنواع. حيث بيّنت اختبارات الحساسية للمضادات الحيوية على عينات المزارع الإيجابية أن نسبة المقاومة للمضادات الحيوية المستخدمة كانت للامبسيلين (95.7 %)، وتبين أن المضاد الحيوي أميبينيم الأكثر كفاءة من المضادات الأخرى ضد عزلات الـ *UPEC* حيث كانت جميع العزلات حساسة ولم تبد أيّة مقاومة. كما و اختبرت جميع عزلات بكتيريا *Escherichia coli* لمعروفة قابليتها لانتاج الإنزيم الحال للدم *hemolysin*، والناتج أظهرت أن (38.5%) 27 من العزلات كانت منتجة لـ *α-hemolysin* و (12.8%) 9 منتجة لنوع *β-hemolysin* و (48.5%) 34 منتجة لنوع *γ-hemolysin*. كما تم دراسة قابلية بكتيريا *Escherichia coli* المعزولة لانتاج إنزيم بيتا لاكتاميز واسع الطيف *ESBLs*، واظهرت الناتج أن (58.5 %) من العزلات كانت موجبة لـ *ESBLs* و (41.4 %) سالبة لـ *ESBLs*. أستخدم الترحيل الكهربائي للهلامي لدراسة محتوى الدنا البلازميدي وكانت البلازميدات موجودة في (91.6%) 64 من العزلات، و بأحجام تراوحت بين 1 إلى أكثر من 10 kbp. للسيطرة على مقاومة عزلات *E. coli* للمضادات الحيوية وبواسطة التحديد البلازميدي ، تم استخدام *SDS* بتركيز (2% وزن / حجم) و الحرارة المرتفعة 46°C ولهذا الاختبار تم اختيار عزلتين مقاومتين للمضادات الحيوية ، حيث تم تحديد المورثات المانحة لمقاومة للعزلة *E3* وبنسبة (50 %) والمورثات المانحة لمقاومة للعزلة *E34* بنسبة (41.6%). كما و أستخدمت درجات الحرارة المرتفعة 46°C وأظهرت الناتج أن لها تأثير واضح على تحديد المورثات المانحة لمقاومة الاميکاسين للعزلة *E3* بنسبة (66.6 %) وللعزلة *E25* بنسبة (58.3 %). أثبتت الدراسة أن التحديد البلازميدي باستخدام درجات الحرارة المرتفعة أكثر كفاءة" من عامل *SDS*. تم التحري عن انتاج إنزيم البيتا لاكتاميز واسع الطيف في المستعمرات التي تم تحبيدها واظهرت الناتج عدم قدرة هذه المستعمرات لانتاج الإنزيم المذكور مؤكداً ان المورثات المسئولة عن انتاج هذه الصفة تقع على بلازميد العزلات. كانت الناتج موجبة في (87.1%) 61 من ضمن 70 عزلة *UPEC* ، من حيث وجود (*aer gene*) في هذه العزلات باستخدام *PCR*.

الكلمات الدالة: اشبرشا كولاي للمجاري البولية، مقاومة المضادات الحيوية، والجين الضارى.



1. INTRODUCTION

Urinary tract infection, or UTI, is an infection that can happen anywhere along the urinary tract. Urinary tract infections have different names, depending on what part of the urinary tract is infected [1]. Uropathogenic *Escherichia coli* (UPEC) is the most frequent agent causing UTI in adults and children [2]. At least 80% of urinary tract infections in children are caused by *Escherichia coli* followed by *Klebsiella spp.*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Enterobacter spp.* [3]. While 10% of UTIs are caused by gram positive bacteria include *Staphylococci*, *Enterococci* and *Streptococci* [4]. Pathogenic potential of *E. coli* strains is thought to be dependent on the presence of virulence factors (VFs), which are located either on bacterial plasmid or on chromosome. The virulence genes most commonly associated with UPEC include P fimbriae (*pap*), afimbrial adhesin I (*afal*), aerobactin (*aer*), type 1 fimbriae, hemolysin (*hly*), S fimbriae (*sfa*), cytotoxic necrotizing factor 1 (*cnf1*), adhesins and fimbriae [5]. Virulence factors are encoded by genes located on the chromosome or on the plasmids. The location of virulence factors on such genetic mobile elements may facilitate the spread of virulence properties within bacterial communities [6]. Increasing rates of resistance to antibiotics among uropathogens have caused growing concern in both developed and developing countries [7]. Understanding antibiotic resistance patterns and molecular characterization of plasmids and other genetic elements is epidemiologically useful [8]. Plasmids allow the movement of genetic material, including antimicrobial resistance genes between bacterial species and genera. Controlling of antibiotic resistance at the molecular levels such as using curing agents may limit or decrease the resistance of pathogenic bacteria [9]. Curing may occur naturally through cell division or by treating the cells with chemical and physical agents [10]. Therefore the aim of this study are isolation and identification of uropathogenic *E. coli* (UPEC), study the antibiotic resistance patterns of these isolates to different antimicrobials, elimination the bacterial resistance to antibiotics for some isolates by curing the plasmid using SDS and elevated temperature, and finally detection of virulence genes (Aerobactin gene) of *E.coli* isolates using Polymerase Chain Reaction (PCR) assay and gel electrophoresis.

