Vol. 07, No. 01, pp. 16 –22 (2026)
ISSN: 2708-1818

JOURNAL OF LIFE AND BIO-SCIENCES RESEARCH

www.jlbsr.org

Investigating Escherichia coli Resistance to Antibiotics and Heavy Metals

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Abstract

Bacterial resistance to antibiotics and heavy metals is an increasing ecological and public health risk. The genes responsible for the resistance are mainly carried on resistant plasmids; the elimination of these plasmids is carried out by curing using physical agents like low pH levels and elevated temperature. In this study, four stool isolates of *Escherichia coli* were obtained from Ibn-Sina Hospital in Mosul city, Iraq. The isolates were diagnosed using appropriate biochemical tests and tested for their resistance against five antibiotics: Ampicillin (Amp), Tetracycline (Tc), Erythromycin (Er), Nalidixic acid (Nal), and Chloramphenicol (Cm); and three heavy metals: Mercury chloride ($HgCl_2$), Cobalt chloride ($CoCl_2$), and Copper chloride ($CuCl_2$). Results showed that all isolates were 100% resistant to (Nal) and ($CuCl_2$), and (75%) for (Amp) and (Cocl2), while (50%) of the isolates were resistant to Tc and (25%) for Er and Cm; however, all isolates were sensitive to ($HgCl_2$). MIC was conducted to examine the ability of bacteria to tolerate heavy metal concentrations, and we found that the MIC for Cobalt was 1000µg/ml and 1700 µg/ml for copper. Using low pH (pH5) as a curing agent showed that the resistance for Tc and Nal was lost in 60-64% and 36-68% of the isolates, respectively, while resistance to cobalt was lost in 40-52% of the isolates. These results suggest that genes responsible for the resistance of Tc, Nal, and Cobalt are probably located on the plasmids; while the resistance of Amp, Er, Cm, and Copper was not affected, which may suggest that they are chromosomally encoded. Using elevated temperature (46°C) as a curing agent showed higher rates for curing of Tc (94-96%), Nal (72-92%), and Cobalt (88-96%), which supports the results for gene location obtained from curing with low pH.

Keywords: *Plasmid Curing, Antimicrobial Resistance, Heavy Metal Resistance, Escherichia coli.*

Received: February 6th, 2026/ Revised: March 30th, 2026/ Accepted: April 5th, 2026/ Online: April 10th, 2026

I. INTRODUCTION

Escherichia coli is a Gram-negative, rod-shaped, facultative aerobic coliform bacterium found in the intestinal tract as a normal flora in humans and animals. *E. coli* is a species belonging to Enterobacteriaceae that causes many diseases, such as urinary tract and bloodstream infections (Martinez – Medina, 2021). The high use of antibiotics has raised a public health problem, leading to multi-antibiotic-resistant bacteria; because of the presence of bacterial plasmid, which has resistant genes that transfer from one bacterium to another by transformation, conjugation, or mobilization (Michaelis and Grohmann, 2023). Heavy metals are used in medicine and agriculture; as a result, they accumulate in the environment and cause the spread of metal-resistant bacteria (Stepanauskas ET AL., 2005).

The increasing use of antibiotics and heavy metals creates a selective pressure for the survival of bacteria by resistance through mutations or plasmid acquisition (Bhattacherjee *et al.*, 1988). Heavy metals and antibiotic resistance genes were found on plasmids together, which transferred to microorganisms (Alav *et al*., 2024). The exposure to low concentrations of heavy metals may select for the resistance of antibiotics and adaptation of bacteria (Gullberg *et al.*, 2014).

