Genetic detection of Resistance Genes among Enterobacter cloacae and Citrobacter spp. isolates

Dhay Haider Salih Abbas and *Nabil* Salim Saaid Tuwaij*

Department of Biology, Faculty of Science, University of Kufa, Najaf, Iraq

Abstract. Internationally, there is an increasing prevalence of diseases caused by germs that are resistant to many drugs, which is often a reason for worry. The objective of the present investigation was to identify specific genes linked to the resistance of beta-lactam, macrolide, and sulfa medications *in Enterobacter cloacae* and *Citrobacter* spp. isolates. A total of 364 patients were included in the study, with 132 (36.26%) being male and 232 (63.74%) being female. The results indicated that 135 samples, accounting for 37.08% of the total, exhibited positive bacterial growth. The findings indicated that Enterobacter cloacae was present in 25 (6.86%) of the patients with urinary tract infections, whereas *Citrobacter* spp. was found in only 2 (0.54%) cases (consisting of 1 (0.27%) *C. freundii* isolate and 1 (0.27%) C. farmer isolate). The PCR findings indicated that the sul-1 gene was present in 23 out of 25 (92%) E. cloacae isolates and in 2 out of 2 (100%) Citrobacter spp. isolates. On the other hand, the sul-2 gene was detected in 22 out of 25 (88*%) E. cloacae* isolates and in 2 out of 2 (100%) *Citrobacte*r spp. isolates. The mph(A) gene was present in 80% (20/25) of E. cloacae isolates and in 100% (2/2) of Citrobacter spp. isolates. The findings showed that 96% of the E. cloacae isolates and 100% of the Citrobacter spp. isolates tested positive for both ctx-m and ctx-m-10 genes. The prevalence of the veb gene in E. cloacae isolates was 76% (19/25), but in Citrobacter spp. isolates it was 100% (2/2). The investigation did not detect the gene ctx-m-14 in any of the local isolates examined. Genes of sul-3 and ctx-m-14 were no recorded in this study.

1 Introduction

Enterobacter species have a significant role in causing infections in both community and institutional settings. Among the genus, Enterobacter cloacae is the predominant species responsible for human illness, out of the more than twenty species in the genus [1, 2]. Citrobacter may cause several disease symptoms in people, including urinary tract infections, bloodstream infections, brain abscesses, respiratory tract infections, and newborn infections such meningitis and bacteremia. Citrobacter species are believed to have low virulence in comparison to other Enterobacteriaceae pathogens. This is because human infections caused

* Corresponding author: nabeel.tuwaij@uokufa.edu.iq

by Citrobacter species are rare in the general population [1, 2]. The β-lactam medicines have formed the fundamental basis of antibiotic treatment for such diseases. Excessive usage of these antibiotics has led to the emergence of β-lactam-resistant Enterobacteriaceae isolates, making β-lactam resistance a significant global problem. The synthesis of enzymes that deactivate β-lactams, namely extended-spectrum β-lactamases and carbapenemases, may result in multidrug resistance patterns that significantly limit treatment choices. Moreover, the development of β-lactam resistance may lead to elevated levels of medication toxicity, death rates, and healthcare expenses linked to Enterobacteriaceae infections [3]. Antimicrobial resistance (AMR) is a worldwide health issue that transcends geographical limitations. This elevates the likelihood of complications and therefore renders the treatment of infections more challenging, leading to escalated healthcare expenses and an increased mortality rate [4]. Sulfonamides are classified as bacteriostatic agents. Despite being used in therapy for almost 70 years, sulfonamides remain the preferred medications for treating many ailments and disorders. The broader use of sulfonamides in treatment is restricted due to bacterial resistance and the adverse effects of sulfonamides [5].

2 Materials and Methods

2.1 Ethical approval

Every the sample of urine utilized in this study was obtained with the consent and authorization of the patients and healthcare institutions [6].

2.2 Patients and Specimens Processing

The current research included a total of 364 individuals gathered from various clinical sources, selected randomly, who were suffering from different disorders. The treatment of burn injuries, wounds, blood-related conditions, and urinary tract infections took place in prominent hospitals and specialised burn centres in Al-Najaf City and Babil City, Iraq. Additionally, clinical labs in Al-Najaf City were involved in the diagnostic process. This medical intervention spanned several months, from October 2023 to February 2024. Each specimen was streaked on MacConkey Agar using cotton swabs and incubated aerobically at 37oC overnight in a sterile environment [7].

