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ORIGINAL ARTICLE

# Prevalence of Virulence Genes Carried on Plasmids versus Chromosomes in *Enterobacter cloacae* Isolated from Various Clinical Samples

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***Key words:***
***Enterobacter cloacae, fepA, aac(6')-Ib, virulence genes, mobile genetic elements***

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## ABSTRACT

***Background:*** *Enterobacter cloacae* ***is*** *a common cause of Hospital-Acquired Infections. Its resistance and virulence are often associated with stable chromosomal regions or mobile genetic elements such as plasmids.* ***Objective:*** *This study aims to investigate the role of bacterial plasmids in contributing to clinical diseases in the Thi-Qar Governorate, Iraq.* ***Methodology:*** *Thirty E. cloacae isolates were collected from blood, urine, wound, and stool samples. Bacterial identification was performed using both conventional and automated methods. Antibiotic susceptibility was assessed using the disc diffusion method. PCR assays were employed to detect the presence of selected virulence genes: fepA, aac(6')-Ib, and csgA.* ***Results:*** *The fepA gene was significantly more prevalent in chromosomal DNA (56.67%) compared to plasmid DNA (40.0%). The aac(6')-Ib gene showed a prevalence of 66.67% in chromosomal DNA versus 53.33% in plasmid DNA. Additionally, the csgA gene, which encodes curli fimbriae, was detected in 60.0% of chromosomal samples and 36.67% of plasmid samples. The differences in distribution for all three genes were statistically significant ($p < 0.05$).* ***Conclusion:*** *The higher prevalence of virulence genes in chromosomal DNA suggests a stable integration that may enhance environmental adaptability and long-term resistance. These findings highlight the importance of gene location in understanding the persistence of antibiotic resistance and bacterial virulence.*

## INTRODUCTION

Hormaeche and Edwards first introduced the genus Enterobacter in 1960. It belongs to the family Enterobacteriaceae and comprises rod-shaped, facultatively anaerobic, Gram-negative bacteria that inhabit a variety of environments, including soil, water, plants, and the tissues of humans and animals[1]. Due to their numerous antibiotic resistance mechanisms and frequent association with hospital-acquired infections, *Enterobacter* species are of significant clinical importance. They possess several virulence factors that enhance their pathogenicity and enable survival in harsh environments, including biofilm formation and iron acquisition system[2]. Common species include *Enterobacter cloacae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter roggenkampii*, *Enterobacter asburiae*, and *Enterobacter ludwigii*[3]. Among these, the *E. cloacae* complex (ECC) is particularly noteworthy due to its genetic diversity and role as a major nosocomial pathogen. It is responsible for approximately 10% of post-operative peritonitis cases, 4% of urinary tract infections (UTIs), and up to 5% of hospital-acquired septicemia.

Beyond its clinical significance, *E. cloacae* is also widespread in terrestrial and aquatic environments and is capable of infecting plants and insects[4,5]. The bacterium's pathogenicity is largely attributed to its array of virulence factors. Key genes include *fepA*, involved in iron acquisition; *qnrB* and *aac(6')-II*, which confer resistance to quinolones and aminoglycosides, respectively; *tetA*, associated with tetracycline resistance; and *csgA*, which facilitates curli fimbriae production, enhancing adhesion and biofilm formation[6]. These resistance and virulence genes are often located on mobile genetic elements such as transposons, integrons, and, notably, plasmids.

Plasmids, as extrachromosomal DNA elements, are particularly important in the horizontal transfer of resistance genes such as *bla<sub>CTX-M-9</sub>* and *qnrA1*, which confer resistance to beta-lactam antibiotics and fluoroquinolones[7]. By carrying clusters of resistance and virulence genes, plasmids enhance *E. cloacae*'s adaptability to hostile conditions, such as those imposed by antibiotic therapy in hospital settings. Plasmids like pQC have been associated with multidrug-resistant strains of *E. cloacae*, significantly contributing to their persistence and spread in nosocomial environments[8]. The presence of mobile

genetic elements, especially plasmids, underscores the need for continuous genetic surveillance and advanced molecular diagnostic techniques. These factors reveal *E. cloacae*'s remarkable resilience across diverse environments, further highlighting its therapeutic and epidemiological significance[2,9].

