Indian Journal of Modern Research and Reviews

This Journal is a member of the *'Committee on Publication Ethics'*

Online ISSN:2584-184X

Research Article

Role of Plasmids in Antibiotic Resistance and Biofilm Formation Among Clinical Bacterial Isolates

Sabreen G. Ghazal *

Department of Medical Laboratory Techniques, College of Health and Medical Techniques
AL-Furat Al-Awsat Technical University, Iraq

Corresponding Author: *Sabreen G. Ghazal

DOI: https://doi.org/10.5281/zenodo.18349948

Abstract

Background: Plasmids are extra-chromosomal circular DNA molecules that often carry virulence and resistance genes, transmitted horizontally among bacteria through conjugation or transformation. Their role makes them central to the global spread of resistance.

Methods: In this study, 32 *Salmonella typhi* isolates were obtained from blood samples of typhoid fever patients. Biofilm formation was assessed by the tissue culture plate (TCP) method, antibiotic susceptibility by disk diffusion (CLSI 2021), and plasmid profiles by electrophoresis.

Results: Results showed that all isolates (100%) were strong biofilm producers, enhancing survival against host defences and antibiotic action. Antimicrobial testing revealed high resistance to multiple drugs: cefotaxime and ciprofloxacin (100%), ampicillin (93.7%), piperacillin, and aztreonam (96.8%). In contrast, isolates were fully sensitive to carbapenems such as meropenem (100%). Thus, all were classified as multidrug-resistant (MDR). Plasmid analysis indicated the presence of 3–5 distinct plasmid bands of varying molecular sizes across isolates, suggesting genetic diversity.

Conclusions. This diversity likely contributes to the carriage of genes associated with resistance and biofilm formation. The findings highlight a strong correlation between plasmid content, biofilm-forming ability, and MDR in *S. typhi*. Such traits complicate treatment, given the bacteria's enhanced survival and resistance capacity. The study underscores the need for detailed plasmid sequencing to identify specific resistance and virulence genes. Understanding this genetic basis may provide insights for novel therapeutic strategies aimed at controlling the spread of resistant *S. typhi* infections and mitigating public health risks.

Manuscript Information

- **ISSN No:** 2584-184X
- **Received:** 20-11-2025
- **Accepted:** 29-12-2025
- **Published:** 23-01-2026
- **IJCRM:**4(1); 2026: 162-168

- **Plagiarism Checked:** Yes
- **Peer Review Process:** Yes

How to Cite this Article

Ghazal S G. Role of Plasmids in Antibiotic Resistance and Biofilm Formation Among Clinical Bacterial Isolates. Indian J Mod Res Rev. 2026;4(1):162-168.

Access this Article Online

www.multiarticlesjournal.com

KEYWORDS: Extraction, Salmonella. S Typhi, Susceptibility test, TCP.

1. INTRODUCTION

India's a rod-shaped, gram-negative pathogen that belongs to a member of the family of Enterobacteriaceae. Every strain of Salmonella is infectious, which implies it can enter, multiply, and endure through human host cells, leading to potentially lethal illness. But when Salmonella infects non-phagocytic human cells that are hosts, it has a particular property. (Yadav *et al*., 2020) A large number of copies of plasmid, including virulence genes, is carried by specific strains of Salmonella. Virulence plasmids must be present to cause systemic sickness, even if their importance in the intestinal phase of illness is not known. Salmonella plasmid virulence (SPV), a 7.8 kb piece that is necessary for the growth of bacteria into the reticulo-endothelium (RE), is found in all virulence plasmids, which vary in size between 50 and 90 kb. (Sultana, 2012; AL-Quraishi, 2018). The non-chromosomal two-strand circular DNA fragments known as plasmids can both survive and replicate without a host chromosome. These plasmids are the primary carriers of contemporary resistance to certain antibiotics, as they have been directly linked to widespread epidemics of antibiotic-resistant illnesses. (Karthikeyan and Santhosh, 2010). One factor influencing the extension of resistant antibiotics (AR) is horizontal transmission of genes (HGT). Mobile genetic elements such as plasmids, phages, and transposons can facilitate HGT through conjugation, transmission, and transformation, respectfully. (Frost et al., 2005).