2.3. Diagnosis *of Enterobacter cloacae* and *Citrobacter spp.* Isolates

The suspected gram-negative bacteria, including Enterobacter cloacae and Citrobacter spp., were streaked on MacConkey Agar Medium. This was done based on microscopic, morphological, oxidase, and motility features, as well as certain important biochemical assays. The ultimate identification was carried out with the automated Vitek-2 compact system employing ID-GP cards [8, 9].

2.4 DNA extraction and PCR assay

Using the guidelines from the manufacturing firm, we used a genomic DNA extraction micro kit (Favorgen, South Korea) to gather all the nucleic acids from 27 clinical isolates of Enterobacter cloacae and Citrobacter spp. This was executed in compliance with the manufacturer's methodology. After confirming the integrity of the whole DNA sample by putting it in a deep freezer set to -20 degrees Celsius, a PCR analysis was conducted to examine the genes indicated in Table 1. The gel documentation equipment was used to

analyse the migration of PCR amplification bands on a 1% agarose gel. Subsequently, the bands were stained with ethidium bromide at a concentration of 0.5 g/ml. [10].

Table 1. Primer Sequence and condition

Gene name	Primer Sequence 5' to 3'	Annealing (°C)	Size of product(bp)	Reference
Sul-1-F	GTGACGGTGTTCGGCATTCT	68	779	[13]
Sul-1 -R	TCCGAGAAGGTGATTGCGCT			
Sul-2-F	CGGCATCGTCAACATAACCT	66	721	[13]
Sul-2-R	TGTGCGGATGAAGTCAGCTC			
Sul-3-F	CAGATAAGGCAATTGAGCATGCTCTGC	55	569	[14]
Sul-3-R	GATTTCCGTGACACTGCAATCATT			
veb -F	CGACTTCCATTTCCCGATGC	55	642	[15]
veb -R	GGACTCTGCAACAAATACGC			
Ctx-m-10-F	GCAGCACCAGTAAAGTGATGG	56	524	[16]
Ctx-m-10-R	GCGATATCGTTGGTGGTACC			
Mph(A)-F	GTGAGGAGGAGCTTCGCGAG	60	403	[17]
Mph(A)-R	TGCCGCAGGACTCGGAGGTC			
Ctx-m-F	AACCGTCACGCTGTTGTTAG	55	766	[18]
Ctx-m-R	TTGAGGCTGGGTGAAGTAA			
Ctx-m-14-F	GAGAGTGCAACGGATGATG	56	941	[19]
Ctx-m-14-R	TGCGGCTGGGTAAAATAG			

2.6 Statistical analysis

The findings were analysed and computed based on numerical data and percentages utilising computer tools [11, 12].

3 Results

3.1 Bacterial growth and patients

The research gathered data from a total of 364(100%) individuals, with 132 (36.26%) being male and 232 (63.74%) being female. Results of this study showed among 364(100%) clinical specimens analyzed, 135 (37.08%) showed positive bacterial growth. Specifically, 22 (6.04%) were identified as gram-positive bacteria, 102 (28.02%) as gram-negative bacteria, and 11 (3.02%) as mixed growth (both gram-negative and gram-positive). The remaining 229 specimens did not show any bacterial growth.

3.2 Identification of Enterobacter cloacae and Citrobacter spp. isolates

The results listed in Table 2, confirmed by Vitek-2 system results, showed that the percentage of Enterobacter cloacae isolated from patients suffering from urinary tract infections was 21 (5.76%) compared to 2 (0.54%) return to Citrobacter spp. (1 (0.27%) C. freundii isolate and 1 (0.27%) C. farmer isolate). At the same time, the percentage of Enterobacter bacteria

isolated from blood sources and burns was 1 (0.27%) and 3(0.82%), respectively. Generally the total rate of *E. cloacae* isolates were 25(6.86%).

