Genetic Detection of Folate Pathway Inhibitors Genes among *Acinetobacter* spp. Isolates

Naseer Flaih Zghair Al-Ghazaly, Nabil Salim Saaid Tuwaij*
Department of Biology, Faculty of Science, University of Kufa, Iraq
***Corresponding author**: Nabil Salim Saaid Tuwaij; E-mail: nabeel.tuwaij@uokufa.edu.iq
Mobile: 009647801280263

ABSTRACT

Background: Exist of folate pathway inhibitor genes among *Acinetobacter* spp. isolates are regarded as a significant mechanism of resistance for sulfa drugs in this pathogen. **Objective:** This study aimed to investigate genes associated with sulfa drugs resistance among *Acinetobacter* spp. using Polymerase Chain Reaction (PCR). **Patients and Methods:** This study included 928 specimens from patients who visited the main hospitals and private clinic laboratories of Al-Najaf City-Iraq. All specimens were cultivated and bacterial diagnosis was done according to standard methods. Antibiotics susceptibility and molecular investigation for sul-1, sul-2, sul-3, dfr-A, dfr-B, dfr-G and dfr-K for all Acinetobacter spp. isolates were done. **Results:** The rate of *Acinetobacter* spp. isolates were 28 (5.4%). Results showed a high resistance towards antibiotics classes, 28 (100%) of isolates were resistance to piperacillin, cefotaxime, ceftriaxone, ceftazidime and cefepime, while the lowest resistance rate was against minocycline reached 42.85. PCR showed 28 (100%) of *Acinetobacter* spp isolates were harbored sul-1 and drf-G genes. 25 (89.29%) and 21 (75%) of isolates were positive for sul-2 and drf-A genes respectively, while sul-3, drf-B and drf-K genes were not detected.
Conclusion: There is a great deal of concern about antimicrobial agent resistance and also about the number of drug-associated resistance genes contained in *Acinetobacter* spp. isolates especially sul-1, sul-2, dfr-A, and dfr-G, which have a significant role in sulfa drug resistance.

Keywords: *Acinetobacter baumannii*, *Acinetobacter lwoffii*, Folate pathway, PCR.

INTRODUCTION

The infection via Acinetobacter genus especially *A. baumannii* has caused concern newly. Furthermore, the expanded resistance of *Acinetobacter* to a different popularly applied antibacterial agent has resulted in incapable drugs and elevated mortality subsequent to the infection[(1)]. Approximately 80% of documented *Acinetobacter* infections are caused by *Acinetobacter baumannii*, as reported by the Centers for Disease Control and Prevention [(2)].

Healthcare-associated Gram-negative infections caused by *A. baumannii* isolates are more likely to be fatal and to cause life-threatening infections and septic shock than those caused by other pathogens [(3)].

In recent years, antibiotic has become a significant and provoking phenomenon with rising costs for healthcare systems. This is in addition to the significant morbidity, mortality, as well as cost increases associated with extended therapy and hospitalization[(4)].

Among the nonclassical antifolates category, the medication sulfonamide is able to block dihydropteroate synthase (DHPS) by entering the para-aminobenzoic acid (PABA) sac of the enzyme, preventing PABA from entering the reaction site, and creating an analog that cannot be used as a substrate in the subsequent step of the folate cycle. Therefore, they become competitive inhibitors for this enzyme as well as significantly lower folate levels as a result. This reduction results in mistakes in DNA synthesis because microorganisms are not able to use exogenous folate [(5)]. This study aimed to investigate genes associated with sulfa drugs resistance among *Acinetobacter* spp. using PCR.

PATIENTS AND METHODS

Patients and Bacterial Identification: The aggregate of 928 clinical samples was obtained from patients admitted to the largest hospitals and private clinical laboratories in Najaf City, these samples involved urine (412), wound swabs (109), burns swabs (71), ear swabs (63), blood (54), sputum (70), seminal fluid (44), peritoneal fluid (19), pleural fluid (13), throat swab (41), and vaginal swab (32). Blood agar and MacConkey agar were used to culture all samples then incubated aerobically at 37oC for 24 hours [(6)]. Morphological characters and biochemical tests were used to initial diagnose the bacteria and all suspected *Acinetobacter* spp. were verified grounded using the Vitek-2 system.

