Molecular study of antibiotic resistance gene among nitrofurantoin- resistant gram-negative bacteria isolates from pregnant women

Zuhair Sabah Mahdi Al-Sallami, and *Nabil* Salim Saaid Tuwaij*

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

Abstract. In pregnancy, urinary tract infection (UTIs) is a significant risk factor for morbidity, and nitrofurantoin is a common UTI therapy. The goal was to identify gram-negative UTI isolates and detect antibiotic resistance genes. The research involved 350 pregnant women hospitalised to Al-Zahraa teaching hospital for children's health and maternity between September and December 2024. Out of 250 pregnant patients, 200 (57.14%) urine samples were bacterial-free, whereas 150 (42.86%) were positive for gram-negative bacteria. The findings showed that 105 isolates (70%) were *Escherichia coli*, 33 (22%) were *Klebsiella pneumoniae,* 8 (5.3%) were *Pseudomonas aeruginosa*, 3 (2%) were *Proteus mirabilis*, and 1 (0.7%) were *Acinetobacter baumannii*. The disc diffusion Kirby-Bauer method determined 150 (100%) gramme negative bacteria's nitrofurantoin sensitivity. 33 (22%) isolates were antibiotic-resistant, 16 (10.66%) intermediate, and 101 (67.33%) susceptible. PCR analysis showed that all 33 out of 33 (100%) gram negative isolates had Ribe, and AcrAB genes. Additionally, a significant prevalence of nfsA-2 genes was discovered in 32 out of 33 isolates (96.96%) the nfsA-1 gene in 78.78% (26/33) of the isolates. The NfsB gene was detected in 30 out of 33 (90.90%) distinct isolates of nitrofurantoin-resistant gram-negative bacteria. The Nucleic acid and amino acid sequence study of NfsA and Ribe genes in some local isolates of *K. pneumoniae* reveals variations in sequence when compared to other global strains with the same genes.

1 Introduction

Urinary tract infections (UTIs) are prevalent bacterial infections that include a wide range of illnesses, from simple cystitis to severe urosepsis. Urinary tract infections (UTIs) are one of the most common bacterial infections, affecting millions of individuals worldwide, with women being disproportionately affected [1]. UTIs are associated with significant morbidity, reduced quality of life, and substantial clinical and economic costs.

Approximately 50% to 60% of adult women may have at least one urinary tract infection (UTI) over their lifetime [2]. Approximately 20% of pregnant women are documented to get urinary tract infections (UTIs) [3]. Untreated urinary tract infection (UTI) during pregnancy

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

is linked to several problems, including pyelonephritis, sepsis, severe sepsis, septic shock, hypertensive illness of pregnancy, anaemia, acute and chronic renal failure, intrauterine growth restriction, preterm birth, death, and an increased likelihood of caesarean delivery [4]. There is a worldwide increase and spread of bacteria that are resistant to antibiotics, which is endangering the effectiveness of these drugs that save millions of lives [5, 6]. Nevertheless, Nitrofurantoin has been used for almost five decades as an alternate therapy for simple urinary tract infections [7]. The rise in the prevalence of uropathogens that are resistant to several drugs, along with a scarcity of new oral antibiotics, has resulted in an increasing use of nitrofurantoin due to its growing resistance [8]. The aim of this study is to isolation and identification of pathogenic bacteria from pregnant women with urinary tract infection and diagnosis of some antibiotic-resistance genes.

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 [9].

2.2 Specimens' collecting and gram-negative bacteria Identification

A total of 350 pregnant women were hospitalised to Al-Zahraa teaching hospital for children's health and maternity between September and the end of December 2024. Urine samples from pregnant women with symptomatic urinary tract infections (UTIs) were collected during the middle of urination. The samples were placed in clean containers and then spun in a centrifuge at a speed of 2000 revolutions per minute for a period of two minutes. The sediment obtained was then introduced into a brain heart infusion broth and subjected to overnight incubation at a temperature of 37 degrees Celsius. Afterwards, all samples were streaked on Blood Agar and MacConkey Agar and incubated aerobically at 37oC overnight. Bacteria were identified based on colony features, gramme stain response, and biochemical assays. Finally, the Vitek-2 system was used for identification [10].