According to Gillings, some of the HGT agents related to AR are being found in Enterobacteriaceae, especially Salmonella., (2014) and (Brown- Jaque *et al*. 2015). Resistance for the modern world. A plasmid outbreak is the presence of plasmids from several kinds of enteric bacteria membrane species; nevertheless, the host strains' contributions to gene-encoded antibiotic-resistant traits make therapy and observation more difficult still. Due to the fast spread of antibiotic resistance caused by plasmids, both prevalent and life-threatening diseases are now inoperable and medical interventions are no longer effective. Salmonella's plasmids contain a few genes, such as virulence genes, sex pilli, and antibiotic resistance, allowing Salmonella to readily adapt to various circumstances. (Karthikeyan and Santhosh, 2010). The rise in antibiotic-resistant bacteria (AR) resulting from Salmonella infections in the United States highlights the need to monitor plasmids carrying AR genes. (CDC, 2019; Tack et al., 2020). Conjugative plasmids may transport AR genes more deeply because they are self-transmitted. The conjugation required a source of transfers ("oriT"), the MOB gene sequences, and mating pair-forming (MPF) genes. (Smillie et al., 2010; Banuelos-Vazquez et al., 2017).

DNA replication and transport to a new cell are helped by the Mobil genes. DNA may travel between the two cells according to the pair of mates formation (MPF) complex. The protein in this complex is part of the subgroup of the Type-4 secretion systems. Fortunately, the genes coding these proteins vary based on the type of plasmid. (Wallden et al., 2010; Li et al., 2019). For conjugation to happen, the relaxasome—the type of protein complex that breaks down plasmid DNA and prepares it for transfer—has to join together at the oriT. (Wong et al., 2012; Waksman, 2019). In the presence of the matching pair structures, the relaxation enzyme then breaks down plasmid DNA and directs it through a translocation route made available by the MPF genes. (Ilangovan et al., 2015). Salmonella typhi's multidrug resistance (MDR) is an important threat to public health. Via gene transfer between cells from bacteria, plasmids are essential to the transmission of this resistance. Genes linked to factors of virulence, such as the capacity to build biofilms, which offer additional defence against antibiotics, may additionally be transmitted by plasmids. Few research have connected plasmid patterns to specific phenotypic traits of S. typhi, even though a few have examined resistance in this bacterium. By connecting plasmid patterns to the capacity to produce biofilm and resistance to several antibiotics in S. typhi isolates, this study hopes to fill this information gap.

2. MATERIAL AND METHOD

Specimens Collection

The public health laboratory, the AL-Furat Health Centre, and other hospitals obtained 32 blood specimens from people suffering from symptomatic enteric fever. Males and females with typhoid-related illnesses provided specimens for study. Blood was extracted from patients who did not receive antibiotics for a week prior to the sample being obtained. The specimens included details about the patients' age, sex, and kind of infection. A form printed on sheet paper was filled out with the date of birth, sex, age, and various other information. When a positive result was obtained, each sample was directly inoculated on artificial media involving MacConkey, XLD, and SS agar; the inoculation growth dishes were quickly kept in an incubator set at 37 °C for a period of between 18 and 24 hours before being stored for a week or until they were requested. A specific screws cup containing four millilitres of pure venous blood was kept inside the bacterial/alert 3D apparatus for a week. (Richard et al., 2007; Cheesbrough, 2010).

Detection of Biofilm Formation Using the Tissue Culture Plate Method (TCP)

Each isolate's capacity to generate biofilm was assessed using the TCP protocol (Stepanovic et al., 2004). After being injected into the broth medium, the isolated cell was diluted one to one hundred with fresh 3% glucose brain-heart infusion medium and incubated at 37°C for roughly eighteen hours at a constant temperature. A sterile polystyrene 96 flat-bottom culturing plate was filled with 0.2 ml portions of the diluted culture, with broth acting as a control, evaluating sterility and non-specific media binding. A tissue culturing plate was incubated at 37°C for 18 to 24 hours. The tissue cultivation plate was incubated for 18 and 24 hours. Following incubation, each well's contents were carefully extracted by tapping the plate. To get rid of free-floating "planktonic" bacteria, the wells were washed four times with 0.2 cc of saline with phosphate buffer (pH 7.2). The biofilm that adhering "sessile" bacteria created on the plate was fixed with sodium acetate (2%) and

stained with crystal violet (0.1% w/v). The plates were allowed to dry after any remaining discolouration was removed with deionised water. Attachment bacteria, which were frequently stained with crystal violet, produced biofilm on each side of the wells. A micro-ELISA auto reader (OD 570) was used to quantify the optical density, also called the OD, of stained adhesion bacteria at wavelengths of 570 nm. Three replicates and three iterations of the study were carried out. The results are presented after the data was averaged. in Table (1).