This study aims to investigate the role of bacterial plasmids in contributing to clinical diseases in the Thi-Qar Governorate, Iraq.

## METHODOLOGY

**Ethical clearance:**

This study is subjected to the qualifications of ethical considerations and according to the form prepared for this purpose by the Iraqi Ministry of Health. The research also got the agreement of the committee of ethical standards at the College of Science, Thi-Qar University, one of the colleges belonging to the Ministry of Higher Education and Scientific Research, Iraq October (2024) in accordance with document number 232/2024. In addition, informed consent was obtained from all patients before we took samples.

**Samples Collection:**

A total of 200 clinical samples were collected from patients at the Public Health Laboratory, Al-Hussein Teaching Hospital, Al-Haboubi Private Hospital, and Al-Turki Hospital between December 9, 2024, and January 31, 2025. The samples included blood, urine, wound swabs, and stool specimens.

**Culture and Isolation:**

Blood samples were initially inoculated into Brain Heart Infusion (BHI) broth and incubated aerobically at 37°C for 24–48 hours. Subsequent subculturing was performed on MacConkey agar plates under aerobic conditions.

Urine samples were directly inoculated onto MacConkey agar and incubated aerobically at 37°C for 24 hours.

Wound swabs were streaked onto both blood agar and MacConkey agar plates and incubated aerobically at 37°C for 24 hours.

Stool samples were cultured on Xylose Lysine Deoxycholate (XLD) agar and MacConkey agar, followed by aerobic incubation at 37°C for 24 hours.

**Identification of Bacteria**

Preliminary identification of bacterial isolates was performed based on colony morphology, Gram staining, and a series of conventional biochemical tests, following the guidelines described by[10]. Final confirmation of the isolates was conducted using the API 20E identification system and the automated VITEK-2 Compact system (bioMérieux**,** France).

**Antibiotic Susceptibility Testing:** The antimicrobial susceptibility of the isolates was evaluated using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar, following the Clinical and Laboratory Standards Institute (CLSI) guidelines[11]. The panel of antibiotics tested included:

Amikacin, Gentamicin, Ciprofloxacin, Cefotaxime, Ceftazidime, Meropenem, Imipenem, Trimethoprim-sulfamethoxazole, and Piperacillin-tazobactam.

Zones of inhibition were measured and interpreted according to CLSI standards to determine the susceptibility profile of each isolate.[12]

**Molecular study**

**Extraction of genomic DNA and Plasmid from bacterial culture**

Genomic and plasmid DNA were extracted from *E. cloacae* isolates using the Geneaid Genomic DNA Purification Kit (Taiwan), following the manufacturer's instructions. Plasmid DNA purification was performed using the Column-Pure Mini-Prep Kit (abm, Canada), which employs a silica spin filter. This method is widely used in modern molecular biology laboratories due to its simplicity, high yield, and suitability for downstream applications such as automated sequencing.

The primers used in this study were supplied lyophilized by Bioneer (South Korea) and were reconstituted in sterile deionized distilled water prior to use.

**Statistical Analysis:**

The samples underwent statistical analysis accordingto Statistical Package for the Social Sciences (SPSS) Chi sequare ($\chi 2$) and p-value indicated level significant between the samples.

## RESULTS

**Detection of virulence genes by PCR assay**

The PCR mixture for each target gene (*fepA*, *csgA*, *qnrB*, *tetA*, and *aac(6')-II*) was prepared in a total volume of 20 µL, consisting of 3 µL of DNA template, 2 µL of each primer, and 13 µL of nuclease-free water ($ddH_2O$), as detailed in Table 1. After combining the reagents in the PCR tube, the mixture was briefly centrifuged, and the tube was placed into the PCR thermocycler. Amplification was carried out following the cycling conditions outlined in Table 2.