2.MATERIAL AND METHODS

Collection of samples:

This study was carried out during the period 25th Feb.2012 to 17th June 2012. A total of two hundred fourteen (214) urine specimens were collected from infants and children, aged one day-14 years attending (Rapareen Teaching Hospital for Children in Erbil City). Provisional diagnosis of urinary tract infections made by pediatrician. All urine specimens were obtained by mid stream clean – catch, catheterization or from urine bags. The specimens were transferred to the laboratory and processed within half an hour of collection.

Microscopic examination:

For microscopic examination, about 5ml of urine was centrifuged (2000 rpm for 5 min.), the supernatant was discarded and a drop (0.1ml) of the sediment was placed on a clean microscopic slide. The preparation was examined under high power (x40) lens. The common finding expected to be seen are: Pus cell (white blood cell), red blood cell (RBC), casts, crystals, epithelial cells, bacteria and parasites.

Leukocyte esterase test (LE):

This test was used to detect leukocyte esterase enzyme released from leukocytes in urine using urine test strip. The positive result depend on changing the color of strip from yellow to deep brown [11].

Urine culture (isolation of *E.coli*):

A loop full of undiluted urine samples was spreaded on appropriate culture media (blood, MacConkey agar and CLED agar) plates. The plates were incubated overnight at 37C°. Well isolated single colonies were sub-cultured on the same media to check for the purity of the isolated bacteria. Purified isolates were identified using microscopical, morphological, biochemical and Vitek 2 system as a more accurate method for identification.

Bacterial count and significant bacteriuria:

Urine culture is based on colony forming units per ml (CFU/ ml). The diagnosis of urinary tract infection is based on a positive urine culture of $\geq 10^5$ colony-forming units/ml [12].The



number of colonies were counted by spreading 2 μ l standard loop full of well mixed urine specimen on nutrient agar plate, incubated overnight at 37C°. The number of colonies were then multiplied by 500 (standard factor) [11].

Antimicrobial Susceptibility Testing:

The antibiotic resistance pattern of seventy (70) *E.coli* isolates from urine specimens and laboratory strain *E.coli* DH5 α were screened for their resistance to thirteen widely used antibiotics. Disc diffusion method, also known as the Kirby- Bauer-method were used [13].The antibiotics were include: amikacin (AK), ampicillin(AMP), amoxicillin-clavulanic acid(AMK), chloramphenicol(C), ciprofloxacin(CIP), cephalothin(CEP), nitrofurantoin(F), ceftriaxone(CRO), cefotaxime(CTX), imipeneme(IMP), gentamycin(GM), nalidixic acid(NA) and trimethoprim-sulphomethaxazole(SXT).

Detection of extended spectrum β -lactamases (ESBLs):

A double disc diffusion test was performed with amoxicillin-clavulanic acid surrounded by aztreonam and third generation cephalosporin discs cefotaxime and ceftazidime [14,15].

Isolation of plasmid DNA content from bacterial isolates understudy:

DNA spin™ Plasmid DNA Purification Kits (Intron/ Korea) procedure is based on alkaline lysis method of bacterial cells followed by adsorption of DNA onto silca in the presence of high salt.

Study of plasmid profile purified from *E.coli* isolates agarose gel electrophoresis technique:

The method described by [16] with few modifications was followed and include the following steps: The edges of a clean, dry glass plate sealed with tape to form a mold. The mold was set on a horizontal section of the bench. Sufficient electrophoresis buffer prepared to fill the electrophoresis tank and to cast the gel. The slurry heated in microwave oven (at 98C° for about 2 minutes). The flask transferred into a water bath at 55C°. When the gel cooled, ethidium bromide were added to a final concentration of 0.5 μ g/ml (8 μ l ethidium bromide to 100 ml agarose). The gel mixed thoroughly. The warm agarose solution poured into the mold. The gel allowed to set completely (left to be solidified at room temperature 30-45 min) then carefully the comb

removed. Just enough electrophoresis buffer added to cover the gel to a depth of about 1mm). Samples of DNA were mixed with appropriate volume of loading buffer (bromophenol blue). The sample mixture slowly loaded into the slots of submerged gels using a disposable micropipette. Size standards was loaded into slots on both the right and left sides on the gels. The DNA will migrate toward the positive anode at 10 volt / cm for 1 hour, the gel run until the samples have migrated an appropriate distance through the gel. The electric current turned-off and the lid was removed. Finally we examined the gel by UV-transilluminator, and then photographed .