Antimicrobial agents susceptibility testing:

The antibacterial agents of all 28 isolates of *Acinetobacter* spp. were done according to Kirby-Bauer method on Mueller-Hinton agar [(7)]. The inoculation of all isolates were prepared by suspending the overnight growth of tested isolates in sterile normal saline adjusted to a 0.5 McFarland standard tube. Commercial antibacterial agent discs were evaluated. The zone diameters were interpreted as per Clinical Laboratory Standards Institute (CLSI) recommendations [(8)].

DNA extraction and PCR assay:

All Nucleic acid for 28 clinical isolates of *Acinetobacter* spp. had been amassed through the usage of a genomic DNA extraction mini kit (Favorgen, South Korea), based on the guide of a manufacturing corporation. Whole DNA was preserved using a deep freezer at -20°C, then, the PCR procedure was used to screening on the genes indicated in the Table 1. The apparatus of gel document was used to be utilized for the migration of PCR amplification (bands) at 1% agarose and subsequent dyeing of ethidium bromide at 0.5 μg/ml was done[(9)].

Received: 15/3/2022
Accepted: 12/5/2022

Table (1): Oligo sequence, annealing and product size of primers applied in this research

Gene name	Primer Sequence 5' to 3'	Annealing	Size of product (bp)	Reference
Sul1-F	GTGACGGTGTTCGGCATTCT	54.7	921	**(10)**
Sul1-R	TCCGAGAAGGTGATTGCGCT			
Sul2-F	CGGCATCGTCAACATAACCT	51.5	721	**(10)**
Sul2-R	TGTGCGGATGAAGTCAGCTC			
Sul3-F	CAGATAAGGCAATTGAGCATGCTCTGC	55	569	**(11)**
Sul3-R	GATTTCCGTGACACTGCAATCATT			
dfrA-F	CACTTGTAATGGCACGGAAA	57	270	**(12)**
dfrA-R	CGAATGTGTATGGTGGAAAG			
dfrB-F	AATTGTGTTAAATTAAAGATAACTT	43	572	**(12)**
dfrB-R	TAAGTATTCTTTAGATAAATCGGAT			
dfrG-F	TGCTGCGATGGATAAGAA	57	405	**(12)**
dfrG-R	TGGGCAAATACCTCATTCC			
dfrK-F	GCTGCGATGGATAATGAACAG	49	321	**(12)**
dfrK-R	GGACGATTTCACAACCATTAAAGC			

Ethical approval:

An approval of this study was obtained from University of Kufa Academic and Ethical Committee.

Informed consent of all the patients was obtained. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

Based on Graphpad-prism V.10, percentages were used to compare between sex, cases, and antimicrobial resistance.

RESULTS

Specimens and bacterial identification:

The results of culture growth indicated that among 928 patients were 522 (56.25%) bacterial growth compared with 406 (43.75%) no growth. The rate of *Acinetobacter* spp. isolates, which were obtained in current study according to bacterial growth were 28/522 (5.4%), which included 26 isolates with *A. baumannii* (9 isolates obtained from wound, while 8 , 5, 3 and 1 isolates obtained from burns, sputum, urine and blood respectively) and 2 isolates with *A. lwoffii* (obtained from wound and urine), The result showed that the study included 487 females and 441 males**,** however, other details are enrolled in table 2.

