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ROLE OF PLASMIDS IN THE MULTIPLE ANTIBIOTICS RESISTANCE OF *E.COLI* ISOLATED FROM THE URINE OF PATIENTS WITH URINARY TRACT INFECTION IN KIRKUK CITY

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Absract
We collected 120 urine samples, and 28 (23.3%) of these samples included *E. coli* isolates. The isolates' susceptibilities to antibiotics varied. It was determined that every sample was incredibly resistant to both beta-lactam and non-beta-lactam antibiotics., although all isolates were resistant to imipenem.
It was discovered that two isolates displayed multiple resistance to 18 antibiotics concurrently (AMC AX, AM, PRL, CL CTX, CRO, FOX, CAZ, CFM, FEP, ATM, GN, TOB, NA, F, TE, TMP) while one isolate was at least simultaneously resistant to three antibiotics (AX, AM, and TME)..
The analysis revealed that five isolates were simultaneously resistant to seventeen antibiotics ((AMC AX, AM, PRL, CL, CTX, CRO, FOX, CAZ, CFM, ATM, GN, TOB, NA, CIP, TE, TMP), which also contributed to the resistance to twelve antibiotics simultaneously. The five antibiotics were dispersed among the five isolates.
It was discovered that one isolate had four plasmid bundles, four isolates had two, and two isolates had one plasmid bundle. These isolates were all confirmed to be antibiotic-resistant.

Keywords: Plasmids , *E.coli,* Antibiotics, Urinary Tract Infection

Introduction

There are various species in the genus Escherichia, including a number *of*, with *E. coli* being most prevalent and recognizable form (Mahon *et al*., 2007). It represent as non-pathogenic bacteria in the large intestine of warm-blooded animals after being isolated for the first time by the German scientist Theodor Escherichia in the farces of healthy neonates in 1885. (Feng *et al*., 2002). Escherichia isolated these bacteria from the urine of girls with UTI in 1894. (Sussman, 1985). Although *E. coli* is thought to be a harmless symbiotic in the intestines of both people and animals, certain of its strains have a number of virulence factor that allow them to infect many other organs, including the intestinal and extra-intestinal
As one of the leading causes of infections in urinary tract, *E. coli* can spread from its natural site in the body and produce a variety of opportunistic infections in various bodily regions. (Zagaglia *et al*., 2022) because to the fact that 90% of girlis first infections are caused by it (Russo and Johnson, 2003), and it can also result in a several illnesses, as infections of wound , newborn meningitis, septicemia, and

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acquired infections (Brooks *et al*., 2010; Nataro and Kaper, 1998; Nimer, 2022). Additionally, opportunistic strains of *E. coli* can induce kidney failure by causing hemolytic uremic syndrome (HUS) and ulcerative colitis (Rolhion and Darfeuille-Michaud, 2007; Kaper *et al*., 2004; Muhammad *et al*., 2017).

Antibiotic resistance in bacteria can either be innate or acquired. —depending on the cellular genetic structure and physiological state of the bacteria or acquired occurring as a result of changes in the cellular structures and physiological state, which are brought on by changes in the genetic content of these bacteria (Nester *et al*., 2004). Bacteria can develop antibiotic resistance by mutations or by acquiring genes encoding the resistance through modes of gene transmission between various bacterial cells, including conjugation, genetic transformation, or genetic transmission (Forbes *et al*., 2007).

The existence of encoding factors for resistance to many antibiotics on plasmids, the majority of which are conjugative plasmids, as well as the presence of these genes on transposons was discovered to allow bacterial strains to develop a variety of mechanisms for antibiotic resistance and facilitate their transmission to other bacterial genera (Cowan and Talaro 2006: Perry *et al*., 2002), according to Qing *et al* (2019), conjugated and donor isolates were resistant to AMP and KFand.