**Prevalence of Enterobacter cloacae According to Type of Sample**

The current study was showed a significant difference at p. value <0.05, according to type of sample, was noted the most isolated E.cloacae from both blood sample and wound sample 33.33%, then from urine sample 23.34%, while the lowest isolated E. cloacae from stool sample 10.0%, as in table 3.

**Prevalence of *Enterobacter cloacae* According to Sex**

The current study was showed a non-significant difference at p. value <0.05, according to sex, was recorded the 50.0% of patient was male and 50.0% of patient was female, as in table 4.

**Table 1: List of the primer that used for screening VGs through PCR technique**

| Gene | Primer sequence (5′ to 3′) | Product size (bp) | Gene Description |
|---|---|---|---|
| ***csgA*** | F CTG ACG ACA GCA CCA TCT CT R TCC ACC GTA CTG GCT CAC AT | 107 | 11 |
| ***fepA*** | F TCT TTT TTC ACC GGC ATG GA R CGT GCG GTG GTC AAT ATC T | 572 | 11 |
| ***aac(6')-II*** | AGCGACCGACTCTTGATGAA/ GGCTTGTCGTGTTTGAACC | 414 | 12 |

**Table 2: Amplification of G enes used in the study (fepA, *CasgA*, and *aac(6)ll*)**

| **No.** | **Amplification ofCasgA gene** | | | |
|---|---|---|---|---|
| 1 | PCR step | Time | Temperature | Cycle |
| | Initial denaturation | 5 min | 95 °C | 1 |
| | Denaturation | 30 sec. | 95 °C | 35-30 |
| | Annealing | 45 sec. | 55 °C | 35-30 |
| | Extension | 1min | 72 °C | 35-35 |
| | Final extension | 5min | 72 °C | 1 |
| 2 | Amplification of fepA gene | | | |
| | PCR step | Time | Temperature | Cycle |
| | Initial denaturation | 2min | 94 °C | 1 |
| | Denaturation | 30sec. | 94 °C | 35-30 |
| | Annealing | 30 sec. | 58 °C | 35-30 |
| | Extension | 1min | 72 °C | 35-30 |
| | Final extension | 5min | 72 °C | 1 |
| 3 | Amplification of aac(6)ll gene | | | |
| | PCR step | Time | Temperature | Cycle |
| | Initial denaturation | 4min | 94 °C | 1 |
| | Denaturation | 60sec | 94 °C | 35 |
| | Annealing | 60sec | 53°C | 35 |
| | Extension | 45sec | 72 °C | 35 |
| | Final extension | 7min | 72 °C | 1 |

**Table 3: Prevalence of *E. cloacae* according to type of sample**

| | **Clinical Sample** | **No.** | **%** |
|---|---|---|---|
| ***E. cloacae*** | Blood | 10 | 33.33 |
| | Stool | 3 | 10.00 |
| | Urine | 7 | 23.34 |
| | Wound | 10 | 33.33 |
| | Total | 30 | 100 |
| $CalX^2$= 14.4 | $TabX^2$= 7.81 | DF= 3 | p. value <0.01 |

**Table 4: Prevalence of *Enterobacter cloacae* according to age**

| | **Age Group** | **No.** | **%** |
|---|---|---|---|
| ***E. cloacae*** | 20-30 years | 12 | 40.00 |
| | 31-40 years | 6 | 20.00 |
| | 41-50 years | 5 | 16.67 |
| | ≥ 51 years | 7 | 23.33 |
| | Total | 30 | 100 |
| $CalX^2$= 12.7 | $TabX^2$= 7.81 | DF= 3 | p. value <0.01 |

**Antibiotics Susceptibility of *Enterobacter cloacae***

The current study was showed a significant difference at p. value <0.05, according to response of *Enterobacter cloacae* bacteria to antibiotics with different mechanism of action, the study showed 100% of bacteria were sensitive to activity of Meropenem, and 90.0% of bacteria were sensitive to activity of Tigecycline and Colistin, with regard intermediate response the study showed 16.67% had a moderate response to Nitrofurantoin antibiotic, 13.33% had a moderate response to Imipenem and Ceftazidime/taz. Regarding resistance response the study showed 60.0% of bacteria were resistance for activity Amp/Sulb and Ceftazidime, and 50.0% for Ciprofloxacin, in addition 65.1% of bacteria sensitive to all antibiotics and 28.63% of bacteria resistance to all antibiotics as in table 5.