Table (2): Distribution the number (percentage) of *Acinetobacter* spp. according to sex

Sex	Number (%)	The case	Number (%)	Number (%) of *Acineto-bacter* spp.
Female	487 (52.47%)	Gram positive	77 (15.8%)	13 (-4.4)
		Gram negative	221 (45.4%)	
		No growth	189 (30.8%)	
Male	441 (47.52%)	Gram positive	52 (11.8 %)	15 (6.7)
		Gram negative	172 (39%)	
		No growth	217 (49.2%)	

Antimicrobial agent's susceptibility test:

It is evident from the results of the current study listed in table 3 that the bacteria showed a resistance rate of 100% to all beta-lactam antibiotics represented by piperacillin, cefotaxime, ceftriaxone, ceftazidime and cefepime, while the resistance rates of *Acinetobacter* spp. were 85.71, 82.1 and 28.57 for ticarcillin/clavulanic acid, piperacillin/tazobactam and ampicillin/sulbactam respectively.

Drugs of carbapenems had less effect against *Acinetobacter* spp. Isolates, which recorded rate of resistance reached 82.1 and 78.57 to imipenem and meropenem antibiotics respectively.

The results of table 3 showed a high resistance of bacteria towards the aminoglycoside group, where the resistance ratios reached 82.1, 78.57 and 57.14 for amikacin, gentamicin and tobramycin respectively.

Minocycline, recorded resistance ratio reached 42.85 while 10.71 and 46.42 of isolates were intermediate and sensitive to this drug respectively, the rate of resistance in tetracycline and doxycycline were 92.85 and 71.42 respectively.

The percentage of resistance of this pathogen were 85.7 and 82.1 for levofloxacin and ciprofloxacin drugs respectively.

Additionally, the current study included evaluating the efficacy of trimethoprim-sulfamethoxazole for inhibiting folate pathway antagonisms, with the bacteria recording 78.57, 3.57, and 17.85 relative resistance ratios.

Table (3): Antimicrobial agent's susceptibility of *Acinetobacter* spp. isolates

Antimicrobial agents	Resistance N (%)	Intermediate N (%)	Sensitive N (%)
Piperacillin	28 (100)	0 (0)	0 (0)
Ticarcillin with clavulanic acid	24(85.71)	0(0)	4(14.28)
Piperacillin/tazobactam	23(82.1)	0(0)	5(17.85)
Ampicillin/sulbactam	28.57	11(39.28)	9(32.14)
Cefotaxime	28(100)	0(0)	0(0)
Ceftriaxone	28(100)	0(0)	0(0)
Ceftazidime	28(100)	0(0)	0(0)
Cefepime.	28(100)	0(0)	0(0)
Gentamicin	22(78.57)	3(10.71)	3(10.71)
Amikacin	23(82.1)	2(7.14)	3(10.71)
Tobramycin	16(57.14)	1(3.57)	11(39.28)
Minocycline	12(42.85)	3(10.71)	13(46.42)
Doxycycline	20(71.42)	0(0)	8(28.57)
Tetracycline	26(92.85)	2(7.14)	0(0)
IPM	23(82.1)	0(0)	5(17.85)
MEM	22(78.57)	0(0)	6(21.42)
Levofloxacin	24(85.71)	0(0)	4(14.28)
Ciprofloxacin	23(82.1)	1(3.57)	4(14.28)
Trimethoprim-sulfamethoxazole	22(78.57)	1(3.57)	5(17.85)

Molecular detection of folate pathway inhibitors genes:

The molecular results of the current research showed that 28 (100%) of *Acinetobacter spp* isolates were harbored positive bands at correct position for Sul-1 and drf-G genes (Figure 1 2). At same respect, data of PCR showed 25 (89.29%) of and 21(75%) isolates were positive for sul-2 and drf-A genes respectively (Figure 3 and 4), while sul-3, drf-B and drf-K genes were not obtained or observed in the present study**.**