Plasmid curing assay

Sodium Dodecyl Sulfate (SDS):

Curing by SDS was done by the procedure mentioned by [17]. Five ml of LB broth containing appropriate antibiotic at final concentration inoculated with single colony of *E.coli* isolate , incubated at 37C° for 24 hours, after overnight 0.1 ml of the broth bacterial culture were inoculated into 5 ml LB broth containing 2% of SDS agents and incubated with shaking at 37C° for 48 hours. Serial dilutions were prepared up to 10⁻⁶, 0.1 ml from the last three dilutions were spreaded onto nutrient agar plates and incubated at 37C° for 24 hours. After incubatin time, fifty colonies were transferred to nutrient agar plate and incubated over night at 37C° representing the master plate. Five colonies were randomly choosen, picked up and transferred to Muller Hinton agar plates and tested for antibiotic sensitivity pattern and ESBLsproduction, incubated at 37C° for 24 hours. After overnight incubation the percentages of curing colonies for both antibiotic sensitivity pattern and ESBL production were calculated.

Elevated temperature (46C°):

This curing was carried out according to [18]. Ten ml of LB broth inoculated with single colony of *E.coli* isolate, after inoculation for 24 hours at 37C°, 10 ml LB broth culture inoculated with 0.2 ml of bacterial culture, incubated with shaking (100 rpm at 46C° for 24 hrs.). The same steps as in above procedure were repeated.



Detection of Aerobactin gene (*aer* gene) in *E.coli* isolates using PCR technique:

Detection of *aer* gene was performed by amplifying the gene by PCR. The primers sequences were obtained from (CINNAGEN company, Germany). Amplification was performed in a thermal cycler (Eppendorf, Germany) according to the methods described by [19]. Expected sizes of the amplicons were ascertained by electrophoresis in 1.2 % agarose gel with an appropriate molecular size marker (100-bp DNA ladder (Genedrix / Tiwan). All the 70 UPEC isolates were tested for presence of *aer* gene (a plasmid sequence associated with Uropathogenic *E. coli*), the primers sequences are: F- 5'-TACCGGATTGTATGCAGACCGT-3' and R 5'-AATATCTCCTCCAGTCCGGAGAAG -3' (19). The protocol for PCR technique include preparing of PCR mixture for each sample as follow:12.5 μ l master mix ,9.5 μ l nuclease free water,1 μ l primer (reverse),1 μ l primer (forward),1 μ l plasmid DNA template, total volume 25 μ l. All the steps were done on ice. These materials were mixed in a sterile amplification tube and transferred to thermal cycler and for PCR program including cycle, time, temperature and volumes, the following program was used: - 1 cycle of 94°C for 60 s, - 30 cycles of 94°C for 60 s, - 63°C for 30 s, - 72°C for 90 s followed by final cycle of 72°C for 30 s. The amplified products were visualized by ethidium bromide staining after gel electrophoresis of 10 μ L of the final reaction mixture in 1.2% agarose.

3.RESULTS AND DISCUSSION

Prevalence of microorganisms isolated from urine samples in relation to gender :

After collection of 214 urine specimens from children aged one day- 14 years expected to have UTIs we obtained 158 urine samples from females (73.8%) and 56 (26.2%) from males, the samples were examined microscopically (General urine examination) then cultured on appropriate media (CLED, MacConky and Blood agar) in order to isolate the bacteria causing infections. Out of the total samples, 134 (62.6%) were culture positive samples. From the positive culture, 98 (73%) were from female and 36 (27%) from male, while 80 (37.3%) of the samples were culture negative, of these, 60 (75%) were female and 20 (25%) were male .



The leukocyte esterase as diagnostic test for UTIs.

The results of leukocytes esterase test in relation to the results of urine culture revealed that out of 134 positive urine culture, 129 (96.2%) were leukocyte esterase positive.

Frequency of microorganisms isolates from urine samples

The most common microorganisms isolated was *Escherichia coli* (52.23%) followed by *Staphylococcus aureus* (20.9%), *Proteus mirabilis* (8.2%), *Staphylococcus saprophyticus* (6%), *Klebsiella pneumoniae* (5.2%), *Pseudomonas aeruginosa* (2.2%) and (1.5%) for each of *lactobacillus* spp., *Candida albicans* and *Streptococcus* spp. while the lowest percent of infection was caused by *Enterobacter* spp. found in only (0.7%) of isolates. Similar findings have been obtained by [20,21] from Nigeria and [22], from Iran, they obtained 51.5 % and 47.6 % respectively. [23 , 24] from Iran, [25] from India [26] from Erbil found that *E. coli* was the most common uropathogen identified at a rate of 32.8 %, 38 %, 30.2 % and 33.8 % respectively. The higher prevalence of *E. coli* may be due to fecal contamination, *E.coli* is a member of human colonic flora and spread to urethra from the anus. The strain that cause UTIs are able to do so because they produce factors that allow them to attach to the urinary epithelial mucosa [27].

