Academic Science Journal

Correlation of Phylogenetic with Antibiotics Resistance Profiles and Integron Class I in Clinical Isolates of *Klebsiella pneumoniae* in Baquba City

Ghada Abdul-Ameer I. Al-Shabender* **and Hadi R. Rasheed Al-Taai**

Department of Biology – College of Science – University of Diyala

scibioms2206@uodiyala.edu.iq

Received: 10 June 2023 **Accepted: 12 August 2023**

DOI: https://doi.org/10.24237/ASJ.01.04.787B

Abstract

Klebsiella pneumoniae is a Gram-negative bacterium that causes serious infections and is resistant to various antibiotics. Fifty isolates of *Klebsiella pneumoniae* were reactivated and reidentified based on Compact System Vitek 2. All the isolates were tested for six antimicrobial agents using the agar diffusion process. The final results were as follows: Ampencillin 96%, Imipenem 38%, Cefotaxime 96%, Tobramycin 48%, Doxycycline 90%, and Azythromycin 66%. *β*--lactamase producers (MBL and AmpC) appeared at 70% and 22%, respectively. Clinical *K. pneumoniae* isolates' RAPD amplification information revealed that 16 isolates were 0% Applying the integrase gene as an indicator for class 1 integron research and PCR analysis revealed that 16 (100%) of *K. pneumoniae* Integrin I dendrograms of *K. pneumoniae* isolates showed two main clusters: A and B. According to our data, resistance to all antibiotic classes was shown to be more widespread in B lineages (100%), followed by A phylogenetic groupings, which were resistant (100%) to cefotaxame and ampencillin.

Keyword: *Klebsiella pneumoniae*, antibiotic resistance, Multidrug-resistance stains, RAPD–PCR, Integrons.

Academic Science Journal

الارتباط بين التنميط الجيني ومقاومة المضادات الحياتية وانتكرون الصنف الاول في العزلات السريرية لبكتريا *Klebsiella pneumoniae* في مدينة بعقوبة

غادة عبد الامير ابراهيم الشابندر وهادي رحمن رشيد الطائي

قسم علوم الحياة – كلية العلوم – جامعة ديالى

الخلاصة

تعد بكتريا الكليبسيلا الرئوية *K. pneumoniae* سالبة لصبغة كرام تسبب اصابات صحية خطيرة ومقاومة لمضادات حياتية مختلفة .قد تم اعادة تنشيط خمسون عزلة تعود لبكتريا *Klebsiella pneumoniae*.ومن ثم اعادة تشخيصها باستخدام نظام Vitek 2 Compact System تم إجراء اختبار حساسية المضادات الحياتية تجاه 6 مضادات حياتية باستخدام طريقة نشر الأقراص (Kirby-Bauer-method) , اظهرت نتائج فحص الحساسية أن عزلات *K. pneumoniae* تمتلك مقاومة عالية تجاه معظم المضادات الحياتية قيد الدراسة حيث كانت :Azythromycin66% و 96%Ampencillin, 96% Impenem38Cefotaxame , 90% Doxycycline, 52% Tobramycin, % عند اجراء الفحص المظهري للكشف عن *Klebsiella pneumoniae* المنتجة لانزيم البيتالاكتاميز للفئات (AMP^C,MBL,).بينت النتائج ان 22%, 86% على التوالي. كما كانت نتائج تضخيم RAPD للعزلات البكتريا 0% . بناء على التحري عن جين Integrase للكشف عن وجود انتكرون الصنف الاول Class I Integron, اظهر اختبارPCR ان 16 عزلة (100%) من *K.pneumoniae* كانت ايجابية لجين Integrse مما يؤكد الانتشار العالي للغاية لانتكرون الصنف الاول Class 1 Integron في مستشفيات بعقوبة في محافظة ديالى. اظهر التطور الشجري Dendogram لتحليل انتكرون الصنف الاول في بكتريا *k.pneumoniae* هناك مجموعتين Aو B وفقا لنتائجنا تبين ان المقاومة لجميع فئات المضادات الحياتية المستخدمة كانت اكثر انتشارا في سلالات (100B %), وتليها مجموعة النشوء والتطور A والتي كانت مقاومة (100%) للمضادات Ampencillinو Cefotaxame