Table 2. Number of Enterobacter cloacae and Citrobacter spp. from different clinical source

Source	Number	growth	No growth	E. cloacae	Citrobacter spp.
UTIs	273(75%)	83(22.80%)	190(52.19%)	21(5.76%)	1(0.27%) C. freundii 1 (0.27%) C. farmer
Blood	31(8.51%)	4(1.09%)	27 (7.41%)	1 (0.27%)	0 (0%)
Burn and wound	60 (16.48%)	48(13.18%)	12(3.29%)	3(0.82%)	0 (0%)
total	364 (100%)	135(37.08%)	229(62.91%)	25(6.86%)	2 (0.54%)

3.3 Molecular assay of sulfa drugs and macrolide resistance genes

PCR results showed the presence of sul-1 gene at a high rate of 23/25(92%) among *E. cloacae* isolates and 2/2(100%) among *Citrobacter* spp. isolates figure (1). Likewise, the gene of sul-2 was recorded 22/25(88%) and 2/2(100%) among *E. cloacae* isolates and *Citrobacter* spp. isolates respectively figure (2). While sul-3gene no observed among the local isolates in this study. Generally, PCR results showed that mph(A) was 20/25(80%) and 2/2(100%) among *E. cloacae* and Citrobacter spp. isolates respectively figure (3).

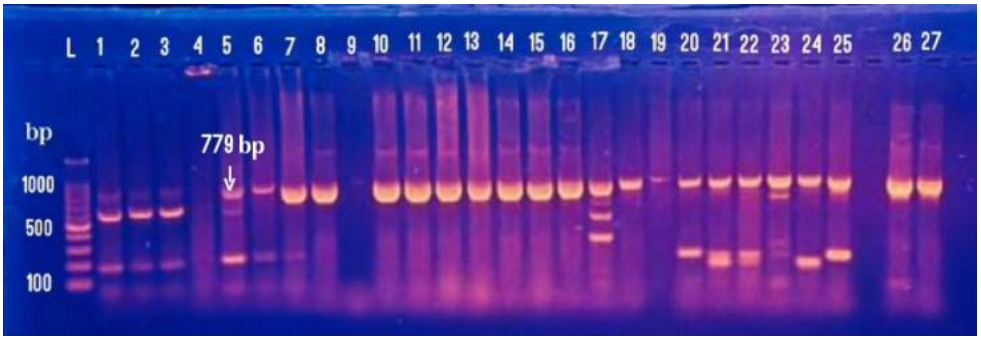

Fig. 1. Frequency of *sul-1* gene among 27isolates (1 to 25 E. clocaea, 26 C. freundii, 27 C. farmer)

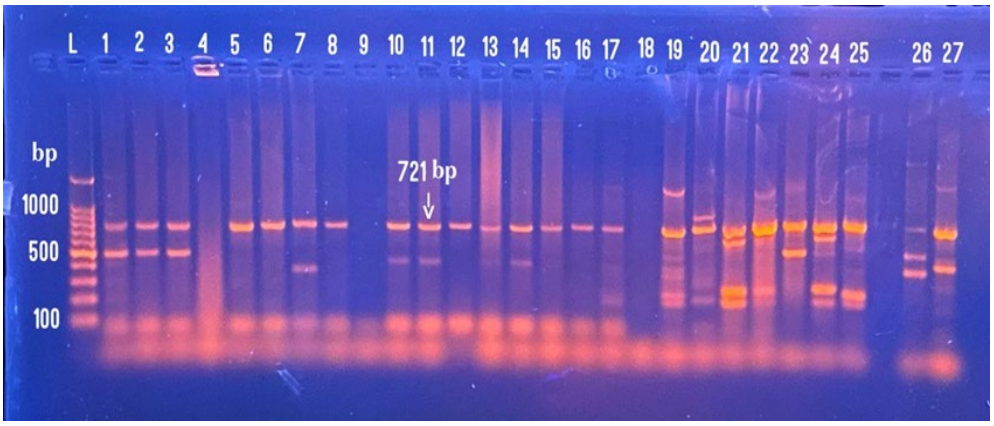

Fig. 2. Frequency of *sul-2* gene among 27isolates (1 to 25 E. clocaea, 26 C. freundii , 27 C. farmer)