Characterization of *E.coli* isolates

Identification of isolates was carried out according to microscopical, cultural, morphological, biochemical tests and the most precious system Vitek 2. Bacterial cells from smear preparation are gram negative short rods, motile, non-spore forming and presumptively are *Escherichia coli* which in accordance with previous observations [28] .In accordance to their pink colony appearance on MacConkey agar demonstrated as lactose fermenters, they produce bright metallic green sheen colonies on Eosine Methylene Blue (EMB) agar and grayish white moderately opaque with or without zone of hemolysis on blood agar, all *E.coli* isolates characterized by producing small, smooth, entire and convex colonies on blood agar. As well as the results of biochemical tests demonstrated that all bacterial isolates under study were negative for oxidase and positive for catalase. On Kligler Iron Agar (KIA) medium, all isolates of *E.coli* under study produce a yellow slant and a yellow butt A/A reaction due to the fermentation of lactose and glucose and no H2S production [29]. Uropathogenic *E. coli* produce alpha- and beta-hemolysins, which cause lysis of urinary tract cells. The results revealed that out of 70 *E.coli* isolates, 27

isolates were α -hemolytic, 9 isolates were β -hemolytic, and 34 isolates were γ - hemolytic representing 38.5 % , 12.8 % , and 48.5 % respectively . Our results agreed with those reported by [30] and [31] that found a large proportion of human extraintestinal *E.coli* isolates produce hemolysin representing (35-50) % and (35-60) % respectively, relative to normal fecal isolates 10 % only. Also they mentioned that epidemiological evidence indicates a role for alpha-hemolysin in extraintestinal human infections.

Antibiotic resistance pattern

Table (1) shows the percentage of resistant bacterial isolates to different antibiotics under study. It's obvious from the Table (1) the percentages of resistant to antibiotics were as follow: 95.7% to ampicillin, 94.2% to cephalothin, 91.4% to each amoxicillin-clavulanic acid and sulfamethaxazole -trimethoprim, 87% to cefotaxime, 74.2% to nalidixic acid, 68.5% to gentamycin, 65.7% to ceftriaxone, 44% to nitrofurantoin, 32.8% to ciprofloxacin, 30% to chloramphenicol and 27% to amikacin, whereas all isolates were sensitive to imipenem. Multiple resistance among isolates were common. The results revealed high resistance to ampicillin 95.7 % , similar results were reported by [32,33,34], they found that resistance of *E.coli* isolated from urinary tract infections to ampicillin were 90 %, 95 % and 92 % respectively. High resistance to amoxicillin - clavulanic acid reported with a percentages of 91.4 %, this result contradicts those reported by [35] who found that most of their *E.coli* isolates were sensitive to amoxicillin- clavulanic acid, which frequently used in the treatment of UTIs. Trimethoprim-sulfamethoxazole (TM-SXT) with high resistance rate 91.4 % was reported in our study, its in agreement with results obtained by [36] who reported 88 % resistance. In present study, the most effective antibiotics against uropathogen isolates were nitrofurantoin, ciprofloxacin and amikacin. These results do agree with those reported by [37] who found that amikacin and nitrofurantoin showed good activity against uropathogen isolates. (38) showed that the resistance to nitrofurantoin which is one of the oldest urinary anti- infective drugs in use remains minima. [39] stated that the amikacin have good anti- *Pseudomonas* activity and other bacteria that are resistant to other antibiotics, and is usually recommended for serious UTI. Our results showed that most isolates were highly sensitive to ciprofloxacin. These results parallel with those reported by [40] and [41]. This is due to that the ciprofloxacin is newly used in treatment in

comparison with other antibiotics. No resistance to imipenem was observed in the studied isolates. The sensitivity to imipenem was 100 %. These findings were similar to the study done by [42] and similar results reported by [43] who obtained 100 % sensitivity of *E.coli* isolates for imipenem. [44] stated that amoxillin is a safe choice for the treatment of urinary tract infection, but in recent years *E.coli* has become increasingly resistant to this antibiotic. These variation of antibiotic resistance in *E.coli* return to the genes that located on conjugant plasmids mostly and transferred by conjugation, transformation or transduction processes, to susceptible recipient *E.coli*, or the resistance genes may be located on bacterial chromosome and jumped to resistant plasmids by transposition process, many composite transposons contain genes for antibiotic resistance, and some bear more than one resistance genes, this is found in both G-ve and G+ve bacteria [45]. To reduce the rate, urine culture and antibiotic sensitivity test prior to the onset of the main treatment seems highly recommended. Also, choice of antibiotic should be based on the site of infection at the patients suspected for resistant strains is another effective measure. To fight against the bacterial resistance gene in their genomes, new generation and semi synthetic antibiotics should be produced or even other therapeutic agents including bacteriophage be suggested [46].