الكلمات المفتاحية: كالبسيلا نيموني، مقاومة المضادات، السلالات متعددة المقاومة، RAPD–PCR، Integrons

Introduction

Klebsiella pneumoniae is an encapsulated Gram-negative bacterium. It has virulence components such as capsule, endotoxin, siderophore, a system for scavenging iron, and adhesins that are necessary to the pathogenicity (1). β-lactamase enzymes, such as Amp^C

βlactamases and carbapenemase, are responsible for resistance to *β*-lactam antibiotics such as penicillins, cephamycin and carbapenem (2). In several gram-negative bacteria, integrons are DNA elements that are important in the transmission of genes conferring multidrug resistance. It is responsible for mobile gene cassettes' site-specific recombination. (3). *K. pneumoniae* isolates originating from various origins can be successfully genotyped using RAPD-PCR, which is regarded as one of the most effective, simple, and affordable methods. (4). Antibiotic resistance is a serious global health concern, resulting in large financial losses caused by antibiotic misuse (5). *K. pneumoniae* strains are increasingly multi- and extensively drug-resistant (MDR, XDR), reducing therapeutic options for treating *K. pneumoniae* infections (6). The study is aimed at detecting integron class I in clinical *Klebsiella pneumoniae* isolates from Baquba hospitals that are resistant to multiple drugs.

Materials and Methods

Reactivation and Reidentification of bacterial isolates

Fifty isolates of *Klebsiella pneumoniae* were reactivated. On the basis of macroscopic, microscopic, and biochemical testing, all of isolates were reidentified, which that Vitek 2 Compact System validated.

Detection of antibacterial agents susceptibility

According to Magiorakos [7], the Kirby-Bauer method was used to test the susceptibility of 6 antibiotics Ampicillin (AM) (10 µg), Cefotaxime (30 µg) (CTX),), Imipenem (IMP) (10 µg), Tobramycin (TOB) (10 µg), Azithromycin (AZM) (15 µg), and Doxycycline (DO) (30 µg). Detection of *K. pneumoniae* phenotypes based on patterns of drug resistance Extensive drug resistance (XDR) makes phenotyping classification depend on the sensitivity test.

Phenotypic detection of MBL: the imipenem-EDTA combined disc test (DDST) was utilized to identify isolates that produced MBL in a study by El-Taie [8].

Phenotypic detection of AMP^{C}: A confirmatory test for the detection of Amp^{C} enzymes by disk antagonism was prepared and spread on the Muller-Hinton agar plates, according to Coudron [9]. Detection of phenotyping virulence factors in *Klebsiella pneumoniae* capsule formation. According to Black [10], the Indian ink method, also known as the negative stain method, was used to test the susceptibility of bacterial isolates to capsule formation.

Polymerase chain reaction (PCR) amplification and DNA extraction

Utilizing extraction kits for genomic DNA and purification in accordance with the manufacturer's instructions, genomic DNA has been isolated from isolates. (Promga USA). Transferring the PCR reaction tubes into the thermal cycles with the following programming: 5 minutes of initial denaturation at 95°C (each cycle's circumstances were: 30 seconds at 94°C, thirty seconds). repeatedly altering annealing temperatures (between 48°C and 60°C based on primers) for 30 seconds. final extension for 5 minutes at 72°C. Agar gel electrophoresis was used to identify amplified PCR products. According to Table 1, the primers were applied.

Table 1: lists the primers for detecting the RAPD and IntI genes

primer name	sequence of primers 5 ` 3	annealing temp (C)	producte size (bp)	Reference
intl-F	CCTCCCGCACGATGATC	58	280	(3)
intl-R	TCCACGCATCGTCAGGC			
RAPD	CGTGGGGCCT	36		(11)

Statistical analysis

The correlation matrix between susceptibility agents, virulence genes, and phylogenetic trees was determined using the Fisher Extract Test.

Results

Total number (N) of Gram-negative clinical isolates reactivated and diagnosed as *Klebsiella pneumoniae* were collected from several medical sources. Isolates were identified by an automated VITEK-2 system according to the manufacturers' protocols to confirm their species. The probability result revealed an agreement rate of 99%. VITEK-2 systems are more reliable and accurate at species-level identification of clinically important microorganisms.