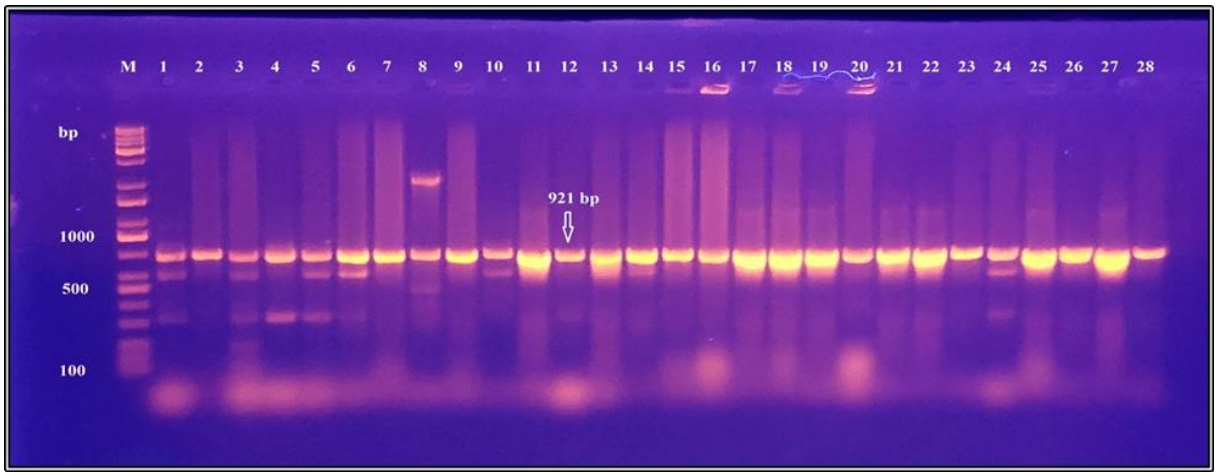

Figure (1): PCR amplification of sul-1gene among 28 clinical *A. baumannii* isolates (all isolates were positive)

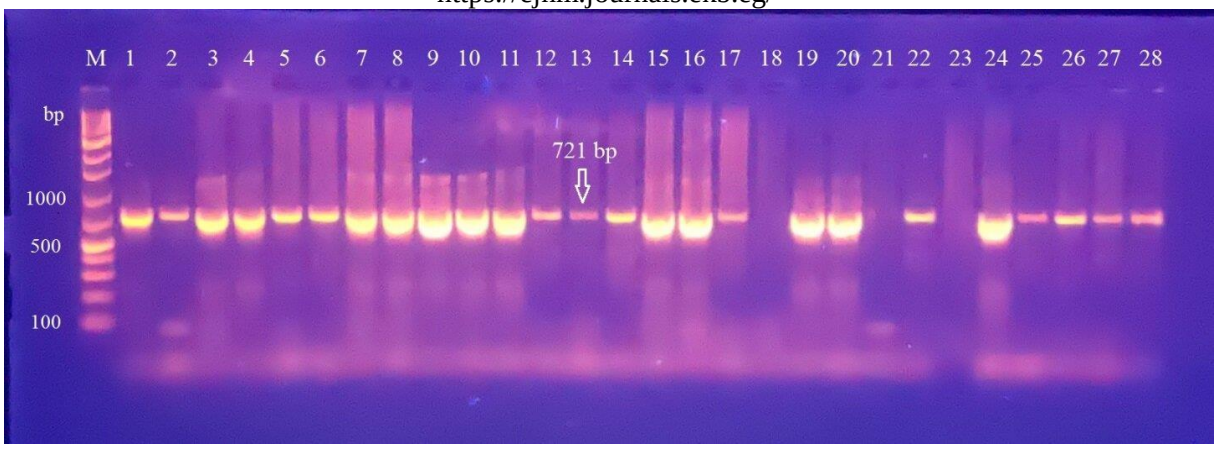

Figure (2): PCR amplification of Dfr-G gene among 28 clinical *A. baumannii* isolates (all isolates were positive)