Table1. Bacterial Biofilm Formation Classification Using the Tissue Culture Plate Method (TCP) (Stepanovic et al., 2004).

Biofilm formation	Adherence	Mean OD Values
Non/Weak	Non/Weak	< 0.120
Moderate	Moderately	0.120-0.240
High	Strong	> 0.240

Determination Susceptibility Test (Disk Diffusion Method)
The in vitro susceptibility of S. typhi isolates to eleven antimicrobial medications was assessed using the disk diffusion method in compliance with the Clinical and Laboratory Standards Institute's standards (2021). Following twenty-eight hours of activation in nourishing broth at 37°C, the resulting colonies were spread out on the Muller-Hinton agar (MHA) using an uncontaminated cotton swab after being adjusted to 0.5 McFarland's® criterion (1.5×108 CFU/ml). Antibiotic disks were placed on MHA, lightly pressed down to ensure their complete interaction with the cultured agar, and then incubated at 37°C for a full day before measurements were taken in millimetres (mm).
Based on the findings, they were classified as resistant, intermediate, or susceptible. CLSI (2021).

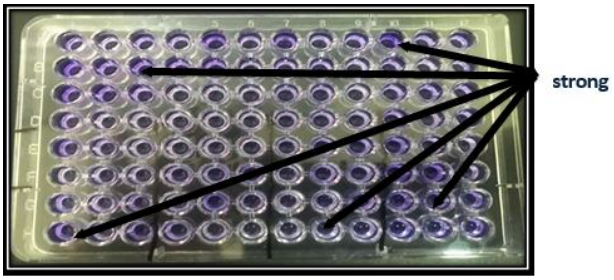
Plasmid DNA Extraction:
It has been extracted and purified in accordance with Favorgen/China's guidelines. The following actions are included in the instructions:

1. A centrifuge tube was filled with 1-3 ml of fresh bacterial culture that had been cultivated in a BHI broth medium. Pellets that were categorised as resistant, intermediary, or vulnerable were obtained by centrifugation at 11,000 rpm for one minute.
2. Carefully dispose of the supernatant using a micropipette after centrifugation to prevent precipitate mixing.
3. To fully resuspend the pellets, 200 µl of the FAPD-One buffer (with RNase A added at initial usage) was added, and the mixture was thoroughly mixed by pipetting.
4. 200 µl of the FAPD solution was added to the buffer, mixed, and inverted five to ten times
5. The sample is followed by incubation at room temperature for two to five minutes. Because using a vortex during this stage could shear the chromosomal DNA, it was avoided. Furthermore, prolonged incubation (more than five minutes) has been avoided since it could denaturize a supercoiled plasmid DNA.
6. Due to the significance of preventing asymmetric precipitation, the lysate was neutralised by adding 300 µl of FAPD-Three buffer and gently mixing it by inverting the tube five to ten times. In order to clear the lysate, this was accompanied by centrifugation at a super speed of about 18,000 rpm for five minutes. During this time, the Column FAPD was placed in the Collection Tubes to prepare the stepcause denature on the supercoiled plasmid genome. To ensure that no white precipitate was being moved or disturbed, the loading stage included transferring the waste product to the FAPD Column utilising a micropipette following centrifugation
7. A centrifugation phase takes place at 11,000 speed for 300 seconds following the loading column. The column returned to the same collecting tube after this step, and the supernatant was disposed of.
8. The FAPD Column was filled with 400 µl of W-One, centrifuged for 30 seconds at 11,000 rpm, the flow was discarded, and the column was then put back into the Collection Tube.
9. To ensure that the column is dry and that any remaining liquid has been removed, this step involves mixing in the 700 µl of washing buffer, centrifuging at 11,000 rpm for 30 seconds, removing the flow, and placing the column in the collection tube before centrifuging at a super-speed of approximately 18,000 rpm for an additional 3 minutes. This step involves adding 96%–100% ethanol to the buffer for washing for the first time.
10. The most crucial step is to transfer the FAPD Column into a fresh 1.5 ml micro centrifuge tube. The elution was performed by adding 50 µl to 100 µl of the Elution Buffer immediately towards the membrane. middle of the FAPD Column without making any contact with the surface of the membrane. The column was then stood to stay one minute to make sure that the elution solution was distributed on the membrane centre and completely absorbed. The plasmid DNA was then eluted and stored at -20°C by centrifugation at a super-speed of 18,000 rpm for one minute.