E. coli contains bla_{TEM}, bla_{CTXM-3}, and bla_{CTXM-9} genes; in contrast, these genes produce extended-spectrum beta-lactamase enzymes (ESBLs), which hydrolyze penicillin, cephalosporins, and monobactams, emerging multidrug-resistant strains that can face environmental contamination with antibiotics. Due to the occurrence of such genes found on conjugative plasmids, this leads to the spread of multidrug resistance between different species of bacteria (Malloy and Campose, 2011; Wolny-Koładka and Zdanie, 2021). *E. coli* enables survival in acidic culture because it has an efflux pump of ions to face the changing of pH, where low pH may break down the plasma membrane or DNA, leaving effects on the activity of enzymes (Bearson *et al.*, 1997; Wilks and

doi: 10.38094/jlbsr70178

Slonczewski, 2007). *E. coli* has three systems of acid resistance: the Gad system (glutamate decarboxylase), the Adi system (arginine decarboxylase), and the Cad system (lysine decarboxylase) (Castanie-cornet *et al*., 1999).

The genes of antimicrobials and heavy metal resistance found on plasmids have high mobility rates, wide host ranges, and a higher percentage of homologous recombination than other genes; therefore, targeting plasmids by curing reduces that resistance (Coluzz and Rocha, 2025). Plasmid curing is defined as the elimination of plasmids from bacteria using curing agents such as ethidium bromide (EB), sodium dodecyl sulphate (SDS), and acridine orange (Ao), or by physical agents like exposure to low pH level or elevated temperature. Plasmid curing was used to determine the location of resistance genes for antibiotics and heavy metals in bacteria; if the resistance is lost after plasmid elimination by one of the curing agents, this indicates that the resistance gene is located on a plasmid DNA (Carlton and Brown, 1981; Liu *et al.*, 2012).

This study aimed to test the sensitivity of *Escherichia coli* against five antibiotics: Ampicillin, Tetracycline, Erythromycin, Nalidixic acid, and Chloramphenicol; and three heavy metals: Mercury chloride, Cobalt chloride, and Copper chloride. MIC was determined to show heavy metals tolerance made by the isolates and compare the efficiency of two curing agents by using low pH (pH5) and elevated temperature (46°C) against bacterial isolates.

II. Materials And Methods

A. Bacterial isolates

Four *E. coli* were obtained from stool specimens from Ibn Sina Hospital in Mosul city, Iraq. These isolates were diagnosed by biochemical tests depending on oxidase, catalase, urease, Indole, and Methyl red (Brown and Smith, 2017).

B. Antibiotic resistance test

Bacterial isolates were tested for resistance to five antibiotics: ampicillin (Amp), tetracycline (Tc), erythromycin (Er), nalidixic acid (Nal) and chloramphenicol (Cm) according to (Timmis and Puhlar, 1984); whereas stock solution was prepared for each antibiotic and added to nutrient agar culture by final concentration (µg/ml) for each antibiotic (Amp 50, Tc 15, Er 15, Nal 30, and Cm 20), the plates were allowed to solidify then bacterial cultures were streaked on the nutrient agar plates and incubated at 37°C for 24 hours, after that we observe for any growth on the medium to detect antibiotics resistance.

C. Heavy metals toxicity test

E. coli isolates were tested for resistance against three heavy metals: Mercury chloride (Hgcl2), Cobalt chloride ($CoCl_2$) and Copper chloride ($CuCl_2$); stock solutions were prepared for each one and was added to nutrient agar culture by final concentration (25µg/ml), the plates were allowed to solidify then bacterial cultures were streaked on the nutrient agar plates and then incubated at 37°C for 24 hours, after that we observe for any growth on the medium to isolate heavy metals resistant bacteria (Groves *et al*., 1975).

D. Determination of minimum inhibitory concentration (MIC)

MIC was conducted according to (Faisal and Younis, 2024) to show heavy metals tolerance by *E. coli* isolates. Metal tolerance was determined as the minimum inhibitory concentration MIC of cobalt and copper, which inhibited growth. MIC concentrations began with (100 µg/ml) of metal concentration in nutrient agar, and then bacterial cultures were streaked on it. After incubation at 37°C for 24 hours, if bacterial growth was observed, the concentration was increased to double until the MIC was reached.