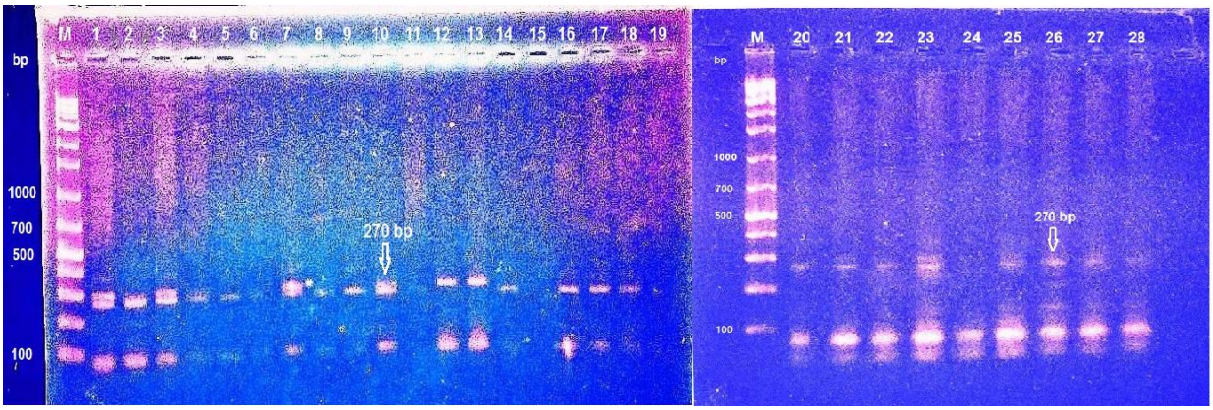

Figure (3): PCR amplification of sul-2gene among 28 clinical *A. baumannii* isolates (isolates number, 18, 21 and 23 were negative).

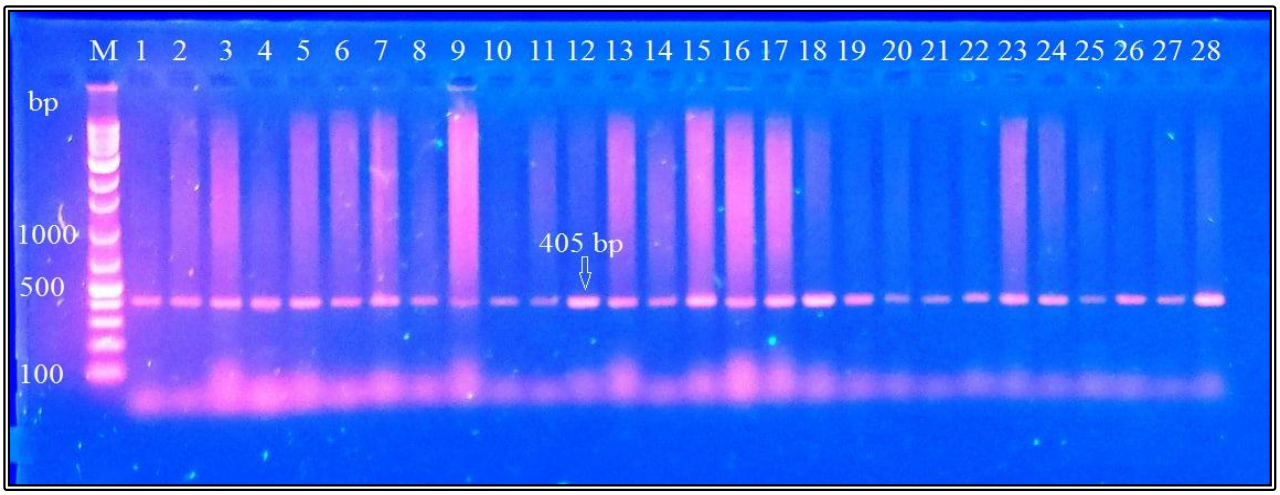

Figure (4): PCR amplification of Dfr-A gene among 28 clinical *A. baumannii* isolates (isolates number, 6, 8, 11, 15, 19, 24 and 28 were negative).