**Comparison of *feBA* Gene Frequency between hromosome and Plasmid**

The present result was showed a significant difference at p. value <0.05, between chromosome and plasmid, was showed the frequency of *feBA* gene in chromosome of *E. cloacae* was 56.67%, while in plasmid 40.0%, also their frequency in chromosome increased 1.98 times than frequency in plasmid as in table 6.

**Table 5: Antibiotics susceptibility of *Enterobacter cloacae***

| Antibiotics | Sensitive | | Intermediate | | Resistance | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| Amp/Sulb | 10 | 33.33 | 2 | 6.67 | 18 | 60.00 |
| Pip/Tazo | 20 | 66.67 | 3 | 10.00 | 7 | 23.33 |
| Cefotaxime | 8 | 26.67 | 0 | 0.00 | 22 | 73.33 |
| Ceftazidime | 10 | 33.33 | 2 | 6.67 | 18 | 60.00 |
| Ceftazidime /av | 15 | 50.00 | 2 | 6.67 | 13 | 43.33 |
| Ceftazidime/taz | 21 | 70.00 | 4 | 13.33 | 5 | 16.67 |
| Cefepime | 18 | 60.00 | 3 | 10.00 | 9 | 30.00 |
| Imipenem | 22 | 73.33 | 4 | 13.33 | 4 | 13.33 |
| Meropenem | 30 | 100 | 0 | 0.00 | 0 | 0.00 |
| Amikacin | 20 | 66.67 | 3 | 10.00 | 7 | 23.33 |
| Gentamycin | 18 | 60.00 | 1 | 3.33 | 11 | 36.67 |
| Ciprofloxacin | 15 | 50.00 | 0 | 0.00 | 15 | 50.00 |
| Tigecycline | 27 | 90.00 | 2 | 6.67 | 1 | 3.33 |
| Colistin | 27 | 90.00 | 0 | 0.00 | 3 | 10.00 |
| Trimethoprim | 24 | 80.00 | 0 | 0.00 | 6 | 20.00 |
| Ertapenem | 26 | 86.67 | 1 | 3.33 | 3 | 10.00 |
| Nitrofurantoin | 21 | 70.00 | 5 | 16.67 | 4 | 13.33 |
| Susceptibility % | 65.1 | | 6.27 | | 28.63 | |
| CalX²= 135.3 TabX²= 43.77 DF= 32 p. value <0.01 | | | | | | |

**Table 6: Comparison of *feBA* gene frequency between chromosome and plasmid**

| *feBA* | Chromosome | | Plasmid | | Total | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| **Positive** | 17 | 56.67 | 12 | 40.0 | 29 | 48.33 |
| **Negative** | 13 | 43.33 | 18 | 60.0 | 31 | 51.67 |
| **Total** | 30 | 50.0 | 30 | 50.0 | 60 | 100 |
| CalX²=5.78 TabX²= 3.84 DF= 1 p. value 0.016 Odds Ratio 1.98 (1.13 – 3.49) | | | | | | |

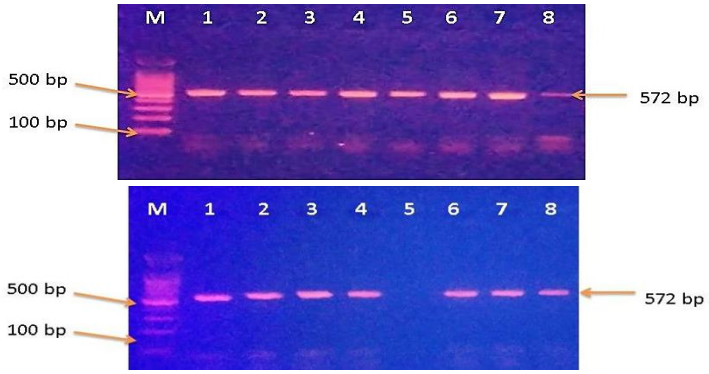


**Fig. 1:** Plat of Agarose gel electrophoresis which explained the amplification product of fepA gene (572bp.) specific to E. clocae. from different sample with PCR analysis, was marker ladder 1500bp., agarose was 2g, TBE buffer (1x), and 100 V in 30 min. then followed by 50V in 45min., stained with red stain, first gene in chromosome and second plasmid