2.3 Nitrofurantoin susceptibility of gram-negative bacteria

The sensitivity of all gram-negative isolates to nitrofurantoin was originally evaluated using a 300µg nitrofurantoin disc on sterile Mueller Hinton agar medium. The suspension of all tested isolates was prepared using a 0.5 McFarland standard and the disc diffusion technique following the Kirby-Bauer method [11]. The zone diameter was determined according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) [12].

2.4 DNA extraction and PCR assay

The genomic DNA of all gram-negative isolates resistant to nitrofurantoin was extracted using a conventional kit for whole genomic DNA extraction (favorgen, Korea). The extraction process followed the manufacturer's instructions and was carried out after overnight liquid growth of the bacteria. The nucleic acid was preserved at a temperature of -20^{o}C using a deep freezing apparatus. The PCR method was used to analyse and identify all the genes listed in table (1).

Table 1. Oligosequence of primers used in this investigation

rimer Target	Sequence (5' to 3')	Annealing (ºC)	Product size	Reference
nfsA-1-F	ATTTTCTCGGCCAGAAGTGC	56	1036	[13]
nfsA-1-R	AGAATTTCAACCAGGTGACC			
nfsA-2-F	TCTTGCCCCACAGCTGATG	58	893	[13]
nfsA-2-R	CTTACACGAATAGAGCGTTCC			
nfsB-F	CAACAGCAGCCTATGATGAC	56	923	[13]
nfsB-R	CTTCGCGATCTGATCAACG			
ribE-F	GCATTTAGTGGGTGCATGATC	58	700	[14]
RibE-R	GGAACTGGTATTCAACATCAGCG			
acrAB-F	ATCAGCGGCCGGATTGGTAAA	53	312	[15]
acrAB-R	CGGGTTCGGGAAAATAGCGCG			

2.5 Analysis of the nfsA and ribE genes

The PCR amplification products including the nfsA and ribE genes were sent to Macrogen (Korea) for nucleotide sequence analysis. The sequencing data was compared to the findings in the GenBank database using BLAST on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The ExPASy translate tool was used to get the amino acid sequences for these genes. The DNA strands of the target gene were sequenced using the Sanger technique, specifically the dideoxy chain termination approach [16].

2.6 Statistical analysis

The findings were analysed and computed based on numerical data and percentages utilising computer tools.

3 Results

3.1 Bacterial growth and patients

The results of the culture medium analysis showed that out of the 350 pregnant individuals, 150 (42.86%) tested positive for gram-negative bacteria, whereas 200 (57.14%) had no bacterial growth in their urine sample. The data from the Vitek-2 system showed that out of 150 positive bacterial growth samples, *Escherichia coli* accounted for 105 out of 150 (70%), followed by *Klebsiella pneumoniae* with 33 samples (22%). *Pseudomonas aeruginosa,* Proteus mirabilis, and *Acinetobacter baumannii* accounted for 8 (5.3%), 3 (2%), and 1 (0.7%) sample respectively, as shown in table (1).

Table 1. Distribution of 150 Gram-negative bacteria among pregnant patients

Gram negative	Number (percentage)
Escherichia coli	105 (70%)
Klebsiella pneumoniae	33 (22%)
Pseudomonas aeruginosa	8 (5.3%)
Proteus mirabilis	3 (2 %)
Acinetobacter baumannii	1 (0.7 %)
Total	150 (100%)

3.2 Nitrofurantoin Susceptibility among gram-negative bacteria

Data of Susceptibility for nitrofurantoin drug recorded among 105 (70%) isolates of *E. coli* were 15 (10%) of them resistant to Nitrofurantoin while 7 (4.66%) of isolates were intermediate and 83 (55.33%) of isolates were sensitive to this antibiotic. Followed by 33 (22%) of *K. pneumoniae* recorded that 11 (7.33%) resistant to Nitrofurantoin while 5 (3.33%)of isolates were intermediate and 17 (11.33) of isolates were sensitive to this antibiotic. At same respect, the of Nitrofurantoin resistance among *P. aeruginosa*, and *P. mirabilis* were 3 (2%) while *A. baumannii* isolate recorded1 (0.66%) as shown in Table 3.