Antimicrobial Sensitivity Test

All 50 *K. pneumoniae* isolates were tested against six antimicrobial agents in this study. The rates of resistance to ampencillin (96%), meropenem (38%), cefotaxime (96%), tobramycin (52%), doxycycline (90%), and azethromycin (66%). In the study period Among these isolates. 8 (16%) were resistant to the medications. XDR patterns. Furthermore, 16 (32%) of the isolates were resistant to all antibiotic classes, implying that they were PDR isolates.

Screening and Confirmation of metallo *β*-lactmase MBL Producers in *Klebsiella pneumoniae*

The existence of MBLs encoding genes on chromosomes or plasmids in *Klebsiella pneumoniae* was evaluated using the imipenem-EDTA synergy test. metallo *β*-lactmase-encoding genes that cause beta-lactam resistance to antibiotics such as imipenem and meropenem. When the inhibition zone with imipenem-EDTA is bigger than seven mm, the result is positive. The positive result the total number of positive *K. pneumoniae* isolates that formed MBLs enzyme was 35 (70%) and 15 (30%) isolates were negative.

Screening and Confirmation of Ambler class C *β*-lactamase (Amp^C) Producers

A disk antagonism test was utilized to identify Amp^C enzymes in all 50 isolates. The isolates displayed inhibition of the cefotaxame inhibitory zone near the cefoxitin disk or lower sensitivity to each of them [18]. The results showed that 11 (22% of the isolates) had the ability to produce Amp^C enzymes.

Phenotyping Detection capsule of *K. pneumoniae*

When studied under oil immersion following capsule staining, all of the isolates showed a distinct capsule as a clear zone enclosing bacterium, as seen in the image.

Genotyping of *Klebseilla pneumoniae* by RAPD –PCR

Agarose gel electrophoresis of the amplicons from the RAPD PCR reaction of the 16 *K. pneumoniae* isolates showed no bands. The target sequence for the study not have been found within the particular local isolates of the study (0%).

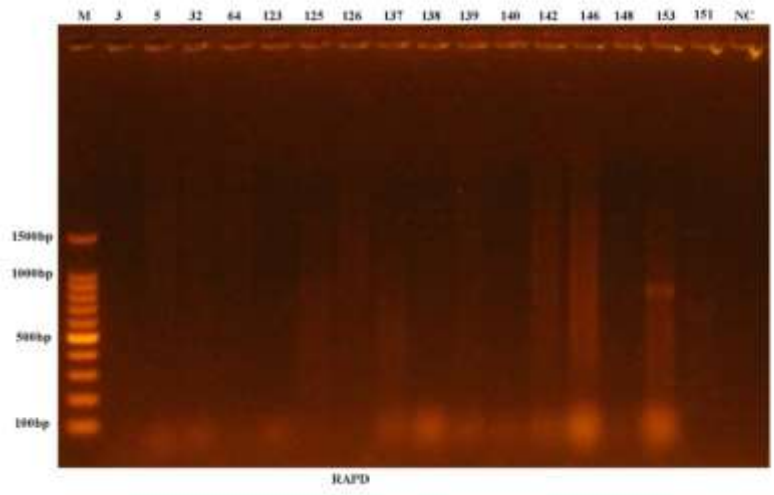

Figure 1: Results of the amplification of RAPD gene of bacterial species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 3-153 resemble PCR products.

Molecular Detection of Integron ClassI Genes Among *Klebsiella pneumoniae* Isolates

The PCR experiment revealed that 16 (100%) of the integrase genes were detected for the investigation of class I integrons.

The isolates tested positive for integrase, as shown in figure 2.

Academic Science Journal

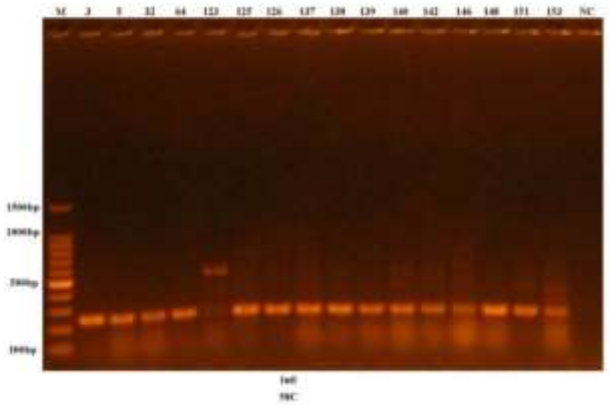

Figure 2: The results of bacterial species' amplification of the Intl gene were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 3-153 resemble PCR products.