E. Spontaneous curing test

According to Meyer (1974), bacterial culture was diluted to obtain single colonies. 1 ml of bacterial broth culture was added to a test tube containing 9ml distil water to make the dilution 10^{-1}. Then, serial dilutions were made until 10^{-5}. Master plates were prepared for each isolate by spreading 100 µl from the dilutions 10^{-3}, 10^{-4}, 10^{-5} on nutrient agar plates and incubating at 37°C for 24 hours to obtain single colonies. 100 colonies were transferred to nutrient agar plates to prepare master plates, and after incubation at 37°C for 24 hours, colonies were transferred to plates containing antibiotics and heavy metals to test spontaneous curing.

F. Curing plasmid by low pH

E. coli isolates were grown in nutrient broth with pH5 as curing agents, then serial dilutions were prepared to obtain single colonies, 1ml of bacterial broth culture add to test tube have 9ml distil water to make the dilution 10^{-1} then we took 1ml of 10^{-1} to test tube have 9ml to make10^{-2} and then we did 10^{-3},10^{-4}, 10^{-5} dilutions then master plates were prepared for each isolate, we took a loop sample from10^{-3},10^{-4}, 10^{-5} dilutions and spread on nutrient agar plates then incubation at 37°C for 24 hours to get single colonies then we transfer 100 colonies to nutrient agar plates to prepare master plates and after incubation at 37°C for 24 hours we transfer these cultures to plates of antibiotics and heavy metals and after incubation at 37°C for 24 hours we test curing plasmid by low PH against antibiotics and heavy metals cultures (Sinha, 1989; Faisal, 2010).

G. Curing plasmid by elevated temperature 46°C

According to Baldwin *et al.* (1969), the isolates of *E. coli* were grown at 46°C in nutrient broth as curing agents, each bacterial culture was diluted, 1ml of bacterial broth culture add to test tube have 9ml distil water to make the dilution 10^{-1} then we took 1ml of 10^{-1} to test tube have 9ml to make10^{-2} and then we did 10^{-3}, 10^{-4}, 10^{-5} dilutions then master plates were prepared for each isolate, we took a loop sample from10^{-3}, 10^{-4}, 10^{-5} dilutions and spread on nutrient agar plates then incubation at 37°C for 24 hours to get single colonies then we transfer 100 colonies to nutrient agar plates to prepare master plates and after incubation at 37°C for 24 hours we transfer these cultures to plates of antibiotics and heavy metals and after incubation at 37°C for 24 hours we test curing plasmid by elevated temperature against antibiotics and heavy metals cultures.

Table 1. Resistance to antibiotics and heavy metals in *E. coli* isolates.

Isolates numbers	Ap50 µg/ml	Tc 15 µg/ml	Er 15 µg/ml	Nal 30 µg/ml	Cm 20 µg/ml	HgCl2 25µg/ml	CoCl2 25 µg/ml	CuCl2 25 µg/ml
E. coli 1	R	R	S	R	S	S	R	R
E. coli 2	R	R	S	R	S	S	R	R
E. coli 3	S	S	S	R	S	S	S	R
E. coli 4	R	S	R	R	R	S	R	R

R: Resistant, S: sensitive, Amp: Ampicillin, Tc: Tetracycline, Er: Erythromycin, Nal: Nalidixic, Cm: Chloramphenicol, HgCl2: Mercury Chloride, CoCl2: Cobalt Chloride, $CuCl_2$: Copper Chloride.

III. RESULTS

A. Biochemical test for E. coli isolates

All isolates were positive for catalase, indole, and methyl red. The isolates were negative for oxidase and urease.

B. Antibiotics and heavy metals resistance test

During the study of *E. coli* sensitivity to antibiotics and heavy metals, many degrees of resistance were obtained. Table 1 indicates that all isolates were resistant to nalidixic acid and copper at a rate of (100%). In the current study, the rate of resistance to ampicillin and cobalt was (75%).

The resistance to tetracycline was (50%), (25%) to chloramphenicol, while all isolates were sensitive to mercury.

C. Determination of minimum inhibitory concentration

The highest concentration (1600 µg/ml) of copper allowed the growth of *E. coli* isolates, while Cobalt was at 900 µg/ml, as shown in Table 2.

D. Spontaneous curing

There was no spontaneous curing in all isolates for the resistance to antibiotics and heavy metals.