Table 3. Susceptibility of Nitrofurantoin antibiotic among gram-negative bacteria

Type of bacteria	Total No.(%)	No.(%) of Resistance	No.(%) of Intermediate	No.(%) of Sensitive
E. coli	105 (70%)	15 (10%)	7 (4.66%)	83 (55.33%)
K. pneumoniae	33 (22%)	11 (7.33%)	5 (3.33%)	17 (11.33)
P. aeruginosa	8 (5.33%)	3 (2%)	4 (2.66%)	1 (0.66%)
P. mirabilis	3 (2%)	3 (2%)	0 (0%)	0 (0%)
A. baumannii	1 (0.66%)	1 (0.66%)	0 (0%)	0 (0%)
total	150 (100%)	33 (22%)	16 (10.66%)	101(67.33%)

3.3 Molecular detection of nfsA-1, nfsA-2 , nfsB, ribE and acrAB genes

PCR results of 33 isolates of nitrofurantoin-resistant gram-negative recorded that 26/33(78.78%) of isolates were harbored NFSA-1 gene, while NfsA-2, recorded rate 32/33(96.96%) however, only 1 isolate return to *E. coli* was lose this gene (figure 1). At same respects, NfsB gene was recorded 30/33(90.90%) as following 10/11(90.90%) among *K. pneumoniae* isolates, 13/15(86.66%) among *E. coli isolates*, while recorded 100% among *P.mirabilis*, *P. aeruginosa* and *A. baumanii* isolates (figure 2).Finally, Results of PCR recorded that all isolates 33/33(100%) were have RibE and AcrAB genes (figure 3,4).

Current work for DNA sequence as well as amino acid sequence for nfsA and ribE genes recorded there are differentiation in DNA alignment among local clinical isolates and global isolates and this may be have a role in nitrofurantion resistance among local gram-negative isolates (table 4, 5).

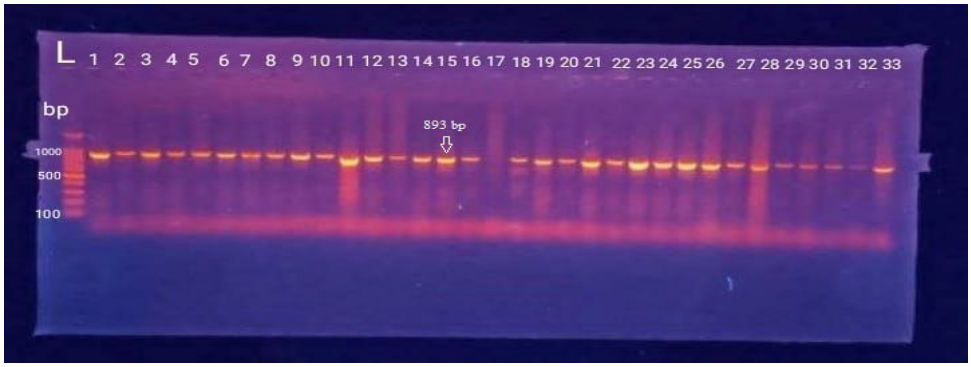

Fig. 1. Frequency of NfsA-2 gene among gram-negative bacteria (1 to 11 *K. pneumoniae*, 12 to 26 E. coli, 27 to 29 *P. mirabilis,* 30 to 32 *P. aeruginosa* and no. 33 is *A. baumannii*

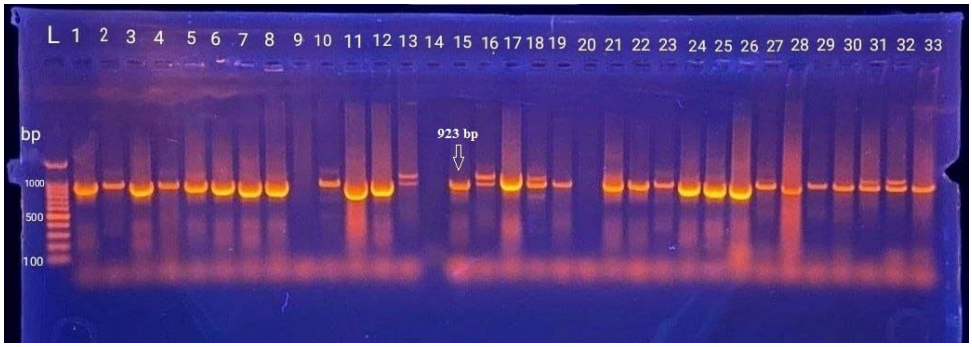

Fig. 2. Frequency of nfsB gene among gram-negative bacteria (no.1 to 11 *K. pneumoniae*, 12 to 26 *E. coli*, 27 to 29 *P. mirabilis*, 30 to 32 *P. aeruginosa* and no. 33 is *A. baumannii*