The majority of bacterial cells as well as some yeasts and fungi include plasmids, which are small double pieces of DNA that are typically circular in shape. It duplicate itself in the bacterial cell (Prescott *et al*., 2005). Although they are not necessary for bacterial growth, they do give bacteria with extra traits that promote their pathogenicity, such as resistance to antibiotics, toxicity, heavy metal production, and adhesion factors (Tortora *et al*., 2010).

The target of the current study was to isolate and identify *E. coli* from urine samples, as well as to examine the bacteria's antibiotic resistance and curing plasmids to determine which antibiotics are more likely to encode resistance in plasmids.

Materials and Methods

According to the recommended methods for collecting samples (Mahon *et al*., 2007; Forbes *et al*., 2007; Koneman *et al*., 1992; Cheesbrough, 2006), Patients' urine samples were taken at Azadi Teaching Hospital and Kirkuk General Hospital. The samples were inoculated on blood agar medium and McConkey agar medium, and the plates were kept in the incubator for 24 hours at a temperature of 37 °C.

Bacterial Diagnosis

Bacteria were diagnosed based on reliable sources in the diagnosis (Benson, 2002; Alexander and Strete, 2001; Steve *et al*., 2004; Brown, 2007; Winn *et al*., 2006). Using the API-20 E system, the diagnosis was verified as it was being done (Biomerieux) as well as using RapID One System (Remel).

Test for Antibiotic Susceptibility

Antibiotic sensitivity tests are carried out using the Standard Kirby-Bauer method on bacterial isolates, as mentioned in (Brown, 2007).

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Plasmid DNA Extraction

The High-Speed Plasmid Mini Kit from Geneaid, USA, was utilised to extract plasmid DNA from bacterial cells using the modified alkaline lysis technique..

Electrophoresis on Agarose Gel

The electrophoresis process for plasmid DNA samples on agarose gels followed the steps given by Sambrook *et al*., (1989)..

DNA Plasmid curing

According to Trevors (1986), curing of plasmid were carried out using SDS (Sodium dodecyl sulphate) and ethidium bromide as curing ingredients with the following concentrations:

concentrations(µg/mL)	The curing substance
(220, 200, 018, 160, 140, 120, 100, 80, 60)	ethidium bromide
(5000,3000, 2000, 1000, 500, 400, 300, 200)	SDS

Antibiotic Susceptibility Test

The Standard Kirby-Bauer method, as described in the reference, was used to assess the susceptibility of bacterial isolates to antibiotics following the plasmid curing procedure. (Brown, 2007).

Results

In this investigation, 120 samples of urine yielded 28 (23.3%) *E. coli* isolates.

Diagnosis

E. coli isolates were found. Isolates showed up as pink medium-sized colonies, lactose-fermented and somewhat opaque white colonies and on blood agar with or without hemolysis, depending on the culture features, microscopic examination, and biochemical assays. On EMB medium, it also manifested as medium colonies and had a black color with a metallic shin, however microscopic analysis revealed that it is a Gram-negative bacillus. Subsequently, biochemical examinations were performed (Table 1). Then the diagnosis was confirmed using a kit API 20E and RapID one system.

Table (1) Biochemical tests for *E.coli* isolates

			Indole	MR	VP	Citrate	TSI Reaction	H2S Production			
E.coli	+	-	+	+	-	-	A/A gas	-	-	+	+

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Antibiotic Susceptibility Test

The antibiotic susceptibility of all *E.coli* isolates was investigated using 22 antibiotics from various groups.. The isolates varied in their sensitivity to antibiotics (Table 2), and showed varying rates of antibiotic resistance (Table 3), it was found that all isolates were sensitive to the Imipenem (100%).

With the exception of imipenem and other antibiotic groups, the clinical isolates used in this study exhibited high resistance to other beta-lactam antibiotics..

The clinical isolates were 100% resistant to ampicillin and 96.4% to amoxicillin + clavulanic acid (AMC). Additionally, it was discovered that the resistance to pipracillin (82.1%) and cephalexin (85.7%), was 71.4 for both cefotaxime and ceftriaxone.