Table (1): Number and percent of resistant *E.coli* to antibiotics used

Antibiotics Used	*AK	AMP	AMC	CIP	CEP	NA	SXT	CRO	IP M	F	C	CTX	GM
No. of resistant isolates	18	67	64	23	66	52	64	46	0	31	21	61	48
Resistance %	27%	95.7	91.4	32.8	94.2	74.2	91.4	65.7	0%	44	30	87%	68.5

Frequency of ESBLs producing *E.coli* (phenotypic detection)

Seventy isolates of *E.coli* were screened for ESBLs production by double disk diffusion method. Of this 41(58.57%) isolates were found to be ESBLs positive, whereas 29 (41.43%) were ESBL negative. The results in agreement with [47] who reported 60 % of *E.coli* isolated from clinical sources were ESBLs positive. [48] in India reported that production of ESBL was noted in *E. coli* at a rate of 66.78 %. [49] showed that multi-drug resistance and ESBLs producing gram negative bacteria are the major cause of infection of the urinary tract. Strains harboring multiple plasmids simultaneously exhibit co resistance to different classes of antibiotics. Association of plasmid-mediated quinolone resistance with ESBLs is well documented by [50] Another study by [51] stated that ESBL producers express their β -lactamase genes from plasmids, these findings suggest that gene coding for ESBLs and resistance to other classes of antibiotics may reside within the same plasmid and therefore be spread together. Plasmid profiling is also an important tool for epidemiological typing and has got diagnostic value as well.

Plasmid profile of *E.coli* isolates

The results revealed that among 70 *E. coli* isolates, 64 (91.6 %) isolates harbored from one to seven bands and the size of the bands ranged from 1Kbp to more than 10Kbp .Figure (1) shows the plasmid profile of nine tested isolates which contain bands with different molecular weights. The reported results indicate the dissemination of plasmids among *E. coli* isolates which may be carrying resistant genes against wide spectrum of clinically used antibiotics, which may explain the reason of evolution antibiotic resistant patterns in studied bacterial cultures, similar results were obtain by (52). Various investigators reported that most *E. coli* isolates contain at least one large plasmid in addition to other smaller plasmids, which encode for several virulence factors such as hemolysin, toxins, siderophores production (aerobactin), in addition to antibiotic resistant markers [53,54]. In a research conducted by [55] on *E.coli*, he demonstrated that the plasmid sizes were from (1.5– 54) Kbp. [56] reported that a range of (2 – 22) Kbp for plasmid sizes among *E. coli* isolates. [57] have also reported plasmid size variations between 3.9 Kbp and 50 Kbp in *E. coli* strains. On the other hand, some of isolates have no plasmids, yet they were resistant to a large number of antibiotics. Possibly, some antibiotic resistance genes may not be

located on the plasmid but may be on the bacterial chromosome or on transposable elements (Transposon) [7].