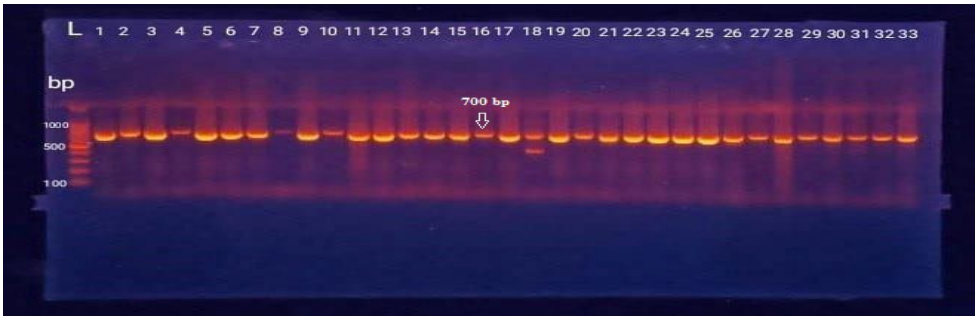

Fig. 3. Frequency of ribE gene among gram-negative bacteria (line 1 to 11 *K. pneumoniae,* 12 to 26 *E. coli*, 27 to 29 *P. mirabilis*, 30 to 32 *P. aeruginosa* and line 33 is *A. baumannii*

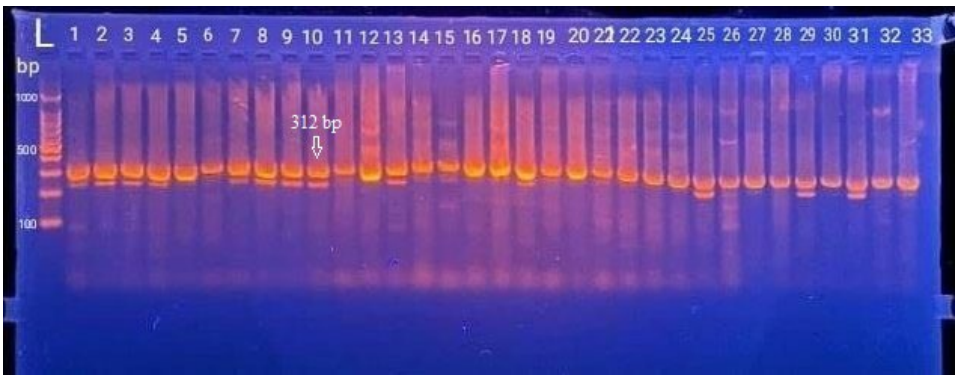

Fig. 4. Frequency of acrAB gene among gram-negative bacteria (line 1 to 11 *K. pneumoniae,* 12 to 26 *E. coli*, 27 to 29 *P. mirabilis*, 30 to 32 *P. aeruginosa* and line 33 is *A. baumannii*

Table 4. Alignment of amino acid sequence of NfsA gene of local *K. pneumoniae* isolate no.3 with *E.coli* sequence ID HAH1353872.1

Score	Expect	Method	Identities	Positives	Gaps
58.2 bits(139)	7e-07	Compositional matrix adjust.	24/26(92%)	26/26(100%)	0/26(0%)

```
Query  10  MTPTIELICGHRSIRHFTDEPISDSR  35
           MTPTIELICGHRSIRHFTDEPISD++
Sbjct  1   MTPTIELICGHRSIRHFTDEPISDAQ  26
```

Table 5. Alignment of amino acid sequence of ribE gene with strain of *K. pneumoniae* sequence ID WP_101999739.1