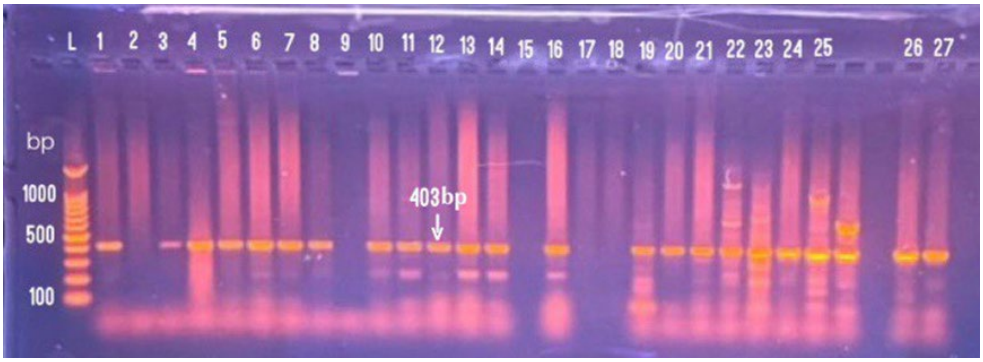

Fig. 3. Frequency of *mph(A)* gene among 27 isolates (1 to 25 E. clocaea, 26 C. freundii , 27 C. farmer)

3.4 Molecular assay of some beta-lactam resistance genes

The results indicated a wide spread of beta-lactam resistance genes among these pathogens, however 24/25(96%) and 2 /2(100%) for both ctx-m and ctx-m-10 genes among isolates of E. cloacae and Citrobacter spp. isolates respectively (figure 4,5). While the frequency of the veb gene in isolates of E. cloacae was 19/25(76%) compared to 2/2(100%) in isolates of citrobacter spp. (figure 6). This study did not record the presence of gene ctx-m-14 among the local isolates in this study.

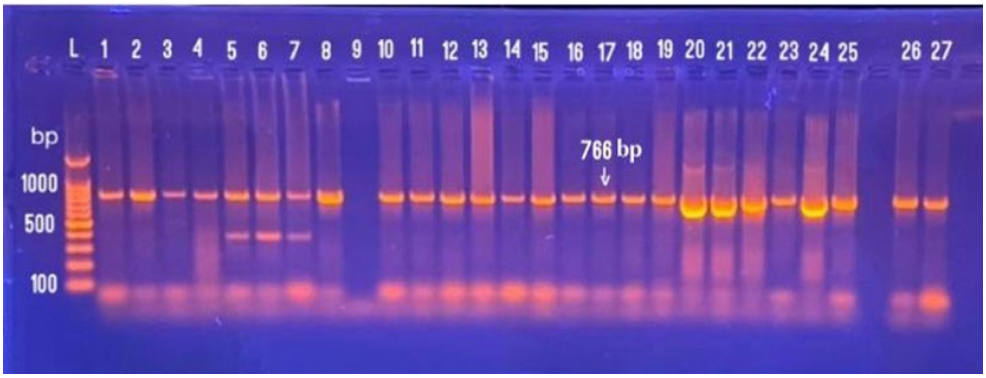

Fig. 4. Frequency of *ctx-m* gene among 27isolates (1 to 25 E. clocaea, 26 C. freundii, 27 C. farmer)

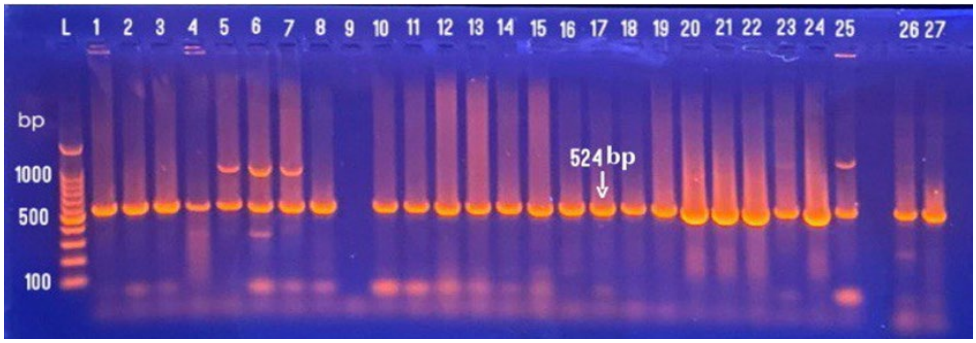

Fig. 5. Frequency of *ctx-m-10* gene among among 27isolates (1 to 25 E. clocaea, 26 C. freundii, 27 C. farmer)