The resistance to both Cefepime and Cefoxitin were (39.2 and 28.5)%, respectively, whereas the resistance to each of the antibiotics Ceftazidime, Cefixime, Aztreonam, Gentamicin, Tobramycin, Nalidixic acid, Ciprofloxacin, Chloramphenicol, Tetracycline and Trimethoprim were (53.5, 75, 53.5, 42.8 , 35.7,71.4, 28.5, 25, 75, and 78.5), respectively.

Table (2) Antibiotics sensitivity of *E.coli* isolates

antibiotics ((µg/disc																						
TMP/ 5	TE/ 30	C / 30	F/300	CIP/5	NA / 30	TOB/10	GN/10	AK/30	IPM/10	ATM/30	FEP/30	CFM/5	CAZ/30	FOX/30	CRO/30	CTX/30	CL/30	PRL/10	Am/10	AX/25	AMC/30	Clinical isolates
R	S	S	R	S	R	R	R	S	S	R	I	R	R	S	R	R	R	R	R	R	R	1
R	R	S	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	2
R	R	S	R	S	R	S	I	S	S	R	I	R	R	R	R	R	R	R	R	R	R	3
R	R	R	R	S	R	R	R	S	S	R	I	R	R	R	R	R	I	R	R	R	R	4
R	S	S	I	S	S	I	R	S	S	R	S	R	R	R	R	R	R	R	R	R	R	5
S	R	S	S	S	S	S	I	S	S	S	S	I	S	I	S	S	R	S	R	R	R	6
S	S	S	S	S	S	I	R	S	S	S	S	S	S	I	S	S	I	I	R	R	R	7
S	R	R	R	I	R	S	I	S	S	S	S	S	S	I	S	S	R	R	R	R	R	8
S	I	S	R	S	R	I	I	S	S	S	S	R	S	I	S	S	S	R	R	R	R	9
S	S	S	R	S	R	S	S	S	S	R	I	R	I	S	R	R	R	R	R	R	R	10
R	R	S	R	S	S	S	R	S	S	R	I	R	R	I	R	R	R	R	R	R	R	11
R	R	S	R	S	S	S	S	S	S	S	S	R	S	R	R	R	R	I	R	R	R	12
R	R	R	S	S	S	R	I	I	S	R	I	R	R	R	R	R	R	R	R	R	R	13
R	R	R	S	S	S	R	I	S	S	R	R	R	R	R	R	R	R	R	R	R	R	14
R	R	S	R	S	R	S	S	S	S	R	S	R	I	S	R	R	R	R	R	R	R	15
R	R	S	R	S	R	R	R	S	S	R	R	R	R	I	R	R	R	R	R	R	R	16
R	R	S	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	17
R	R	R	I	S	R	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	18
R	R	S	R	S	R	R	R	I	S	R	R	R	R	I	R	R	R	R	R	R	R	19
R	S	R	R	S	R	I	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	20
R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	21

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R	R	S	R	S	R	S	S	S	S	S	S	S	S	I	S	S	R	R	R	R	R	**22**
R	R	R	R	S	R	S	S	S	S	I	S	R	R	R	R	R	R	I	R	R	R	**23**
S	R	S	S	S	R	S	R	S	S	S	S	R	R	S	R	R	R	R	R	R	R	**24**
R	S	S	S	S	I	S	S	S	S	S	S	I	S	I	I	S	I	S	R	R	S	**25**
R	R	S	R	S	R	R	R	S	S	R	R	R	R	I	R	R	R	R	R	R	R	**26**
R	R	S	R	S	R	R	R	S	S	I	R	R	I	R	R	R	R	R	R	R	R	**27**
R	R	S	R	S	R	S	I	S	S	S	S	R	I	I	R	R	R	R	R	R	R	**28**

Table (3) Percentage of antibiotic resistance of isolates

The percentage of resistance	Antibiotic
96.4	AMC30
100	AX25
100	AM10
82.1	PRL100
85.7	CL30
71.4	CTX30
71.4	CPO30
39.2	FOX30
53.5	CAZ30
75	CFM5
28.5	FEP30
53.5	ATM30
0	IPM10
100	AK30
46.4	GN10
35.71	TOB10
71.4	NA30
28.5	CIP5
39.2	F300
25	C30
75	TE30
78.5	TMP5

.