Score	Expect	Method	Identities	Positives	Gaps
158 bits(399)	8e-47	Compositional matrix adjust.	78/79(99%)	78/79(98%)	0/79(0%)

```
Query  18  MNIIEANVATPDARVAITIARFNNFINDSLLEGAIDALKRIGQVKDENITVVWVPGAYEL  77
           MNIIEANVATPDARVAITIARFNNFINDSLLEGAIDALKRIGQVKDENITVVWVPGAYEL
Sbjct  1   MNIIEANVATPDARVAITIARFNNFINDSLLEGAIDALKRIGQVKDENITVVWVPGAYEL  60

Query  78  PLGAGALAKTGKYNAVIAL  96
           PL AGALAKTGKYNAVIAL
Sbjct  61  PLAAGALAKTGKYNAVIAL  79
```

4 Discussion

The findings of a recent local study conducted in Karbala City, Iraq by Al-Daamy [17] revealed that among the 50 bacterial isolates obtained from pregnant women with urinary tract infections (UTI), 84% were identified as gram-negative bacteria, while the remaining 16% were gram-positive bacteria. *E. coli* was the predominant bacteria, comprising 60% of the samples, whereas *P. mirabilis*, *P. aeruginosa*, and *K. pneumoniae* had prevalence rates of 12%, 6%, and 4% respectively. In a prior research done in Uganda by Johnson et al. [18], different findings were found compared to the present work. In that study, *K. pneumoniae* accounted for 37.41% of the cases, *E.coli* for 28.78%, and *P. aeruginosa* and *P. mirabilis* each made up 5.04%. The lack of growth in this study is likely attributed to fungus, viruses, parasites, or gram-positive bacteria. Neelima and Kiranmai[19] reported that out of 153 isolates of ESBL-producing gram-negative bacteria isolated from UTIs, 44 (28.75%) showed resistance to nitrofurantoin. Significant heterogeneity in resistance to the nitrofurantoin antibiotic has been seen across different geographical locations.The research done by Shaifali *et al* [20], found that The UTIs prevalence rate was determined to be 45.32% (63 out of 139

cases). The pathogens most often isolated were *E. coli* (33.1%) and followed by *K. pneumoniae* (7.9%). Nitrofurantoin was the most efficacious antibiotic for both. In a recent study conducted by Nedbal *et al*. [21], it was shown that among patients suffering from urinary tract infections, the incidence of resistance to the drug nitrofurantoin among isolates of gram-negative bacteria was 14.18%. A research conducted by Sabarinathan *et al*. [22] found that out of 508 isolates of gram-negative bacteria from patients with urinary tract infections, the incidence of resistance to the nitrofurantoin antibiotic was 50.8%. Nitrofurantoin is the recommended drug for treating uncomplicated urinary tract infections (UTIs) and its use has greatly increased in recent years. However, Gautam et al. [23] did a study that showed that out of a total of 500 samples, 20.17% (94) were found to be resistant to nitrofurantoin, while 9.01% (42) were categorised as intermediate. *Klebsiella sp*. had the highest level of resistance, measuring 44.61%, whereas *E. coli* showed a resistance rate of 8.12%. Acinetobacter sp. shown a notable degree of resistance, as 80% of the samples showed resistance. Currently, there is a scarcity of research that have explored the processes behind the development of resistance to nitrofurantoin among uropathogens. Nitrofurantoin resistance in *E. coli* is primarily caused by genetic changes in the nfsA and nfsB genes, as well as to a lesser degree in the ribE gene which encodes lumazine synthase. Lumazine synthase is an essential enzyme involved in the production of flavin mononucleotide, an important cofactor for NfsA and NfsB. Detrimental mutations that disable the genes nfsA and nfsB, together with the associated gene ribE, have been found in the genomes of confirmed nitrofurantoin-resistant *E. coli* UTI isolates in England [26]. The data of the present investigation was similar to a previous local study conducted by Abid [27]. The PCR approach revealed the detection of the acrA and acrB genes in 100% of the *K. pneumoniae* isolates. Bacterial resistance to antibiotics is a critical problem, and efflux mechanisms play a vital part in this phenomenon. Efflux systems are essential in giving innate resistance in certain illnesses. As they are located on the plasmids, they may be acquired by other microbes.