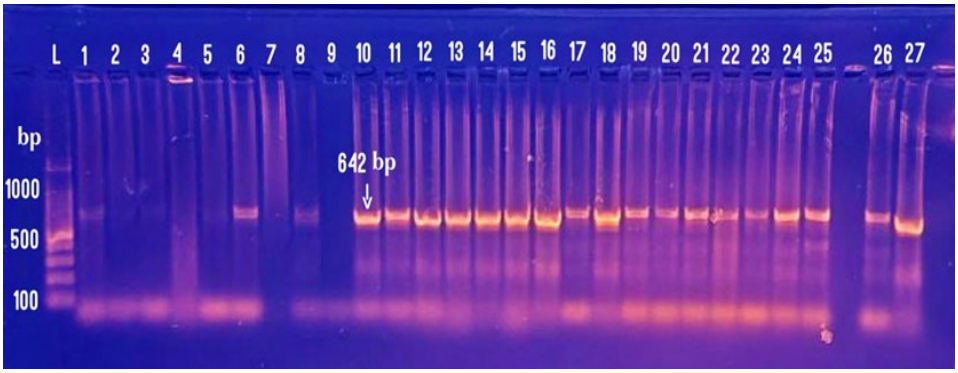

Fig. 6. Frequency of *veb* gene among among 27isolates (1 to 25 E. clocaea, 26 C. freundii, 27 C. farmer)

4 Discussion

Multiple studies have reported the potential of Enterobacter and Citrobacter to cause infections in humans. However, a previous study conducted by Mbula et al. [13] found that Enterobacter cloacae had the highest infection rates, reaching 47 (28.1%), among pregnant women with urinary tract infections compared to other types of gram-negative bacteria. In a previous local study done by Hayder and Aljanaby [14], it was stated that 461 urine samples were collected from patients diagnosed with urinary tract infections. The age of the patients included in the study varied from 18 to 60 years old. Out of the total isolates, 30 (6.5%) were identified as Citrobacter freundii. A local study conducted in Al-Najaf City by Tuwaij [15] found that out of 97 burn swabs collected from treated burn patients, only 2/75 (2.66%) specimens tested positive for Citrobacter freundii. These positive specimens were obtained aseptically from patients admitted to the burn unit. Sulfa medicines have revolutionised the treatment of bacterial infections, enabling the rescue of many lives. These drugs inhibit the growth and reproduction of bacteria, allowing the body's defensive systems to eliminate them. Sulfonamides are currently employed in the treatment of various human conditions, such as urinary system infections [16]. When the pathogen possesses sul-tye genes, it becomes susceptible to sulfonamide drugs like trimethoprim-sulfamethoxazole, which are commonly used for treating UTIs. Previous studies conducted in Al-Najaf City have documented the prevalence of sul-type genes in different gram-negative bacteria, indicating their distribution among these pathogens. In a research done by Ghazaly [17], it was discovered that the occurrence of sul-1 and sul-2 genes among Acinetobacter spp. was 100% and 89.28% respectively. Nevertheless, the sul-3 gene remained undiscovered.

In a subsequent study done by Tuwaij, et al., [18], it was shown that the sul-1 and sul-2 genes were present in K. pneumoniae isolates at frequencies of 11 (36.66%) and 22 (73.33%) respectively. According to a study done by El-Kazzaz et al. [19], the sul2 gene was shown to be the predominant gene in E. coli strains that exhibited resistance to co-trimoxazole. The occurrence of sul2 was higher, reaching 73%, in comparison to sul1 at 31% and sul3 at 4%. Nevertheless, the original strain of E. coli Tf481A was shown to produce Mph(A), an effective inactivator of 14-member ring macrolides such as erythromycin and oleandomycin [20]. A study done by Carey et al., [21], shown a frequent co-occurrence of the resistance gene mph(A) with the blaCTX-M variant group 1 among E. coli isolates. Within the local context, several instances were documented of the widespread dissemination of genes that are known to confer resistance to a majority of beta-lactam medicines. Souna et al. [23] did a research at an Algerian hospital and discovered that genetic analysis identified the presence of resistance genes from the CTX-M-1 group in 32 strains (88.9%).