The Relationship between Antibiotic Susceptibility Test and Phylogeny among Isolates

The JMP program was used to calculate the genetic distance and create a phylogenetic tree of Integron class I (IntI). Integrin I dendrograms of *K. pneumoniae* isolates show two major cluters: A and B. Cluster A was the largest, with 15 isolates. While B clusters consisted of one isolate, as shown in figure (3).

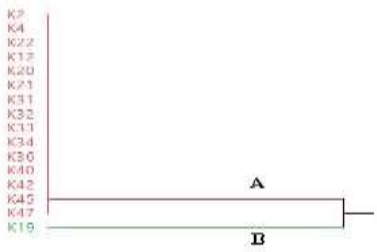

Figure 3: Dendrogram of 16 *K. pneumoniae* isolates based on the results of Integron ClassI.

This study referred to distinct phylogenetic groupings. Carbamenems, aminoglycosides, macrolids, and flouroquinolone groups show a sensitivity pattern in phylogenic A. As a result, we assume that it is conceivable to employ these therapeutics to treat infections caused by *K.*

pneumoniae. In comparison to the antibiotics mentioned above, these drugs have reduced resistance rates, as shown in Figures (4 a b c d e f).

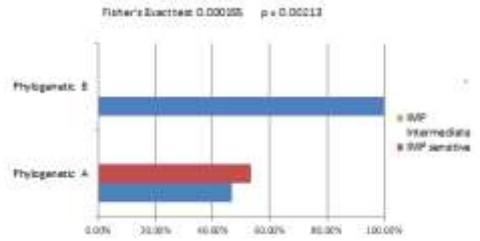

Figure 4a: Comparison of Impenem susceptibility among phylogeny of K. pneumoniae

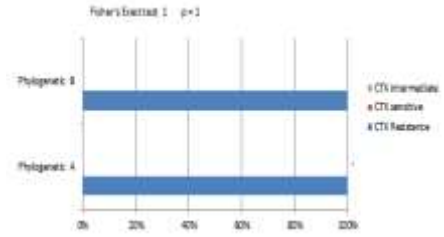

Figure 4b: Comparison of Cefotaxame susceptibility among phylogeny of K. pneumoniae

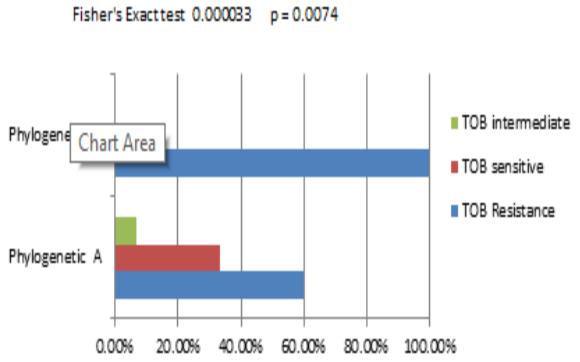

Figure 4c: Comparison of Tobramycin susceptibility among phylogeny of *K. pneumoniae*

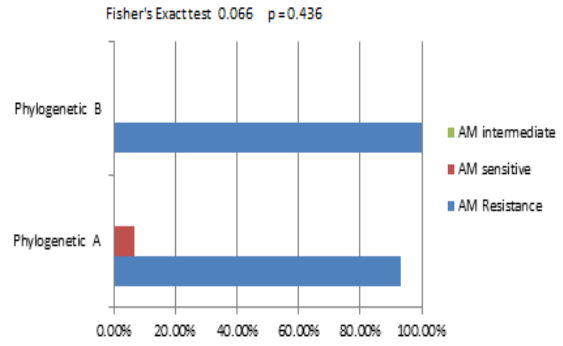

Figure 4d: Comparison of Ampencillin susceptibility among phylogeny of *K. pneumoniae*