DISCUSSION

There have been numerous reports indicating that *A. baumannii* is a pathogen associated with healthcare such as urinary tract infections (UTIs), bacteremia, burn and wound sepsis and several other infections [(13)]. There are several studies that indicates ability of Acinetobacter spp. to cause human infections and correspond with results of current works. In a local study in the Hospital of Al-Kafeel / Kerbala City, done by **Mohammed *et al.*** [(14)], they observed the rate of *Acinetobacter* spp. isolated from different clinical specimens were 52/568 (9.2%). At same respect, 111 (3.36%) of 3298 samples infected with Acinetobacter were found by **Gupta *et al.*** [(15)]. Blood samples were the most commonly isolated *Acinetobacter* species (41%), followed by pus in 25 samples (22.5%), respiratory samples in 16 samples (14.4%), urine in 13 samples (11.7%), other body fluids in 10 samples (9%) and various catheter tips in 6 samples (5.4%).

While in Egypt a study done by **Nageeb *et al.*** [(16)], they obtained 10 (2.9%) isolates of *A. baumannii* from 350 patients suffering different diseases. Another previous work reported that rates of *Acinetobacter* isolates from whole infected

specimens were 4.8%. There were 76 cases of urinary tract infection (39.64%), 38 cases of wound infection (29.55%), and (29.84%) were found in the intensive care unit. The dominant species was *A. baumannii* [17].

Acinetobacter spp. has possession of various virulent factors that help it adhere to and invade, as well as its ability to form biofilm and its great resistance to antibiotics, which makes it a dangerous pathogen that exists for a long time, challenging difficult conditions in a hospital environment, and this may be one of the main reasons that justify its spread and caused it for various diseases [16].

Results of present study observed that the males were infected with *Acinetobacter* spp. isolates more than females. However, a recent work done by **Mohammed *et al.*** [14], found a significant difference in distribution of A. baumannii isolates according to sex, they found ratio of male to female was 3:1. Another study done in Pakistan by **Khurshid *et al.*** [18] on 160 isolates of *A. baumannii*, which were isolated from different clinical sources, found that 64% of the infected patients were males and 36% were females. Current study recorded elevation of antibiotics resistance among Acinetobacter spp. isolates and this results were closer to a previous study conducted by **Al-Tamimi *et al.*** [19] on *A. baumannii* isolates in Jordan. It was noted that most carbapenems, cephalosporins, and fluoroquinolones had high resistance rates among patients from whom *A. baumannii* was isolated, whereas tetracyclines and aminoglycosides had low resistance rates, while trimethoprim/sulfamethoxazole had middle resistance levels as well as the majority of isolates were recorded as multi-drug-resistant (MDR) (76.8%) was observed.

Another recent study done by **Kadhom and Ali** [20] reported that *A. baumannii* isolates recorded resistance rate reached 74%, for piperacillin, ceftazidime and ciprofloxacin, while 32%, 70%, 88%, and 98% for imipenem, meropenem, cefotaxime and ceftriaxone respectively, as well as 62%, 68%, and 76% of isolates were resistance to levofloxacin, amikacin and gentamicin respectively and finally 98% of isolates were observed resistance to sulfa drug.

In the past decade, antibiotic resistance among *Acinetobacter* species has increased significantly, and various mechanisms are employed by the pathogen to combat antibiotic effects, including the production of enzymes that cleavage drugs structure. The organism's relatively impermeable outer membrane may be partly responsible for this, and multidrug efflux pumps [21-23]. It is important to understand mechanisms of antibiotic resistance and develop new agents to overcome it since antibiotic resistance is a rapidly evolving health concern. Trimethoprim (TMP), an oral inhibitor of dihydrofolate reductase (DHFR), continues to be one of the most important antibiotics used today [24]. A study done by **Khurshid *et al.*** [18] found that frequency of sul-1 and sul-2 genes among 131 isolates of SXT-resistant A. baumannii were 10.7% and 72.5% respectively, while 16% of isolates have been both sul-1 and sul-2. However, this result were closer to a local study done by **Muhammed *et al.*** [25] in Al-Najaf City, they observed high spreading of sul-1 and sul-2 but sul-3 was not detected among SXT-resistant *E. coli* isolates.