Multiple Antibiotic Resistance

It is clear from Table (4) that one isolate was resistant to three antibiotics at the same time (AX, AM, TME) as a minimum, while two isolates demonstrated simultaneous resistance to 18 antibiotics (AMC AX, AM, PRL, CL CTX, CRO, FOX, CAZ, CFM, FEP, ATM, GN, TOB, NA, F, TE, TMP,).

Five isolates were observed to be resistant to seventeen antibiotics at the same time. ((AMC AX, AM, PRL, CL, CTX, CRO, FOX, CAZ, CFM, ATM, GN, TOB, NA, CIP, TE, TMP), which took part in the resistance to twelve antibiotics simultaneously while the five antibiotics were distributed among the five isolates. This was determined to be the maximum number of isolates with multiple antibiotic resistance.

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Table (4) Multiple antibiotic resistance in *E.coli* isolates

number Clinical isolates	Number Antibiotics
1	3
1	4
1	5
1	7
1	8
3	9
2	11
2	12
2	13
2	14
3	15
2	16
5	17
2	18

PLASMID PROFILE

To identify the plasmid content of the isolates, a group of 7 isolates were tested. The process of plasmid DNA extraction was carried out and then electrophoresis used. The isolates were divided into groups according to their possession of plasmid bundles, as we note in Table (5) and Figure (1). The first group included two isolates, each containing one plasmid bundle, while the second group included 4 isolates, each containing two plasmid bundles, and the third group included one isolate that contained 4 plasmid bundles.

Table (5) Plasmid profile of clinical isolates and their antibiotic resistance

Resistance to antibiotics (number of antibiotics)	plasmid bundles	isolation source	isolation number	The groups
17 8	1	Urine Urine	16 21	2
18 5 18 17	2	Urine Urine Urine Urine	2 6 17 20	3
11	4	Urine	12	4

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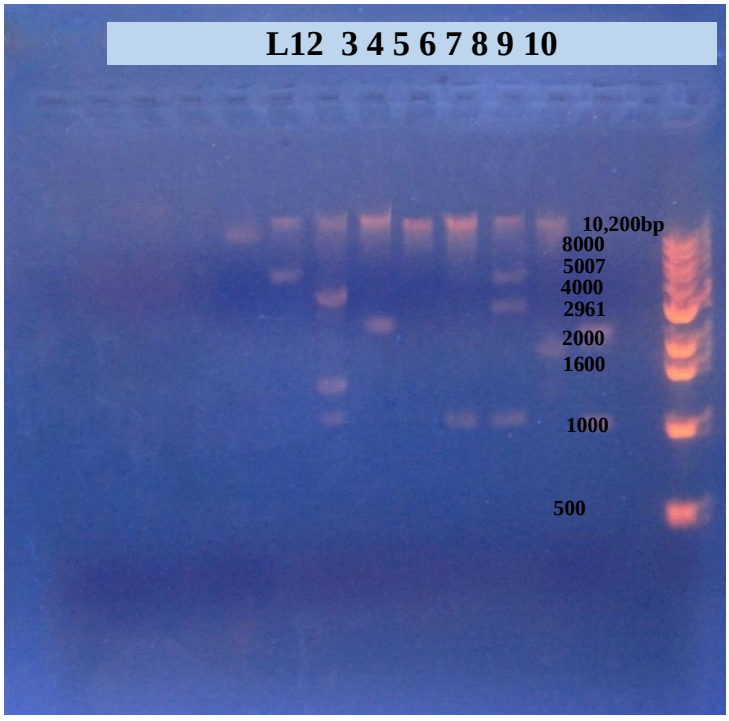

the shape (1) Electrophoresis of plasmids of clinical isolates using agarose gel concentration of 0.7%

path (L): DNA Ladder Marker
Path (2) isolate (2) Path (3) isolate (17)
Path (4) isolate (12) Path (5) isolate (6)
Path (6) isolate (16) Path (7) isolate (20)