3. RESULTS AND DISCUSSION

Biofilm Formation in S. typhi
The TCP technique for biofilm formation showed that 32/32 (100%) of the tested isolates were strong biofilm formers, as shown in Table 2 and Figure 1. Microcolony production and the next batch of a mature biofilm typically follow the first attachment phase of biofilm growth. These extracellular polymer substances (EPS), which encourage cell-cell interaction and the development of biofilms, were present in both phases. (Geoffrey, 2013).

Figure 1. Biofilm formation of S. typhi



Many studies have shown similar results of biofilm development (Chauhan et al., 2016). The ability of these isolates to create biofilms was similarly linked to increased antibiotic resistance; the most prolific biofilm makers possessed numerous antimicrobial-resistance cassettes (Raza et al., 2011). The ability of bacteria to form biofilm through complex interactions is essential to their survivability in the human host. Bacterial biofilms were shown to be the most prevalent form of bacterial growth, accounting for around 80% of all bacterial infections (Hoiby et al., 2010; Visvalingam et al., 2019). It was believed that biofilm supported the causing factor and resistance and had a protective role, providing the body with a basis for prolonged (Aly et al., 2018).

Table 2. Classification and Percentage of Bacterial Biofilm Formation by TCP Method.

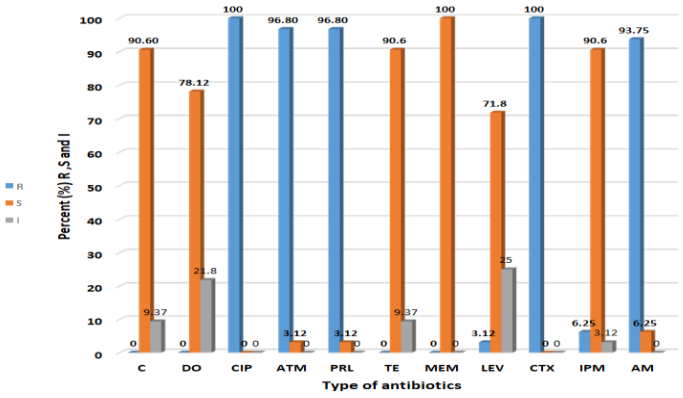
OD	Adherence	Biofilm No (%)	
<0.120	Non	Non\weak	0(0)
0.120-0.240	Moderate	Moderate	0(0%)
>0.240	Strong	Height	32(100)

Bacterial biofilms are organised groups of bacteria that use a self-made polymeric matrix to adhere to inert or living surfaces as well as to one another. They have been increasingly identified as risks to foods and public safety worldwide during the last 20 years (Monds and O'Toole, 2009). Because biofilm formation shows enhanced resistance to immune system responses like antimicrobial agents, nutrition disruption, and chemicals, causing its distribution as well as endurance both inside and outside of the host, they are very challenging to remove. (Hoiby et al., 2010; Tabak et al., 2009). The initial stage of the sequential, strictly regulated procedure of biofilm formation occurs when free-swimming planktonic bacteria adhere to a surface. A self-generated extracellular matrix made of nucleic acid, protein, and exopolysaccharide is created during biofilm maturity, encasing the microbial community and providing structure and defence (Monds and O'Toole, 2009). This sessile, matrix-bound population's continuous planktonic cell shedding may result in the microbe's discharge towards the surrounding environment or the biofilm's reconnecting and fortifications (Pasmore and Costerton, 2003). Biofilms, which are often thought of as a response to stress, have been connected to several acute and chronic illnesses. A biofilm component is present in around 80% of all bacterial infections. (Stoodley and Hall-Stoodley, 2009). According to a study by AL-Quraishi (2018), there were 11 (31%) robust biofilm formations, 21 (60%) intermediary biofilm formations, and 3 (8.5%) weaker biofilm formations of S. typhi. Nineteen (63.3%) of S. typhi develop intermediate biofilm, two (6.6%) create weak biofilm, and nine (30%) form strong biofilm, according to Flyyiha (2017). These outcomes aligned with their conclusions.