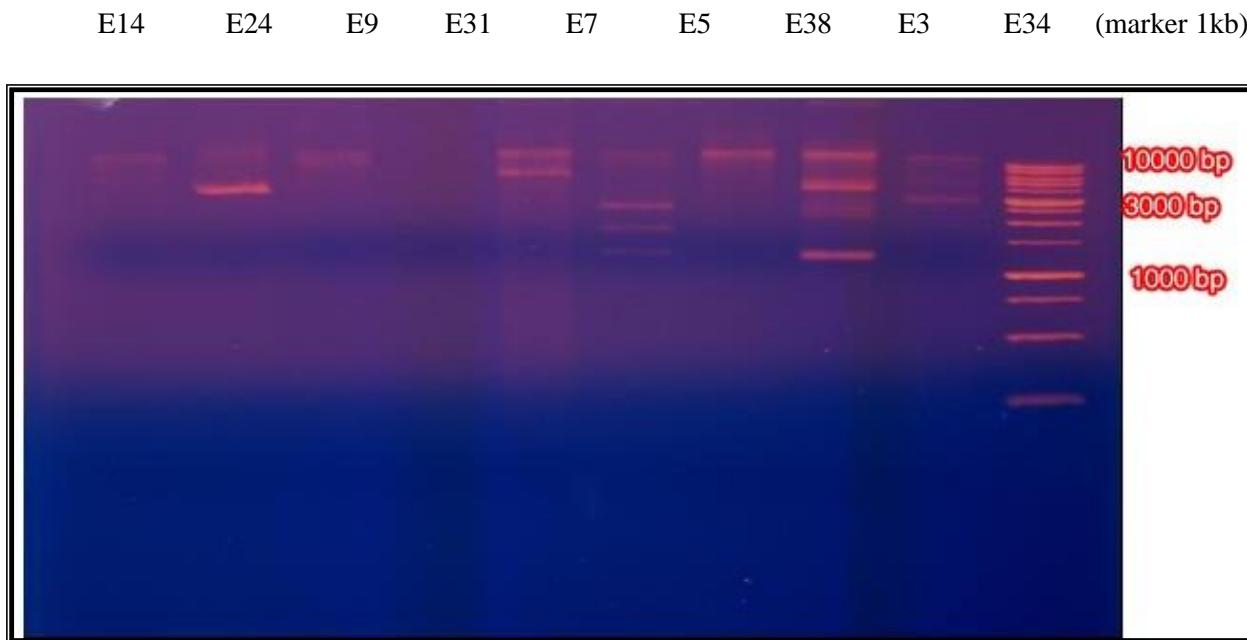


Figure (1) :Plasmid profile of nine tested *E.coli* isolates

Plasmid curing

Curing by sodium dodecyle sulphate (SDS) and elevated temperature

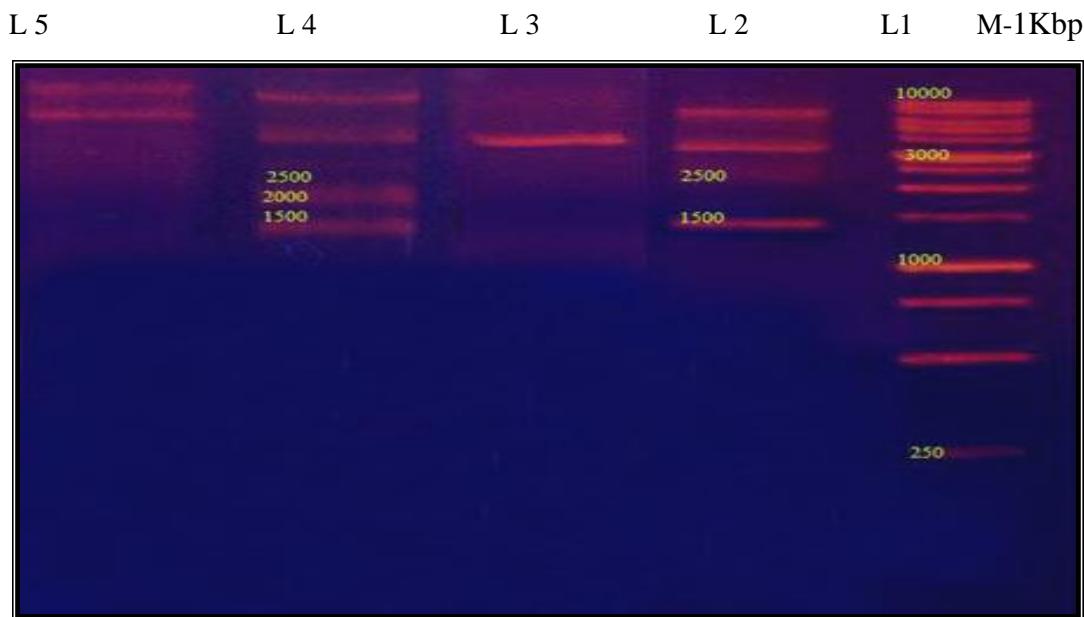
Table (2) show the curing percent of plasmid DNA from *E.coli* isolates by SDS. For E3 isolate, SDS affect CIP, CRO, C, CTX, GM and AK genes, the resistance rate of E3 isolate decreased and the percent of curing reached (50%), while for E25 isolate, it affect the genes which were responsible for CIP, CRO, F, GM and SXT and the curing percent was (41.6%).

Figure (2) shows the plasmid DNA profile for *E.coli* isolates E3 and E25, and it is clear that different plasmid DNA exist in the E3 and E25 isolates before treating with SDS and after treating and revealed that the SDS reduced the plasmid DNA content for E3 to (2) bands and showed missing of 2 plasmid bands (1500 and 2500 bp in size), while for E25, 3 plasmid bands (1500, 2000 and 2500 bp in size) were lost after using 2% SDS with exceeded incubation time to 48 hours. [58] found that studied *E. coli* isolates that were resistant to gentamicin, neomycin, rifampicin and imipenem when cultured in absence of 1% SDS solution but became

sensitive when cultured in presence of 1% SDS solution. It may be noted that copies of the plasmid lying closer to the membranes are completely eliminated by chemical agents while those lying closer to the nucleus may escape the curing effect, thereby; one may observe partial curing [59].The effectiveness of curing methods depends on the nature of the bacterial host and/or plasmids where some may work better than the others. Plasmid loss in cured *E. coli* cells resulted in the disappearance of the outer membrane components and a concomitant change in the thickness of the peptidoglycan layer [60].