E. Plasmid curing by pH5

There were no eliminations for resistance to each of: Ampicillin, Erythromycin, Chloramphenicol, and Copper after growing bacteria in low pH as curing agents; while resistance was cured for Tetracycline by a range of 60-64%, Nalidixic acid 36-68%, and Cobalt from 40% to 52%.

Isolate 1 was cured for Tc, Nal, and $CuCl_2$ by percentage 60 %, 52% and 40%, respectively, as shown in Figure 1A. Also, isolate 2 cured for Tc, Nal, and $CuCl_2$, but the percentage was 64%, 68%, and 44%, respectively, as shown in Figure 1B. The isolate 3cured 48% only for Nal (Figure 1C), while isolate 4 cured for Nal 36% and CoCl2 52% as in Figure 1D, by using pH5.

Table 2: Determination of MIC for *E. coli* isolates against CoCl2 and $CuCl_2$.

Concen. µg/ml	100	200	300	400	500	600	700	800	900	1000	1200	1400	1600	1700
$CoCL_2$	R	R	R	R	R	R	R	R	R	S	-	-	-	-
$CuCL_2$	R	R	R	R	R	R	R	R	R	R	R	R	R	S

R: resistant, S: sensitive

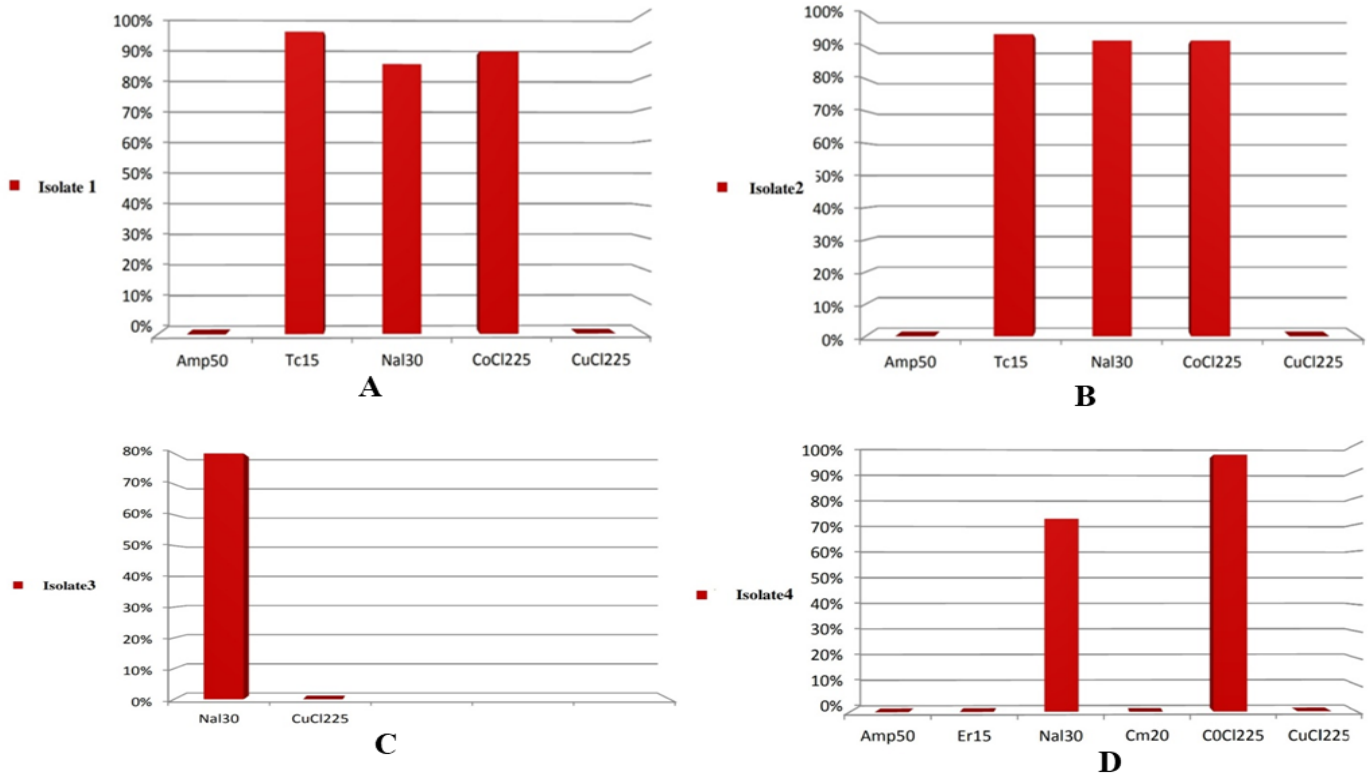

Figure 1. Percentage of plasmid curing of *E. coli* isolates using pH5. A, isolate 1, B, isolate 2, C, isolate 3, D, isolate 4

F. Plasmid curing by elevated temperature 46°C

We obtained high results for losing resistance according to Tetracycline in a rate of 94-96%, Nalidixic acid (72-92%), and 88-96% for Cobalt Chloride, as shown in Table 3.

Isolate 1 was cured by using 46°C, for Tc (94%), Nal (84%), and $CoCl_2$ (88%). Isolate 2 was cured for Tc (96%), Nal (92%), and $CoCl_2$(92%). The isolate 3 cured only for Nal (72%), while the isolate 4 cured for Nal (72%), then CoCl2(96%).

Table 3. Curing plasmids of *E. coli* isolates by elevated temperature 46°C.

E. coli isolates	Amp 50 µg/ml	Tc 15 µg/ml	Er 15 µg/ml	Nal 30 µg/ml	Cm 20 µg/ml	HgCl2 25µg/ml	CoCl2 25 µg/ml	CuCl2 25 µg/ml
E. coli 1	0%	94%	S	84%	S	S	88%	0%
E. coli 2	0%	96%	S	92%	S	S	92%	0%
E. coli 3	S	S	S	80%	S	S	S	0%
E. coli 4	0%	S	0%	72 %	0%	S	96%	0%

Percentage of losing antibiotics and heavy metals resistance by growing bacteria at 46°C, the concentrations of antibiotics and heavy metals by µg/ml.

IV. DISCUSSION