**Comparison of *aac(l)6* Gene Frequency between Chromosome and Plasmid**

The present result was showed a significant difference at p. value <0.05, between chromosome and plasmid, was showed the frequency of *aac(l)6* gene in chromosome of *E. cloacae* was 66.67%, while in plasmid 53.33%, also their frequency in chromosome increased 1.8 times than frequency in plasmid as in table 7.

**Comparison of *caga* Gene Frequency between Chromosome and Plasmid**

The present result was showed a non-significant difference at p. value <0.05, between chromosome and plasmid, was showed the frequency of *caga* gene was equal in both chromosome and plasmid, also, there is no advantage to frequency the gene in the chromosome than plasmid. as in table 8.

**Table 7: Comparison of *aac(l)6* gene frequency between chromosome and plasmid**

| *aac(l)6* | Chromosome | | Plasmid | | Total | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| **Positive** | 20 | 66.67 | 16 | 53.33 | 36 | 60.0 |
| **Negative** | 10 | 33.33 | 14 | 46.67 | 24 | 40.0 |
| **Total** | 30 | 50.0 | 30 | 50.0 | 60 | 100 |
| CalX²= 4.08 TabX²= 3.84 DF= 1 p. value 0.043 Odds Ratio 1.80 (1.01 – 3.19) | | | | | | |

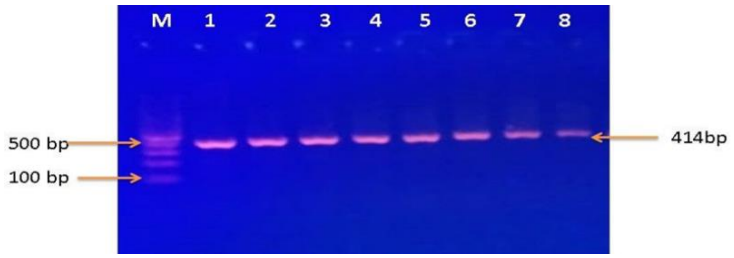


**Fig. 2:** Chromosomal Detection of *aac(6')-II* Gene (572 bp) in *E. cloacae* by Agarose Gel Electrophoresis

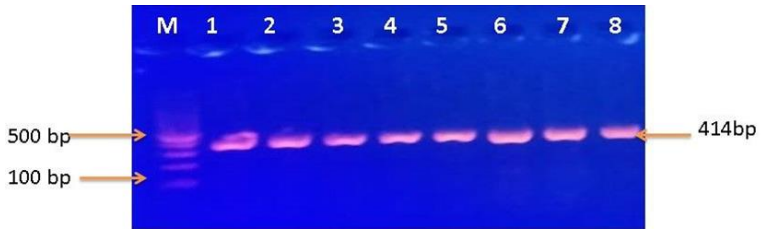


**Fig. 3:** Plasmid Detection of *aac(6')-II* Gene (572 bp) in *E. cloacae* by Agarose Gel Electrophoresis

**Table 8: Comparison of *caga* gene frequency between chromosome and plasmid**

| Caga | Chromosome | | Plasmid | | Total | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| **Positive** | 3 | 10.0 | 3 | 10.0 | 6 | 10.0 |
| **Negative** | 27 | 90.0 | 27 | 90.0 | 54 | 90.0 |
| **Total** | 30 | 50.0 | 30 | 50.0 | 60 | 100 |
| CalX²=0.00 TabX²= 3.84 DF= 1 p. value 1.00 Odds Ratio 1.00 (0.39 – 2.51) | | | | | | |