Table (2): Curing of plasmid DNA from E3 and E25 using 2% SDS agent

Bac. Isolates	Antibiotics resistant pattern														% of curing
	AM P	CIP	AM C	N A	CR O	F	C	CT X	G M	A K	SXT	IPM	CEP		
E3	R	R	R	R	R	R	R	R	R	R	R	S	R		
E3+ SDS	R	S	R	R	S	R	S	S	S	S	R	S	R		50%
E25	R	R	R	R	R	R	R	R	R	R	R	S	R		
E25+S DS	R	S	R	R	S	S	R	R	S	R	S	S	R		41.6%

**Figure (2):** Plasmid profile of cured E3 and E25 using 2% SDS

Lane 1 Ladder 1Kb Lane 2 plasmid profile of E3 Lane 3 plasmid profile of E3
after curing with SDS Lane 4 plasmid profile of E25 isolate Lane 5 plsmid profile of
E25 after curing with SDS

Elevated temperature at 46C° was used for elimination of plasmid DNA in *E.coli* isolates E3 and E25. Table (3) shows that E3 when treated with elevated temperature at 46C°, the CIP, CRO, F, C, CTX, GM, SXT and AK genes were affected and the curing percent was (66.6%), while when E25 isolate treated with elevated temperature at 46C°, genes which responsible for CIP, CRO, F, CTX, GM, SXT and AK resistance were affected with the percentages of (58.3%). Figure (3) showed missing of 3 bands of plasmid after treating E3 with elevated temperature (1500, 2500 and 3000bp in size), while it showed missing of 4 bands of plasmid after treating E25 with elevated temperature (1500, 2000, 2500, 5000 bp in size). Our finding agreed with results obtained by [61] that they studied the effect of high temperature on plasmid DNA of two *P.aeruginosa* isolates and they found sensitivity of isolates to all used antibiotics except lincomycin after incubating at high temperature and they concluded that curing by elevated temperature is the most efficient method among others. [62] studied the effect of elevated temperature on eliminating plasmid from *E. coli* isolates and the results indicated that CEP, CTX

and CRO determinant were plasmid encoding and they concluded that this may due to the existence of two separate R-plasmids or one plasmid is carrying these antibiotic resistance genes. Many researchers demonstrated the mechanism by which the elevated temperature create curing of plasmid DNA, of these, the effect of elevated temperature on plasmid curing may be due to decreasing the amount of synthesized DNA. This may be due to the fact that the enzymes which contribute in the DNA replication processes are more affected by this high temperature.

Table (3) :Curing of plasmid DNA from E3 and E25 isolates by using elevated tempreature

Antibiotic resistant pattern															
Bac.	AMP	CIP	AMC	NA	CRO	F	C	CTX	GM	AK	SXT	IPM	CEP	% of curing	
E3	R	R	R	R	R	R	R	R	R	R	R	S	R	66.6%	
E3+e.T emp.	R	S	R	R	S	S	S	S	S	S	S	S	R		
E25	R	R	R	R	R	R	R	R	R	R	R	S	R	58.3%	
E25+e. Temp.	R	S	R	R	S	S	R	S	S	S	S	S	R		