References

1. A. Davin-Regli, J. P. Lavigne & J. M. Pagès, Clinical microbiology reviews, **32(4),** 10-1128 (2019)
2. W. Stokes, G. Peirano, Y. Matsumara, D. Nobrega & J. D. Pitout, European Journal of Clinical Microbiology & Infectious Diseases, **41(1),** 119-125 (2022)
3. M. A. Ariza-Prota, A. Pando-Sandoval, M. García-Clemente, R. Fernández & P. Casan, Case Reports in Pulmonology, **2015(1),** 670373 (2015).
4. M. T. Anderson, L. A. Mitchell, L. Zhao & H. L. Mobley, Scientific reports, **8(1),** 11792 (2018)
5. A. Tačić, V. Nikolić, L. Nikolić & I. Savić, Advanced technologies, **6(1),** 58-71 (2017)
6. Z. A. A. Al-Hamdani & N. S. S. Tuwaij, International Journal of Pharmaceutical Research, **12(4),** 09752366 (2020)
7. J.G. Collee, A.G. Fraser, B.P. Marion and A.C. Simmon, Mackie and McCarteny. Practical Medical Microbiology, 4th ed. (Churchill Livingstone, New York, 1996) 263-298.
8. J.F. MacFaddin, *Biochemical tests for identification of medical bacteria* (3rd edition) (The Williams and Wilkins-Baltimor, USA, 2000)
9. F. H. A. Shallal & N. S. S. Tuwaij, BIO Web of Conferences, **65,** 05004 (2023)
10. M. A. F. Al-Salami & N. S. S. Tuwaij, BIO Web of Conferences, **84,** 03017 (2024)
11. B.S. Mohammed and A.A.J. Aljanaby, Assessment of the Interleukin8 and Interleukin12 indices in individuals with *Helicobacter pylori* infected with gastric ulcer, **43** (special issue, Part 2), 662-6 (2024)
12. B.S. Mohammed and A.A.J. Aljanaby, Interleukin-22 plays a crucial role in patients infected with gastric ulcer and Helicobacter pylori, **43** (special issue, Part 3), 752-5 (2024)
13. J. Mbula, M. S. Abdulsalami, P. A. Vantsawa, K. Onuh, U. O. Ozojiofor, S. B. Ayodele... & I. A. Oyong, J. Adv. Microbiol., **32(5),** 1-15 (2023)
14. T. Hayder & A. A. J. J. Aljanaby, International Journal of Research in Pharmaceutical Sciences, **10(2),** 1481-1488 (2019)
15. N. S. Tuwaij, Al-Kufa University Journal for Biology, **8(3),** 81-95 (2016)
16. S. J. Mohammed, A. A. AL-Hadedi & S. A. Abood, Rafidain Journal of Science, **31(3),** 64-74 (2022)
17. N. F. Al-Ghazaly, *M.Sc. Faculty of Science* (University of Kufa, 2022) 1-117
18. N. S.S. Tuwaij, H. J. B. Al-khilkhali & H. M. Mohsen, Int. J. Res. Pharm. Sci, **11**, 2424-2432 (2020)
19. S. S. El-Kazzaz, G. E. S. Mashaly, A. M. El-Sabbagh & D. S. Abd Elmagid, African Journal of Microbiology Research, **10(33),** 1363-1370 (2016)
20. K. O. J. I. O'Hara, T. Kanda, K. Ohmiya, T. Ebisu & M. Kono, Antimicrobial agents and chemotherapy, **33(8),** 1354-1357 (1989)
21. A. M. Carey, S. F. Capik, S. Giebel, C. Nickodem, J. M. Piñeiro, H. M. Scott ... & K. N. Norman, Microorganisms, **10(2),** 411 (2022)
22. A. A. Shlash, N.S. S. Tuwaij, Iraq.Biochem. Cell. Arch, **18(2),** 2511–2522 (2018)
23. D. Souna, M. Drissi, I. Almahmoud & M. Maurin, Microbial Drug Resistance, **28(3),** 346-354 (2022)