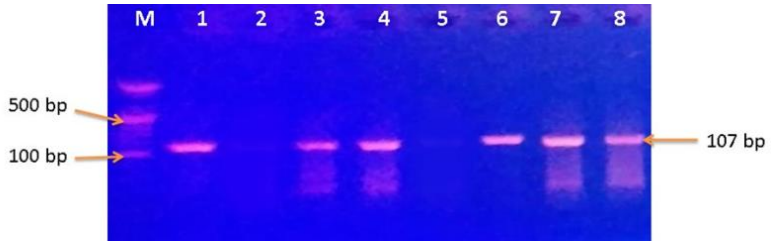


**Fig. 4:** Plat of Agarose gel electrophoresis which explained the amplification product of aac(6')-II gene (572bp.) specific to E. clocae. from different sample with PCR analysis, was marker ladder 1500bp., agarose was 2g, TBE buffer (1x), and 100 V in 30 min. then followed by 50V in 45min., stained with red stain, 1, 3, 4 bands is a gene in chromosome the other in plasmid.

## DISCUSSION

The current study aimed to compare the distribution of selected virulence genes between plasmid and chromosomal DNA in clinical *Enterobacter cloacae* isolates collected from Thi-Qar Province. The results demonstrated a significant difference in the prevalence of these genes between the two genetic loci, reflecting distinct genetic characteristics and potential for horizontal gene transfer. These findings underscore the importance of differentiating gene location when evaluating bacterial virulence and antibiotic resistance, with critical implications for infection control and clinical management.

The *fepA* gene showed a significantly higher prevalence in the chromosome (56.67%) compared to the plasmid (40%) ($p = 0.016$). This suggests that *fepA* is likely part of the stable genetic makeup of *E. cloacae*, contributing to environmental adaptation and long-term resistance. These findings are consistent with [13], who reported that certain virulence genes are integrated into the chromosome, providing stable bacterial traits. Similarly Benredjem et al.[14] noted that chromosomal genes generally exhibit greater long-term stability than plasmid-borne genes, making them less prone to rapid mutation. In line with this, Romero et al.[15] found comparable results in Algeria, with *fepA* expressed in 60% of chromosomal DNA samples versus 42% in plasmids. This convergence of evidence supports the role of the *fepA* gene as part of *E. cloacae*'s environmental adaptation system, where its chromosomal location enhances bacterial stability and persistence, providing an evolutionary advantage by maintaining baseline pathogenicity and fitness.

A recent study by Lee et al.[16] demonstrated that siderophore-related genes integrated into the chromosome play a vital role in maintaining iron homeostasis and survival under oxidative stress, particularly in Hospital-Acquired strains of *Enterobacter* spp. Similarly Seeberg et al.[17] found that chromosomal localization of iron acquisition genes in *Klebsiella pneumoniae* was associated with enhanced biofilm formation and persistence in the urinary tract, whereas plasmid-located variants were more commonly linked to acute infections. Moreover Dimitriou et al.[18] reported that *fe*-related chromosomal genes exhibited less variability across clinical isolates collected over five years, indicating genetic stability and vertical transmission. These findings support the interpretation that the chromosomal integration of *feBA* may provide long-term survival advantages, especially under antibiotic pressure or nutrient-limited conditions.

Interestingly, in contrast to your study's 56.67% chromosomal presence, Frasson et al.[19] found a 65% chromosomal versus 35% plasmid distribution of the *fepA* gene in *E. cloacae* isolated from bloodstream infections in Greece, further confirming the trend of chromosomal dominance. This supports the idea that the chromosomal *fepA* gene is likely a marker of environmental adaptation and contributes to sustained resistance mechanisms.

The *aac(6')-Ib* gene was more prevalent on the chromosome (66.67%) than on the plasmid (53.33%), with a significant difference ($p=0.043$). This distribution suggests that aminoglycoside resistance may be primarily inherited from the chromosome, complicating the treatment of these infections in clinical settings.[6] emphasize that analyzing the gene's location is a crucial step in assessing the risk of transmission. They also explain that chromosomal genes may play a significant role in drug resistance due to their stability within the bacterial genome.