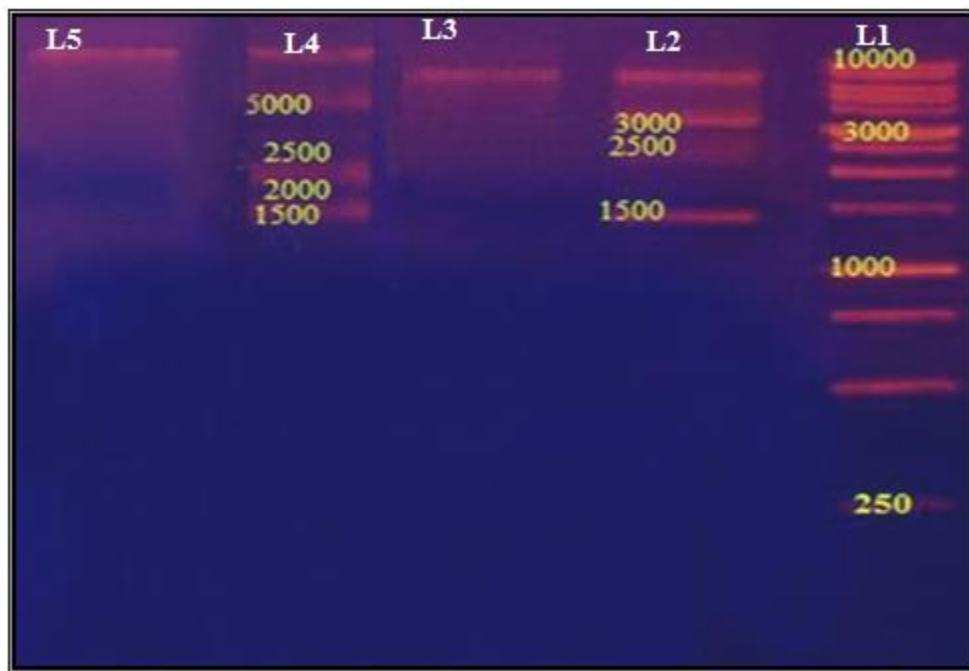


Figure (3): Plasmid profile of cured E3 and E25 by elevated Temperature

Lane 1 Ladder 1Kb, Lane 2 plasmid profile of E3 ,Lane 3 plasmid profile after curing E3 with elevated temperature, Lane 4 plasmid profile of E25 isolate , Lane 5 plasmid profile of E25 after curing with elevated temperature.

The prevalence of Aerobactin gene among *E.coli* isolates

Results in Figure (4) indicated that *E.coli* isolates which contain *aer* primer, exhibit positive PCR product on gel electrophoresis for 61 (87%) samples among 70 samples tested for the presence of *aer* gene. Aerobactin is a bacterial iron sequestration and transport system which enables *E. coli* to grow in iron- poor environments such as dilute urine. Bacterial siderophores compete for iron with host iron-binding proteins. When bound by the siderophore, the iron is taken up by special bacterial surface receptors and can be utilized by the pathogen; many strains of *E. coli* associated with UTI produce siderophore [63]. Our results were in agreement with other investigations. Rate of 79 % was reported by [64], and 78 % reported by [65] , also [66]

who reported (93.3 %) of isolates that were gave positive results for aerobactin production . In the study of [67], the prevalence of siderophore aerobactin were 69 % of their *E.coli* isolates causing pyelonephritis . In the study of [68] 76.6 % of UPEC isolates were positive for aerobactin production. Such differences in prevalence of UPEC aerobactin (*aer* gene) might be due to divergences in geographical variation or differences in association with host characteristics and even weather climate of each regions. It seems that the epidemiology and prevalence of virulence factors of UPEC strains isolated from patients with UTI are different .Probably, customs, food diets, the levels of public health, hospital's health and even methods of sampling have great rules in prevalence of virulence genes of UPEC strains.

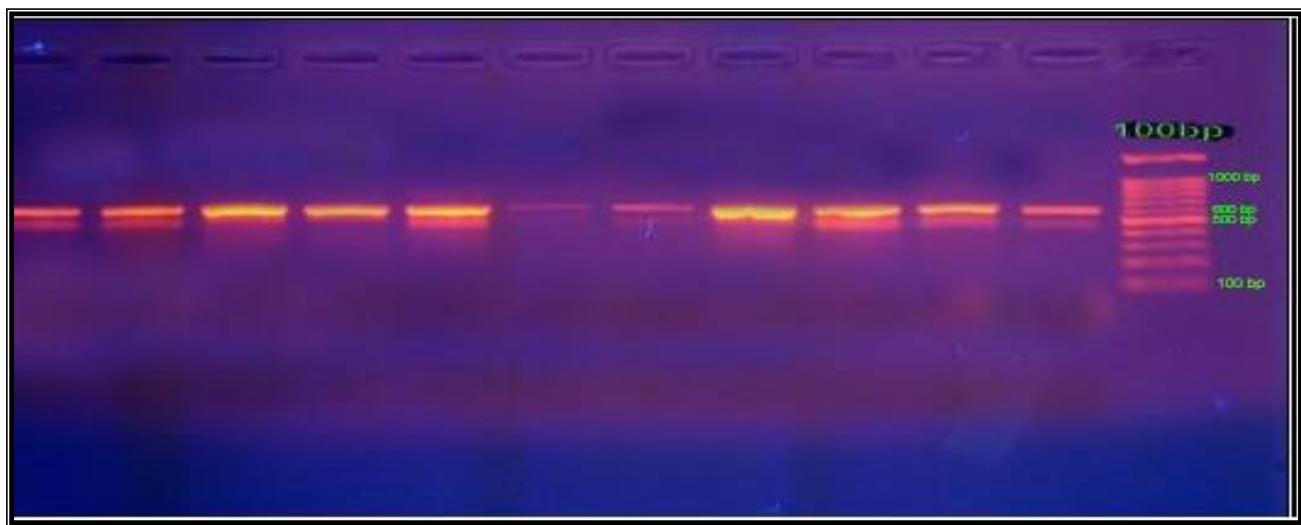


Figure (4): Aerobactin gene amplification product by polymerase chain reaction

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