Antibiotic Susceptibility Test in S. typhi

CLSI's assessment of susceptibility to antibiotics (2021). Eleven antibiotics were tested for their capacity to stop the development of thirty-two isolates of S. typhi utilising the Kirby- Bauer Disc diffusion method. The study showed that the 32 isolates had a high rate of resistance to quinolones and fluoroquinolones (Ciprofloxacin (CIP) was 32/32 (100%)), penicillins (Ampicillin (AM) was 30/32 (93.7%)), β-lactam inhibitors (Piperacillin (PRL) was 31/32 (96.8%)), cephalosporin (Cefotaxime (CTX) was 32/32 (100%), and monobactam (Aztronam (ATM) was 31/32 (96.8%). ß-lactamase enzymes were the main cause of Salmonella resistance to the two commonly used antibiotics (cephalosporins and penicillin), which made the medications ineffective. (Micheal et al., 2006). The World Health Organisation declared penicillins, third-generation cephalosporins, and other antibiotics to be "critically significant" in human medicine (World Health Organisation, 2012). Carbapenems (29/32 (90.6%) and Meropenem (MEM) 32/32 (100%)), tetracycline antibiotics (29/32 (90.6%) and doxycycline (DO) 25/32 (78.1%)), quinolones, and fluoroquinolones (Levofloxacin was 14/32 (43.7)), and antimicrobials (29/32 (90.6%)). Isolates from this study were therefore categorised as resistant to several drugs (MDR) due to, as Figure 2 shows, the S. typhi isolates were resistant to more than three groups of antibiotics.

Figure 2. Antibiotic Resistance, Sensitive, and Intermediate among S. typhi



These results align with those of Ali (2015). According to the new study, the variations in virulent factor levels generated by S. typhi may be caused by a plasmid factor that is either present or not in the isolates of bacteria, or it could be caused by mutations that take place among the bacterial isolates. The findings revealed that all of the S. typhi isolates had greater resistance than normal to the antibiotic penicillin (ampicillin), which could be due to the generation of β-lactamases or the inability of the

antibiotic to reach its target (PBPs). (Harwood and others, 2000). The majority of S. typhi isolates are resistant to cephalosporins, which can be explained by the drug's limited bacterial penetration, the absence of PBPs, or the drug's breakdown by β-lactamases (Brooks et al., 2007). Enzyme which open the ß-lactam rings by adding water molecules to the shared ß-lactam bond are collectively referred to as ß-lactamases. This inactivates ß-lactam antibiotics, such as carbapenems and penicillin (Abrescia et al., 2012). According to Michael et al. (2006), ß-lactamase enzymes are primarily responsible for Salmonella resistance to β-lactam antibiotics, like cephalosporins and penicillins, which make the medications ineffective. Research by Wang et al. (2003) in China, as well as Cortez et al. (2006) in Brazil, revealed that 78% of the isolates had ampicillin resistance. Only 33.3% of the isolates of Salmonella recovered from blood had ampicillin resistance, according to an Italian study by Bacci et al. (2012).

The findings show that the third class of carbapenems, which include synthesised β-lactam antibacterial agents, are different from penicillin-like antibiotics in that they have a 6.2% imipenem resistance rate because the sulfur-rich atom found in the thiazolidine ring has been externalised and replaced by a carbon atom. Since most β-lactam resistance was caused by β-lactamases, which enzymatically break the β-lactam ring and prevent it from binding to and activating cell wall enzymes, most of the bacterial isolates in this study were vulnerable to imipenem. By changing the chemical groups around the β-lactam ring, this resulted in the development of new β-lactams, which became resistant to β-lactamases. (Fadil, 2019; Giguere, 2013). This medication is useful in empirical treatment since it was effective against both Gram-positive and Gram-negative bacteria that produce β-lactamases. A dehydropeptidase located in the brush edge of the intermediate renal tubule cleaves imipenem. This enzyme produces a possibly nephrotoxic inactive metabolite (Karen, 2015). The results of the fourth group showed that monob actams, which also disrupt the production of bacterial cell walls, were different because the -lactam ring was not linked to another rings that increased the rate of resistance 96.8% Aztreonam Aztreonam is usually safe, but it can occasionally cause phlebitis, skin rashes, and abnormal liver function tests, skin rashes and phlebitis. This drug demonstrated little immunogenic potential and little cross-reactivity with other β-lactam-produced antibodies. For individuals who are resistant to carbapenems, cephalosporins, or other penicillins, this drug may offer a safe alternative. Karen (2015). Additional changes further enhanced their effectiveness against particular microbes or their accessibility to 100% resistant illness areas. These include cephalosporins like cefoxitin, cefotaxime, as well as cefazidime, which are the second and third generation cephalosporins, respectively, and modified penicillins such as ampicillin and amoxicillin-clavulanate (Giguere, 2013). Skin rashes and phlebitis