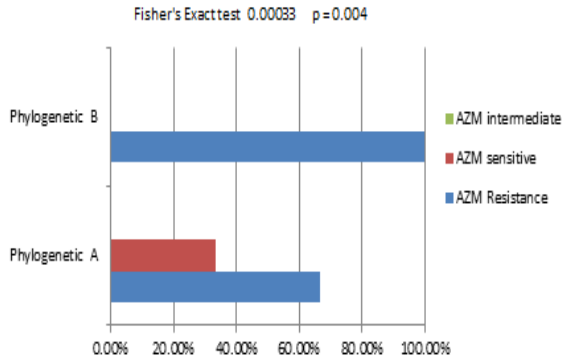

Figure 4e: Comparison of Azythromycin susceptibility among phylogenof *K. pneumoniae*

Figure 4f: Comparison of Doxycycline susceptibility among phylogeny of *K. pneumoniae*

Figures (4 a b c d e f): The Relationship between Antibiotic Susceptibility Test and Phylogeny among Isolates

Discussion

Klebsiella pneumoniae the isolates from different countries are becoming gradually resistant to an expanded variety of antibiotics [12]. A study by Muslims [13] in Iraq revealed a 100% resistance percentage to ampencillin. This high degree of ampencillin resistance is due to the intrinsic expression of a chromosomally encoded lactase (bla_{SHV-1}) that gives resistance to ampencillin [14]. The current study's results are consistent with Mohamad [15], who stated that *Klebsiella pneumoniae* isolated from Baquba Hospitals acquired antibiotic resistance to a variety of antibiotic classes, including cephalosporines, and that the high resistance rate was 96.66% for isolates resistant to ceftazidime. Antibiotic resistance in isolates Cefotaxime may be caused by the creation of extended-spectrum beta-lactamases (ESBLs), which hydrolyze these antibiotics. In addition, increased resistance in the cephalosporin class could be linked to uncontrolled antibiotic use, which contributed to the spread of resistant isolates among hospitalized patients. And resistance to imipenem According to the results of susceptibility testing, 19 (38%). Which stated that Impenem were the most effective antibiotics, this result is probably due to the less frequent use of carbapenems to treat infections, this resistance may be due to the production of the carbapenems enzyme. They are known as carbapenem-resistant *K. pneumoniae* (CRKP), which hydrolyzes these antibiotics.

MBL accountable for antimicrobial beta-lactam group resistance, such as meropenem and imipenem. The result was similar to the test for Antibiotic Susceptibility, in which 17 isolates were resistant to imipenem and meropenem and produced MBL, while two isolates (k23 and k33) were resistant to them but did not possess MBL. It is disagreed with Devi [16] that 15% of *K. pneumoniae* isolates were phenotypically MBL-producers; the presence of carbapenem-resistant genes in hypervirulent strains of *K. pneumoniae* isolated from hospitalized patients.

Resistance to broad-spectrum antibiotics is conferred by the carbapenemase enzyme by preventing the creation of cell walls, and it is one of the important carbapenem-resistant mechanisms in gram-negative bacteria. The variation in occurrence may be caused by strain and time changes, although the overall prevalence of MBLs among bacteria is high [17].

Among the phenotypic methods examined, the "Amp^{C} disk method" is a very specific and sensitive method that can distinguish resistance to cefoxitin induced by the presence of Amp^{C} from resistance caused by a reduction in the external permeability of the membrane [22]. The result of this study is consistent with a study conducted by Mohamed [19] in which positive results (24%) were reported. While I disagree with the results (75% and 68%, respectively), they have been reported in many other studies by Moghadam and Ejikeugwu [20, 21]. Ambler class C β -lactamase (Amp^{C}) synthesis of enzymes is one of the major drug resistance mechanisms in Enterobacteriaceae, providing resistance for all β -lactams except fourth-generation cephalosporins and carbapenems [23]. There are two types of Amp^{C} ß-lactamases: chromosomal and plasmid-mediated Amp^{C} [24].

The capsule is the external cover and virulence factor of *K. pneumoniae* and many other pathogenic bacteria, and it is thought to be a barrier against host innate immunity. *K. pneumoniae* creates around 80 different polysaccharides [25]. Capsule production is a known microbial strategy used by bacterial pathogens to survive adverse conditions in the hospital environment [26]. RAPD is the target sequence for the study not have been found within the particular local isolates of the study. This result disagrees with Aboulela [27], in which results were poative in 96%.