Similarly, a study by Wang et al.[20] reported comparable results, with the *aac(6')-Ib* gene detected on the chromosome at a frequency of 70% compared to 58% on the plasmid. Our findings align with those studies, demonstrating that the *aac(6')-Ib* gene is more commonly located on the chromosome, highlighting the potential for chromosomally mediated aminoglycoside resistance and the resulting challenges in clinical treatment.

Wang et al. [20] conducted a study on clinical isolates of Enterobacteriaceae and found that the *aac(6')-Ib* gene, particularly the subvariant *aac(6')-Ib-cr*, was present on both the chromosome and plasmids. However, they observed that the gene was more stable on the chromosome, which aligns with our results. The stability of chromosomal genes plays an important role in the persistence of resistance, suggesting that aminoglycoside resistance may be inherited chromosomally, providing a more stable form of resistance compared to plasmid-mediated transfer.

Jacoby et al. [21] also reported that the *aac(6')-Ib-cr* gene was present on both the chromosome and plasmids in clinical isolates of Enterobacteriaceae. They found that in certain strains, the gene integrated into the chromosome and became stably incorporated, a finding consistent with our results. The stability of the gene within the chromosomal genome may contribute to its long-term persistence, complicating treatment efforts and underscoring the significance of chromosomal inheritance in resistance mechanisms.

In contrast Al-Azzawi et al.[22] focused on *E. coli* isolates and reported that the *aac(6')-Ib-cr* gene was predominantly plasmid-borne. They emphasized that plasmid transfer was the primary mechanism for the spread of this gene, particularly in strains producing Extended Spectrum Beta-Lactamases (ESBL). This finding differs from our study, where we observed a greater prevalence of the gene on the chromosome. The discrepancy may be due to differences in bacterial species, suggesting that plasmid-mediated resistance

may be more prominent in certain strains, such as *E. coli*, while chromosomal inheritance plays a more significant role in others.

The *csgA* gene plays a crucial role in the production of curli fimbriae, which are essential for biofilm formation in *E. cloacae*. These fimbriae facilitate bacterial adhesion to surfaces and are important for colonization and environmental persistence. In the present study, the presence of *csgA* was associated with enhanced biofilm-forming ability, supporting its significance as a virulence factor in *E. cloacae*.

Similar findings have been reported in several recent studies. For instance Sondh et al.[23] observed that 75% of *E. cloacae* isolates collected in Al-Hilla City, Iraq, harbored the *csgA* gene, and these isolates exhibited significantly higher biofilm production compared to *csgA*-negative isolates.[24] demonstrated that deletion of *csgA* in strain SBP-8 led to a marked reduction in biofilm formation, even at physiological temperature (37°C), confirming the functional importance of the gene in biofilm assembly. Furthermore, Maddela et al. [25] found that the SBP-8 strain could form robust biofilms on multiple surfaces (plastic, glass, stainless steel), and this ability was linked to *csgA*-mediated curli production.

Environmental regulation of *csgA* expression has also been reported Aidi et al.[26] showed that acidic pH levels significantly down-regulated *csgA* transcription, thereby reducing curli biogenesis and impairing biofilm development in clinical isolates of *E. cloacae*. However, findings across different regions and studies have shown variability. A study from Najaf, Iraq, reported the presence of *csgA* in only 13% of isolates (3 out of 24), suggesting limited distribution of the gene in certain populations or potential gene suppression due to environmental or clinical factors. In a study by Kim et al.[27], *csgA* was detected in 42.9% of *E. cloacae* isolates, and while some gene-positive isolates formed biofilms, others did not, indicating that additional regulatory elements or gene-gene interactions may influence the phenotypic outcome. Additionally, [28] found the gene in 78.6% of isolates but noted that its presence was not always correlated with strong biofilm production, suggesting that *csgA* may be necessary but not solely sufficient for biofilm formation. Taken together, these findings highlight the important yet sometimes context-dependent role of the *csgA* gene in *E. cloacae* biofilm formation. While there is strong evidence supporting its contribution, variability in its prevalence and expression suggests that biofilm regulation is multifactorial and influenced by both genetic and environmental conditions. Further investigation is needed to explore gene expression dynamics, regulatory pathways, and their interaction with antimicrobial resistance mechanisms.