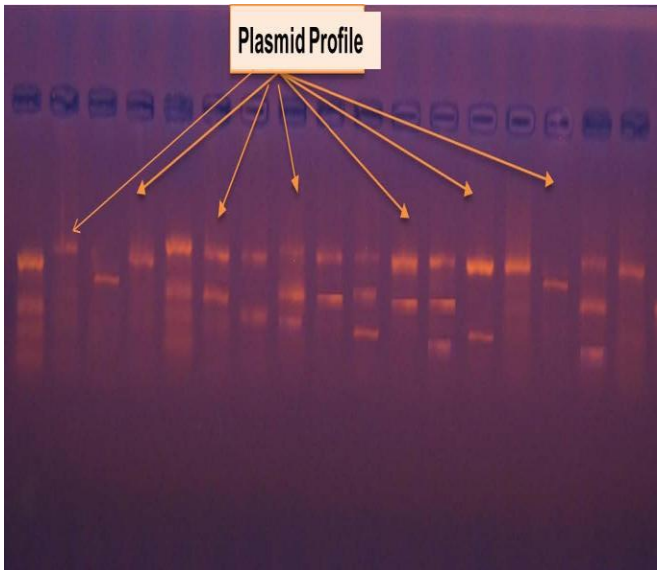
Multiple-antibiotic-resistant pathogens have become a serious threat to human health. Multi-drug resistant (MDR) Salmonella and other Enterobacteriaceae isolates were characterised by resistance to at least one agent in three or more antibiotic categories. Therefore, increased and non-cause typhoid fever is considered multidrug- resistant if it is resistant to the first-line drugs recommended for treatment, including ampicillin, cephalosporins, aminoglycosides, and other common drugs used as routine treatment for typhoid patients. Since the mid-1980s, MDR typhoid disease outbreaks have occurred in several developing countries, increasing rates of death and morbidity, especially in malnourished and affected children under five. (2019, Fadil).

Molecular Study

Detection of Plasmid Profiles

In this investigation, thirty-two S. typhi isolates underwent plasmid profile analysis. Ethidium bromide-stained agarose gel electrophoresis with a concentration of was used to detect the plasmid bands in these isolates, revealing the plasmid profile of S. Typhi. Each isolated Salmonella has a unique total of plasmids with different molecular weights, as shown in plasmid analysis. (Figure. 3).

Figure 3. Isolated plasmid profiles were electrophoresed on a 1% agarose gel at a voltage o80 V for 90 minutes and coloured with ethidium bromide (which contained three to five bands).



According to the results, S. typhi possessed three to five bands, which were in line with earlier discoveries (Edris et al., 2011). Most studies found that various Salmonella serotypes have varying plasmid counts and molecular weights. The results matched those of Ali (2015), who found that S. typhi possessed between 3 and 5 bands. According to Edris et al. (2011), S. typhi and other species share an MW band, and their total number of plasmids varies from three to five bands. Additionally. Typhi belonged to the same band as S. typhimurium. Plasmid profile analysis has been used to examine diarrheal illness outbreaks and to identify pandemic strains of S. typhi. Plasmid-free strains of Salmonella could be less virulent since the pathogenicity of these strains was reliant on the availability of a virulence plasmid (Hassanin et al., 2007)

4. CONCLUSION

This work demonstrates that plasmid patterns, the capacity to produce biofilms, and resistance to multiple drugs in the typhi isolates are strongly correlated. This relationship emphasises how crucial it is to research plasmids to comprehend the mechanisms underlying elevated resistance. Future research aiming at translating such plasmids to recognise the particular genes they carry may be based on these findings, which could aid in the creation of novel therapeutic approaches.

Conflict Of Interest Statement

Regarding the study, writing, and publication, the authors attested to having no conflicts of interest.

Acknowledgements

The research is dedicated to the University of Kufa's College of Science, the Central Laboratory Director, and the participating professors.

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About the corresponding author

Sabreen G. Ghazal is affiliated with the Department of Medical Laboratory Techniques, College of Health and Medical Techniques, Al-Furat Al-Awsat Technical University, Iraq. Her academic and professional interests include clinical laboratory diagnostics, microbiology, biomedical analysis, and quality practices in medical laboratory sciences.