A. Biochemical test for E. coli isolates

Catalase enzyme releases oxygen from hydrogen peroxide H2O2, the indole release from tryptophan amino acid by tryptophanase enzyme, also all isolates were able to ferment glucose therefor all isolates were positive for catalase, indole, and methyl red test (Brown and Smith, 2017; Tille, 2017).

B. Antibiotics and heavy metals resistance test

Bacteria can be resistant to nalidixic acid when a mutation in the gene coding for DNA gyrase or topoisomerase occurs. As a result, Nalidixic acid is prevented from binding to this enzyme (Bansal and Tandon, 2011). Delmar *et al.* (2014) showed that *E. coli* bacteria were resistant to copper due to the copper-transporting efflux pump system; also, there was an efflux pump for resistance to any heavy metals or any drugs. Bacterial cells adapted to the increased Cobalt concentration by inducing a modified mixed acid fermentation pathway under aerobiosis (Majtan *et al*., 2010). In a study where 433 isolates of *E. coli*, Manadhar *et al*. (2020) pointed out that most resistance was to ampicillin at a rate (48.03%). Ampicillin resistance in *E. coli* is the dominant type among the isolates*;* this could be attributed to the synergistic effect of mutations responsible for resistance (Turkyilmaz and Darcan, 2024; Sheet *et al*., 2024). Also, extended spectrum β-lactamases (ESBLs) can be produced by *E. coli* to hydrolyze β-lactam antibiotics such as Ampicillin; therefor they can resist them (Park *et al.*, 2022).

Sun *et al.* (2014) showed that *E. coli* bacteria have Acr AB-TOIC multidrug efflux pumps, which can get antibiotics out of the cell, such as Tetracycline. In a study of Mohamad *et al.* (2013) on *E. coli* and *Staphylococcus,* they showed that all isolates were sensitive to Mercury (25µg/ml) at a rate (100%); therefore, it was used as a bacteriostatic in the past. Puzari and Chetia (2017) showed that *E. coli* bacteria have chromosomal or plasmidic efflux pump give bacterial cells a trait of resistance to antibiotics or heavy metals, while (29.5%) for Nalidixic acid.