Trimethoprim and sulfamethoxazole (SXT) are antifolates that act synergistically to inhibit different steps of bacterial folic acid synthesis. The SXT drug is recommended for the treatment of uncomplicated infections of the skin, infections of the urinary tract, soft tissues, and also respiratory infections [12]. The existence of SXT-resistant *Acinetobacter* spp. isolates can pose a serious threat to the development of new antifolate drugs. A study done in Malaysia by **Al-Marzooq *et al.*** [26] observed that the presence of sul1 and dfrA genes were 53.8% and 59.1% respectively, and in 93 isolates of MDR *K. pneumoniae* was documented.

While another study by **Torkan *et al.*,** [27] found rate of dfr (A1) gene was 35.7%. At same respect, the DfrG gene was responsible for 92% of trimethoprim-resistant *Staphylococcus aureus* [28].

CONCLUSION

There is a great deal of concern about antimicrobial agent resistance and also about the number of drug-associated resistance genes contained in *Acinetobacter* spp. isolates especially sul-1, sul-2, dfr-A, and dfr-G, which have a significant role in sulfa drug resistance.

Financial support and sponsorship: Nil.
Conflict of interest: Nil.

REFERENCES

1. **Li S, Duan X, Peng Y *et al.* (2019):** Molecular characteristics of carbapenem-resistant *Acinetobacter* spp. from clinical infection samples and fecal survey samples in Southern China. BMC Infect Dis., 19(1):1-2.
2. **Howard A, O'Donoghue M, Feeney A *et al.* (2012):** *Acinetobacter baumannii*: An emerging opportunistic pathogen. Virulence, 3(3): 243–50
3. **Aydın M, Ergönül Ö, Azap A *et al.* (2018):.** Rapid emergence of colistin resistance and its impact on fatality among healthcare-associated infections. J Hosp Infect., 98(3):260-263.
4. **Kyriakidis I, Vasileiou E, Pana Z *et al.*(2021):** *Acinetobacter baumannii* antibiotic resistance mechanisms. Pathogens,10(3):373.
5. **Fernández-Villa D, Aguilar M, Rojo L (2019):** Folic acid antagonists: antimicrobial and immunomodulating mechanisms and applications. Int J Mol Sci., 20(20):4996.
6. **MacFaddin J (2000):** Biochemical tests for identification of medical bacteria .(3rd edition).The Williams and Wilkins-Baltimor. USA.
7. **Bauer A, Kirby W, Sherris J *et al.*(1966):** Antibiotic susceptibility testing by astandardized single disk method. Am J Clin Pathol., 45(4):493-6.
8. **Melvin P, James S, April M *et al.* (2021):** Performance Standards for Antimicrobial Susceptibility Testing

(CLSI). 30th ed supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute. p: 316.