Ethidium bromide was able to curing the plasmids of the isolates (17 , 12 , 6 , 2) When the concentration under the minimum inhibitor (Sub-MIC) was 200 µg / ml and as shown in table (6) the isolate (2) became sensitive to antibiotics (TMP, TE, GN, CRO, AM, AMC) after curing, isolate (6) became sensitive to antibiotics (CL, AM, AX, AMC), and isolate (12) became sensitive to antibiotics (TMP, CIP, TE, CRO, CTX, AM) after curing, Isolate (17) became sensitive to (AM, TMP, CIP, FOX, ATM, FEP, CL, CRO, TE, PRL).

Electrophoresis results showed that these isolates lost their plasmids and changed their antibiotic resistance pattern (Fig.2), While ethidium bromide failed to cure the plasmids of the two isolates (16, 20) at the same concentration used, and its antibiotic resistance pattern did not change.

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Schedule (6) Antibiotic resistance pattern of *E.coli* isolates both before and after curing

after curing		**before** curing		isolation number
number of plasmids	resistance pattern	number of plasmids	resistance pattern	
Nothing	NA/ CTX/ ATM/ CAZ	2	AMC/AM/CRO/GN/TE/TMP NA/ CTX/ ATM/ CAZ	**2**
Nothing	TE/RA/E	2	AMC/AX/AM/CL/TE/ RA/E	**6**
Nothing	AMC/RA/E	4	AMC/AM/CTX/CRO/TE CIP/RA/E/TMP	**12**
1	AMC/CRO/CAZ/CTX/ ATM FEP/ CIP/ NA/ GN/ TMP/ TE / TOB / RA	1	AMC/CRO/CAZ/CTX/ ATM FEP/ CIP/ NA/ GN/ TMP/ TE/ TOB /RA	**16**
Nothing	AMC/NA/CTX/CAZ/RA/ TOB/GN	2	AMC /AM/ CRO/ CL/ PRL CTX/FER/ATM/FOX/CIP/NA/TMP/CAZ/RA/TOB/TE/ GN	**17**
2	AMC / AM / CL / CRO / CTX NA / CIP / TMP	2	AMC/AM/CL/CRO/CTX/ NA/CIP/TMP	**20**

GN = Gentamicin
TOB = Tobramycin
NA = nalidixic acid
CIP = Ciprofloxacin
TE = Tetracycline
RA= Rifampin, E = Erythormycin

PRL = piperacillin
CL = Cephalexin
FOX = Cefoxitin
FEP = Cefepime
ATM = Aztreonam
TMP = Trimethoprim

AMC= Amoxicillin+ clavulanic acid
AX = Amoxicillin
AM = Ampicillin
CTX = cefotaxime
CRO = ceftriaxone

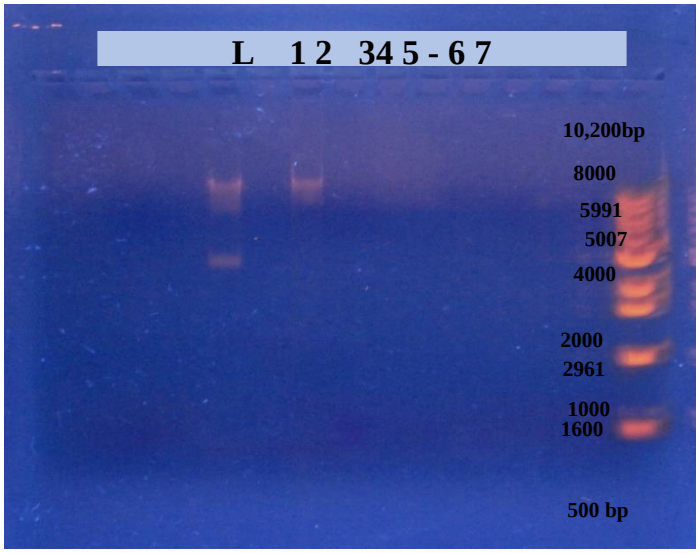

Figure 2: Electrophoresis of plasmids from clinical isolates using an agarose gel0.7% concentration after curing