In another study, Wolny-Koładka and Zdaniewicz (2021) showed that most isolates of *E. coli* were resistant to ampicillin and tetracycline. Park et al. (2022) got resistance of a high percentage to Nalidixic acid (44%), and (41.3) for tetracycline, ampicillin (40%); while a very low percentage was for chloramphenicol (5.3%), when they studied 75 isolates of *E. coli*. Essa and Abdulah (2018) studied 15 isolates of *E. coli* showed the rate of resistance to Ampicillin was (93%), Copper (73.3%), and Cobalt (53.3%).

Al Nuimi (2021) isolated 32 isolates of *E. coli* from urinary tract infection; his study showed a high resistance for Ampicillin (100%), Erythromycin (90%), Nalidixic acid (90%), and Tetracycline (87%). There are many mechanisms of resistance in bacteria that may appear by producing hydrolytic enzymes such as β-lactamase, which hydrolyses β-lactam rings in Penicillin and Cephalosporins, or by Chloramphenicol acetyl transferase, which in active Chloramphenicol, or by alternation of the target through mutation so the antibiotic cannot bind it, like Erythromycin, or by efflux pumps (Bobate *et al*., 2023).

C. Determination of minimum inhibitory concentration

Copper ions are required as cofactors by many enzymes in low concentrations by genes located on chromosomes, while genes of copper resistance are located on plasmids (Cooksey, 1993; Liu *et al.*, 2007). Cobalt resistance genes may be located on plasmids, and these plasmids have low copy number; therefor the tolerance of *E. coli* against Cobalt was lower than Copper (Sengupta and Austin, 2011). If bacteria have heavy metal tolerance and show high resistance to high concentrations, we can use them in bioremediation to remove low concentrations of heavy metals (1mg/L), and that is very important in biological remediation of antibiotics because chemical and physical mechanisms used today are only capable of removing high concentrations of antibiotics (Al-Shamary and Taha, 2017). If bacteria were isolated from polluted sites of heavy metals, they would have higher tolerance than bacteria isolated from a normal and not polluted environment (Ezzouhri *et al.*, 2009). On the other hand, Quintelas *et al.* (2008) had used *E. coli* to remove Chromium from liquid solution, and Quiton *et al.* (2018) had removed Zinc and Chromium by *E. coli*.

D. Plasmid curing by pH5

The results showed that genes responsible for resistance to Ampicillin, Erythromycin, Chloramphenicol, and Copper may be located on the chromosome**,** while the genes of Tetracycline, Nalidixic acid, and Cobalt may be located on the DNA plasmid. Churchill and Romanus (2018) ensured that when they pointed if the resistance of antibiotics or heavy metals was lost after curing that means the resistance was mediated by a plasmid, while if the resistance was not affected, it means chromosomally borne. Zhang *et al.* (2007) pointed out that resistance to antibiotics may remain unaffected after plasmid curing because of the high copy number of the plasmid. *E. coli* has genes that resist ampicillin located on plasmid and chromosomal DNA; Philippon *et al.* (1994) pointed out that *E. coli* have (ESBLs) enzymes (extended–spectrum β–lactamases) that hydrolyze β-Lactam antibiotics, and these enzymes were plasmid–encoded, while Peter *et al.* (2011) showed that *E. coli* has the chromosomal AmpC gene, which is responsible for

Ampicillin resistance by AmpC β-lactamase. Yang *et al.* (2020) showed that *E. coli* have genes for copper resistance located on the DNA chromosome and the DNA plasmid. Possibly, genes responsible for resistance to cobalt are located on the DNA plasmid; Bhattacherjee *et al.* (1988) pointed out that resistance to heavy metals in bacteria is spread by genes located on the R plasmid in general, and sometimes by mutation and selection. Also, in another study, Bennet (2008) pointed out that resistance of antibiotics and heavy metals found on mobile genetic elements: plasmids, transposons, integrons, and gene cassettes, these elements distribute resistance genes among bacteria.