9. **Al-Hamdani Z, Tuwaij N (2020):** Molecular screening of virulence genes among uropathogenic *Enterococcus faecalis* isolated from patients in Al-Najaf City, Iraq. Int. J Pharm Investig., 12(4):331-41.
10. **Tamocha L, Oyinloye M, Rahube T (2019):** Airborne bacterial diversity and antibiotic resistance patterns in operating theatre and paediatric wards in Botswana hospitals. Afr J Microbiol Res., 13(21): 341-352.
11. **El-Kazzaz S, Mashaly G, El-Sabbagh A *et al.* (2016):** Resistance genes to sulphonamide in commensal *Escherichia coli* isolated from stool of patients in Mansoura University Children Hospital. Afr J Microbiol Res., 10(33): 1363-1370.
12. **Coelho C (2016):** Trimethoprim-sulfamethoxazole resistance in *Staphylococcus aureus* in Africa: Distribution of resistance genes and evaluation of the success of major MRSA clones (Doctoral dissertation). https://biblios.ciencias.ulisboa.pt › detalhes › 42253?lang=eng
13. **Almasaudi S (2018):** *Acinetobacter* spp. as nosocomial pathogens: Epidemiology and resistance features. Saudi J Biol Sci., 25(3): 586-596
14. **Mohammed S, Ahmed M, Abd Alameer A *et al.* (2022):** Prevalence of *Acinetobacter* species isolated from clinical samples referred to Al-Kafeel Hospital, Iraq and their antibiotic susceptibility patterns from 2017-2021. IJMM., 16(1):76-82.
15. **Gupta N, Gandham N, Jadhav S *et al.* (2015):** Isolation and identification of *Acinetobacter* species with special reference to antibiotic resistance. J Nat Sci Biol Med., 6(1): 159–162.
16. **Nageeb W, Kamel M, Zakaria S *et al.* (2014):** Phenotypic characterization of *Acinetobacter baumannii* isolates from intensive care units at a tertiary-care hospital in Egypt. EMHJ., 20(3): 203-211.
17. **Lone R, Shah A, Kadri S *et al.* (2009):** Nosocomial multi-drug-resistant *Acinetobacter* infections-clinical findings, risk factors and demographic characteristics. Bangladesh J Med Microbiol., 3(1):34-8.
18. **Khurshid M, Rasool M, Siddique M *et al.* (2019):** Molecular mechanisms of antibiotic co-resistance among carbapenem resistant *Acinetobacter baumannii*. J Infect Dev Ctries., 13(10):899-905.
19. **Al-Tamimi M, Albalawi H, Alkhawaldeh M *et al.* (2022):** Multidrug-Resistant *Acinetobacter baumannii* in Jordan. Microorganisms, 10(5):849.
20. **Kadhom H, Ali M (2022):** Epidemiological molecular analysis of Acinetobacter baumannii isolates using a multilocus sequencing typing and global lineage. Revis Bionatura,7(1): 29.
21. **Eliopoulos G, Maragakis L, Perl T (2008):** *Acinetobacter baumannii*: epidemiology, antimicrobial resistance, and treatment options. Clin Infect Dis., 46(8):1254-1263.
22. **Tiku V (2022):** *Acinetobacter baumannii*: Virulence strategies and host defense mechanisms. DNA Cell Biol., 41(1):43-48.
23. **Al-Husseini M, Tuwaij N (2020):** Molecular determination of efflux pump and aminoglycoside resistance genes among isolates of *Acinetobacter baumannii* isolated from patients in Al-Najaf City, Iraq . Biochem Cell Arch., 20 (1): 1207-1220.
24. **Reeve S, Scocchera E, Narendran G *et al.* (2016):** MRSA isolates from United States hospitals carry dfrG and dfrK resistance genes and succumb to propargyl-linked antifolates. Cell Chem Biol., 23(12):1458-67.
25. **Muhammed A, Alshara J , Tuwaij N *et al.* (2022):** Study of antibacterial chemical substances and molecular investigation among sulfamethoxazole-trimethoprim (SXT)-resistant *Escherichia coli* isolates. Reports of Biochemistry and Molecular Biology, 166-175.
26. **Al-Marzooq F, Mohd Y, Tay S (2015):** Molecular analysis of antibiotic resistance determinants and plasmids in Malaysian isolates of multidrug resistant *Klebsiella pneumoniae*. PLoS ONE, 10(7): e0133654.
27. **Torkan S, Bahadoranian M, Khamesipour F *et al.* (2016):** Detection of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from diarrhoiec dogs in Iran. Arch Med Vet., 48(2):181-90.
28. **Nurjadi D, Schäfer J, Friedrich-Jänicke B *et al.* (2015):** Predominance of dfrG as determinant of trimethoprim resistance in imported Staphylococcus aureus. Clin Microbiol Infect., 21(12):1095-e5.