path (L): DNA Ladder Marker path (1): isolate (2)
path (2): isolate (17) Track (3): isolate (12)
path (4): isolate (6) path (5): isolate (16)
path (6): isolate (20)

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DISCUSSION

According to the study's findings, a total of 120 urine samples included 28 *E.coli* isolates, 23.3% of the total. Several studies' findings revealed varying rates of isolation from urine samples. According to AL-Salamy (2012), the percentage of *E. coli* isolated from urine samples was 44.2%., whereas the percentage reported by Ozbakir and his team (2010) was 79.17%. The number of samples used in the study, the time of year when samples were collected, and the location of the samples, in addition to using antibiotics, may all be contributing factors to these variations in isolation rates.

Antibiotic Sensitivity Test

Clinical isolates of *E.coli* varied In their sensitivity to the antibiotics used under the current study, in general, the clinical isolates were highly resistant to antibiotics.

All clinical isolates showed 100% sensitivity to an Imipenem (from the Carbapenem group) and these findings are consistent with those of a previous study in this field., as Japoni and his group (2008) indicated that 98% of *E.coli* isolates isolated from different clinical sources were sensitive to Imipenem, also Naqid and his group (2020) indicated the sensitivity of these bacteria by 96.4%. Relatively high sensitivity to Carbapenem, such as Imipenem and Meropenem, is due to the high permeability that these antibiotics possess across Gram-negative bacterial cell wall (Labormbardi, 2007). These antibiotics are also considered effective against bacteria that can produce ESβLs enzymes (Paterson *et al*., 2004). The clinical isolates under the current study were greatly resis penicillin whereas, the resistance to (AMC) was 96.4%, and these results are close to the study of Ibraheem (2006), which indicated that 88% of *E. coli* isolated from urine samples were resistant to AMC.

Resistance may be attributed to antibiotics such as AMC leads to an abundant production of beta-lactamase enzymes to overcome the inhibitory action of beta-lactamase inhibitors (Chambers, 2001; Nossair *et al*., 2022).

The current study's findings were also consistent with many studies that indicated *E.coli* isolates with high resistance rates to penicillin antibiotics, as the resistance to ampicillin reached 100% (Kadhim *et al*., 2011) and to amoxicillin 97.7% (Ibrahim *et al*., 2012).). The resistance to penicillin antibiotics is because of the widespread and randomly use of these antibiotics, as well as the transmission of resistance through plasmids or transposons (Normark & Normark, 2002).

Clinical isolates demonstrated resistance to the first generation of cephalosporin, Cephalexin, as well as Cefotaxime, Ceftriaxone, and Cefixime (which represents modern cephalosporins). These findings are comparable to those of Kadhim and colleagues (2011), who found that clinical *E. coli* isolates were 95.1% resistant to the cephalexin, and that cefotaxime resistance was 83%. (Ibraheem, 2006). The main mechanism of infection for Gram-negative bacteria is the production of beta-lactamase carried on chromosomes or encoded by genes carried on plasmids (Pitout, 2010; Johnson and Nolan, 2009).

The findings of the present investigation are consistent with those of Abd-alsttar (2004), who showed that 55% of the isolates were resistant to gentamicin. Bacteria existence of plasmids that encode the creation of enzymes that can modify these antibiotics or change the target site is the cause of their

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resistance to aminoglycosidic antibiotics. These antibiotics act on the small ribosomal unit (30S), which serves as their target site, by blocking them from binding to ribosomes (Brooks *et al.*, 2010).