A study by Ramirez-Bayard *et al*. (2023) showed genes of tetracycline resistance found on the plasmid in *E. coli* bacteria by efflux pump, which could favor their dissemination by horizontal gene transfer. Al-khayyat (2008) studied 18 isolates of *E. coli* isolated from infections of the urinary tract and used low pH (pH 5.5) to cure plasmid of these isolates, and he showed elimination of antibiotics and heavy metals resistance at a rate (2-82%). Changes in pH affect cell function, the synthesis of proteins, lipids, and energy production, and also affect enzyme activities and all biochemical reactions (Padan and Schuldiner, 1986). Rosas *et al.* (1983) conducted a study on the *E. coli* RC424 strain and showed a high degree of resistance to chloramphenicol and tetracycline; however, after curing by Sodium Dodecyl Phosphate (SDS) and Ethidium Bromide EB, the resistance was lost due to the loss of the plasmid p424. This experiment ensured that the genes for resistance were located on its plasmid. p424 plasmid was isolated from non-cured *E. coli* RC424 strain and transformed to chloramphenicol and tetracycline-sensitive *E. coli* HB101 strain; they showed that *E. coli* HB101 became resistant to both antibiotics.

There are many ways to cure plasmid, such as: inhibition of plasmid replication, like acridine orange and ethidium bromide, or inhibition of enzymes responsible for DNA replication, like elevated temperature, pH, urea, or may affect, such as the plasma membrane, like SDS (Spengler *et al.*, 2006; Paul *et al.*, 2020).

E. Plasmid curing by elevated temperature 46°C

Low pH as a curing agent showed high elimination of resistance. This ensured that genes responsible for resistance to tetracycline, Nalidixic acid, and Cobalt may be located on the DNA plasmid; if the resistance was lost after plasmid curing that means genes responsible for these resistances were plasmid-mediated (Churchill and Romanus, 2018). Bacterial isolates remained resistant to Ampicillin, Erythromycin, Chloramphenicol, and Copper after curing by elevated temperature at 46°C, as curing by pH5, which probably ensures that genes of these resistances are located on the DNA chromosome. If the resistance was unaffected by plasmid curing, that means chromosomally borne (Carlton and Brown, 1981). In this study, the results showed that elevated temperature was more successful in curing plasmid than using low pH as a curing agent of plasmid in *E. coli* isolates studied. Ozdemir (2019) studied on 25 bacteria isolates of *E. coli* O157:H7 which were isolated from the stool; and he used elevated temperature 46°C to cure plasmid of isolate number 15, also he showed losing in resistance antibiotics as to each of Gentamicin, Ciprofloxacin, Amikacin and Trimethoprim in a rate of 35.71%; while when he used Ethidium bromide as curing agents showed that the rate was 28.57%; Ozdemir pointed that elevated temperature was more effective than ethidium bromide in curing plasmid. The mechanism of curing plasmid by elevated temperature takes place because high temperature affects the enzymes involved in DNA Replication which leads to a decrease in plasmid replication.

In another case of (Zaaen *et al.*, 2013) studied on 10 isolates of *E. coli* curing by elevated temperature showed that all isolates remained resistant for Ampicillin and all isolates lost resistance to Streptomycin except two isolates; and some isolates lost resistance to Nalidixic acid; while all isolates became sensitive to Erythromycin, and he ensured that Ampicillin resistance didn't affect because of production of alternative Penicillin Binding Protein (PBPs) encoding from *mecA* gene which locate on chromosome.

V. Conclusions

In this study, *E. coli* isolated from stool exhibited high resistance to Nalidixic acid and $CuCl_2$. Curing agents like pH5 and 46°C made *E. coli* lose the resistance to Tetracycline, Nalidixic acid, and Cobalt. These results indicated that the genes responsible for that resistance are located on plasmids. while those isolates which remained resistant to Ampicillin, Erythromycin, Chloramphenicol, and Copper after curing by both agents pointed to the fact that the genes of this resistance are located on chromosomal DNA.

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