In this study, *Enterobacter cloacae* isolates exhibited notably high resistance rates to third-generation cephalosporins, particularly ceftazidime (73.3%) and ceftriaxone (66.7%). This aligns with a multicenter study by Vock et al.[30] which reported ceftazidime resistance rates exceeding 32.8% among clinical isolates in Chinese hospitals. The elevated resistance is primarily attributed to chromosomal expression of AmpC β-lactamases, a common resistance mechanism within the *E. cloacae* complex. Contrastingly Wang et al.[29] reported no significant difference in treatment outcomes between patients treated with third-generation cephalosporins and those receiving other antimicrobials, reflecting potential regional variations in prescribing habits, infection control practices, and genetic diversity among *E. cloacae* strains[30].

Regarding carbapenems, resistance to imipenem (13.3%) and ertapenem (10%) was observed, while all isolates remained fully susceptible to meropenem. These findings are consistent with Tanaka et al.[32]., who documented rising carbapenem resistance in *E. cloacae* isolates from tertiary care hospitals in eastern China, largely linked to the presence of *bla<sub>NDM</sub>* and *bla<sub>IMP</sub>* metallo-β-lactamase genes[31]. Aminoglycosides retained moderate efficacy, with amikacin and gentamicin showing sensitivity rates of 66.7% and 60%, respectively. This is supported by Kim et al. [31] who highlighted the sustained activity of amikacin against ESBL-producing Enterobacterales in Swiss hospitals, where resistance rates remained below 25%[32].

Resistance to fluoroquinolones was also notable, with 50% of isolates resistant to ciprofloxacin, consistent with Maddela et al.[25], who associated fluoroquinolone resistance in *E. cloacae* with plasmid-mediated *qnr* genes, especially in intensive care unit isolates. Their study emphasized the importance of monitoring plasmid-borne resistance genes due to their potential for rapid horizontal transfer[33]. Encouragingly, colistin and tigecycline demonstrated strong in vitro activity, with 90% of isolates susceptible. This corresponds with Tanaka et al. [32] who reported 88.2% colistin susceptibility among *E. cloacae* isolates in Japan, although the detection of *mcr-9* genes in some isolates raised concerns about emerging colistin heteroresistance[32].

Notably, 28.6% of isolates exhibited multidrug resistance (MDR), being resistant to all antimicrobial classes tested. This alarming trend reflects the growing global challenge of antimicrobial resistance and is supported by the 2023 joint report from the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO). The report highlights the increasing prevalence of MDR Enterobacterales and calls for intensified molecular surveillance, infection control measures, and antimicrobial stewardship strategies to curb the spread of resistant pathogens[33].

## CONCLUSION

The study's findings indicate that the chromosomal DNA of *E. cloacae* clinical isolates more frequently harbors the *fepA*, *csgA*, and *aac(6')-Ib* genes compared to their plasmid counterparts. The higher chromosomal prevalence of *fepA* suggests its role in maintaining iron uptake as a stable and essential metabolic process. Similarly, the chromosomal presence of *csgA* underscores its importance in biofilm formation and the long-term survival of bacteria in clinical environments. Due to its chromosomal linkage, *aac(6')-Ib* is likely inherited rather than acquired, which may have significant implications for therapeutic strategies. These findings enhance our understanding of the maintenance and expression of virulence and resistance genes in *E. cloacae*. Chromosomal integration provides stability and persistence, making these traits more difficult to eradicate and more critical in infection control efforts. This underscores the need for ongoing molecular surveillance, focusing not only on resistance patterns but also on the genetic platforms that sustain them.

**Declarations:**

**Consent for publication**: Not applicable

**Availability of data and material:** Data are available upon request.

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