References

1. R. Öztürk & A. Murt, World journal of urology, **38**, 2669-2679 (2020)
2. J.I. Alós, Infectious Diseases and Clinical Microbiology, **23**, 3-8 (2005)
3. S. J. Bacak, W. M. Callaghan, P. M. Dietz, & C. Crouse, American journal of obstetrics and gynecology, **192(2),** 592-597 (2005)
4. S. Taye, M. Getachew, Z. Desalegn, A. Biratu & K. Mubashir, BMC research notes, **11,** 1-7 (2018)
5. C. L. Ventola, Pharmacy and therapeutics, **40(4),** 277 (2015)
6. S. B. Zaman, M. A. Hussain, R. Nye, V. Mehta, K. T. Mamun & N. Hossain, Cureus, **9(6),** e1403 (2017)
7. R. Singh, M. Devi, K. Singh, H. Singh, C. Keisham & K. Singh, Annals of Tropical Medicine and Public Health, **8(6)** (2015)
8. A.A.J. Aljanaby, N. S. S. Tuwaij and H. J. B. Al-khilkhali, J Pharm Sci & Res, **10(5),** 1093-1097 (2018)
9. Z. A. A. Al-Hamdani & N. S. S. Tuwaij, International Journal of Pharmaceutical Research, **12(4),** 09752366 (2020)
10. F. H. A. Shallal & N. S. S. Tuwaij, BIO Web of Conferences, **65,** 05004 (2023).
11. A. W. Bauer, W. M. M. Kirby, J. C. Sherris, and M. Turck, Am. J. Clin. Pathol., **36**, 493-496 (1966)

12. Performance Standards for Antimicrobial Susceptibility Testing (CLSI), 31st ed. CLSI supplement M100 (Clinical and Laboratory Standards Institute, 2021) 1-352
13. L. Sandegren, A. Lindqvist, G. Kahlmeter, D.I. Andersson, J. Antimicrob, Chemother, **62**, 495–503 (2008).
14. P-L Ho, K-Y Ng, W-U Lo, PY Law, EL-Y Lai, Y Wang, et al., Antimicrob Agents Chemother, **60**, 537–43 (2016).
15. M.R. Azeez, A.A.J. Aljanaby, I.Ç. Öcal, Journal of Experimental Biology and Agricultural Sciences, **9(3)**, 378-387 (2021)
16. M. A. F. Al-Salami & N. S. S. Tuwaij, BIO Web of Conferences, **84**, 03017 (2024).
17. K. Al-Daamy, A. A. Hussein, Journal of Obstetrics, Gynecology and Cancer Research, **8(6),** 620-628 (2023)
18. N.Y. Abdulla, I.A.J. Aljanaby, T.H. Hasan, A.A.J. Aljanaby, Archives of Razi Institute, **77 (2),** 669-673 (2022)
19. Neelima A, Kiranmai, Trop J Path Micro, **2(3),** 159-163 (2016)
20. A.A.J. Aljanaby and I.A.J. Aljanaby, F1000Research, **7**, 1157 (2018) (doi: 10.12688/f1000research.15088.1).
21. C. Nedbal, N. Mahobia, D. Browning & B. K. Somani, Ther Adv Infect Dis ., **11**, 1–10 (2024).
22. T. Sabarinathan, P. Bavani, M. Saleem & R. Ramya, GJMEDPH, **12(6),** 1-8 (2023)
23. G. Gautam, S. Gogoi, S. Saxena, R. Kaur & M. S. Dhakad, Journal of Laboratory Physicians, **13(03),** 252-256 (2021)
24. D. McCalla, C. Kaiser, M. Green, J Bacteriol, **133**, 10–6 (1978)
25. J. Vervoort, B.B. Xavier, A. Stewardson, S. Coenen, M. Godycki-Cwirko, N. Adriaenssens, et al. Antimicrob Agents Chemother, **58**, 7225–33 (2014).
26. Y. Wan, E. Mills, R. C. Leung, A. Vieira, X. Zhi, N. J. Croucher. & S. Sriskandan, Microbial Genomics, **7(12),** 000702 (2021)
27. S. Abid Fazaa ALmiyah, Archives of Razi Institute, **78(1),** 269-276 (2023)