The isolates under examination had a 71.4% resistance rate to nalidixic acid. Similar findings to those of the current investigation were presented by Kadhim and his team (2011), who found that the isolates' antibiotic resistance was 70.9%.

despite of that Ciprofloxacin is the more recent agents , the isolates still displayed a high level of resistance to it (28.5%). These findings are in line with those of Ibrahim and his team (2012), who found that 58.4% of the the isolates exhibited a high level of resistance to the same antibiotic.

Resistance to quinolines is attributed to the evolution of resistance genes carried on conjugative plasmids (Martinez-Martinez *et al.*, 1998; Pitout *et al.*, 2008), or resistance to these antibiotics may be due to mutations that cause changes in the DNA gyrase enzyme or mutations that lead to the production of active efflux pumps systems (Wang *et al.*, 2004).

On the other hand, Chloramphenicol is still as one of the effective antibiotics against *E.coli*. The isolates showed low rates of resistance to this antibiotic. One study indicated similar results in this regard. The resistance of the isolates to this antibiotic was 18% (Japoni *et al.,* 2008).

The resistance of the isolates to Tetracycline 75%, which is similar to the study of Ibrahim and his group (2012), where the rate of resistance of clinical isolates of *E. coli* to Tetracycline was 77.1%. Tetracycline antibiotic resistance can be linked to a number of processes, including the Active Efflux, which reduces the amount of antibiotics that accumulate inside cells. TetA protein-encoding plasmids, which encode resistance proteins, are responsible for encoding this mechanism. The second process involves the enzymatic inhibition of these antibiotics, which results in the production of proteins that stop tetracyclines from attaching to ribosomes (Craig and Stitzel, 2004). Clinical isolates were resistant to Trimethoprim at 78.5% and these results agree with Eldydamouny and his group (2010) where the percentage of clinical isolates of *E.coli* was 69.9%.

Multiple antibiotic resistance

The current study's findings indicated rising rates of multi-resistance to antibiotics in general and penicillins and cephalosporins in particular. This increase in resistance may be due to an increase in random uses of antibiotics (Levy and Marshall, 2004). Several studies have indicated an increase in the resistance of isolates to antibiotics, Ibrahim and his group (2012) indicated that 92.2% *E.coli* isolated from a variety of clinical sources were multi-antibiotic resistant. Ibrahim (2006) also showed that all his isolates isolated from urine samples were multi-resistant to antibiotics.

In general, 90% of the known antibiotics are unable to prevent growth of many genera of Gram-negative bacteria, including *E.coli* this is because of the of their cell walls nature and their possession of many resistance mechanisms such as active efflux systems and the production of ESβLs enzymes that contribute to resistance to many types of antibiotics (Demain, 1999; Brooks *et al.*, 2010).

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Plasmid Profile Study

The current study's findings were in line with several investigations that demonstrated that *E. coli* has plasmid bundles, including at least one large plasmid bundle called Mega plasmid as well as other small plasmid bundles with various molecular weights (Ibraheem, 2006; AL Moosawi, 2005).

Plasmid DNA Curing

According to the results of the plasmid curing, SDS was unable to cure the plasmids of numerous isolates while ethidium bromide succeeded.

It was found in a study conducted on *Acinetobacter baumanii* the ability of several curing substances such as acridine orange and ethidium bromide to cure the plasmid in these bacteria (Pahwa *et al*., 2012). Radi and Rahman (2010) reported several agents for curing *Pseudomonas aeruginosa* plasmids, including ethidium bromide and SDS, as well as a heating at 46 C. They indicated that the latter was more efficient in curing than ethidium bromide and SDS. The optimal and most effective curing concentration for any curing substance varies with the different types of bacterial isolates treated and depends on the efficiency of the curing substance and the nature of its work (Trevors, 1986).

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