High prevalence of class I integron at hospitals in the Baquba and Diyala provinces. This result agrees with Sabbagh [28]. Showed that 100% of *K. pneumoniae* possessed a class I integron; however, it differed from the Iranian investigation presented by Laibi [29] that revealed 39% integron-positive *K. pneumoniae* isolates. Also agree with the study of Yakout [30], who found that all isolates of *K. pneumoiae* carried this gene. This mobile genetic factor is critical to the spread of resistance genes among bacteria [31]. The presence of two different fragment sizes of roughly 250 and 700 bp was discovered during analysis of the class I integron variable sections (Figure 2). Fifteen isolates (93.7%) carried sizes of approximately 250 bp class I integrons, and one isolate (6.25%) had sizes of approximately 700 bp. Class I integrons assist in establishing the problem of antibiotic resistance in humans by acquiring, exchanging, and expressing resistance genes encoded within gene cassettes [32].

Class I integrons are related to a variety of antibiotic resistance genes and have played a significant role in the evolution of antibiotics. According to the results, resistance to all antibiotic classes was found to be more prevalent in B lineages (100%), followed by A phylogenetic groups that were resistant (100%) to the cephalosporin group and ampencillin. According to the result, there is statistically significant difference between phylogenics A and B (p value ≤ 0.05).

Conclusions

In general, the findings of this investigation demonstrated a high incidence of integrons, particularly class 1 among MDR*K. pneumoniae* isolates from nosocomial infections in Baqubah, resulting in the fast spread of MDR strains. Also, there is a correlation between the phylogeny of an isolate and antimicrobial resistance.

References

1. M. Ahmadi, R. Ranjbar, P. Behzadi, T. Mohammadian, Expert Review of Anti-infective Therapy, 20(3), 463-472(2022)
2. S. Kuinkel, J. Acharya, B. Dhungel, S. Adhikari, N. Adhikari, U. T. Shrestha, P. Ghimire, Biofilm formation and phenotypic detection of ESBL, MBL, KPC and Amp C enzymes and their coexistence in Klebsiella spp. isolated at the National Reference Laboratory, Kathmandu, Nepal. Microbiology Research, 12(3), 49.
3. F. Jahanbin, M. Marashifard, S. Jamshidi, M. Zamanzadeh, M. Dehshiri, S. A. A. M. Hosseini, S. S. Khoramrooz, Southwestern Iran During 2015–16. Avicenna journal of medical biotechnology, 12 (2), 124(2020)
4. Z. H. Mahmud, S. Z. Uddin, M. Moniruzzaman, S. Ali, M. Hossain, M. T. Islam, S. Parveen, Pharmaceuticals, 15(9), 1116(2022)
5. M. Caniça, V. Manageiro, H. Abriouel, J. Moran-Gilad, C. M. Franz, Trends in Food Science and Technology, 84, 41-44(2019)
6. R. Li, J. Cheng, H. Dong, L. Li, W. Liu, C. Zhang, S. Qin, International journal of antimicrobial agents, 55(6), 105952(2020)
7. A. P. Magiorakos, A. Srinivasan, R. B. Carey, Y. Carmeli, M. E. Falagas, C. G. Giske, S. Harbarth, J. F. Hindler, G. Kahlmeter, B. Olsson- Liljequist, D. L. Paterson, Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired, (2012)
8. M. I. El-Taie, M. Z. El-Hasso, Journal of Education and Science, 22 (35),149-158(2009)
9. P. E. Coudron, Journal of clinical microbiology, 43(8), 4163-4167(2005)

10. J. G. Black, Microbiology: principles and explorations (Vol. 1). Wiley,(2004)
11. A. Saadatian Farivar, J. Nowroozi, G. Eslami, A. Sabokbar, Canadian Journal of Infectious Diseases and Medical Microbiology, (2018)
12. J. Lu, J. Chen, C. Liu, Y. Zeng, Q. Sun, J. Li, R. Zhang, Microbial Biotechnology, 15(4), 1270-1280(2022)
13. R. E. Muslim, Phenotypic and Genotypic Characterization of Extended-Spectrum-β-Lactamase, Amp C, and Carbapenemase Producing Gram negative Bacteria Isolated from Intensive Care Unit Patients in Wasit Province, Wasit University/College of Medicine Department of Medical Microbiology, (2022)
14. A. B. Mohammed, K. A. Anwar, PloS one, 17(9), e0267221(2022)
15. I. Q. Mohamad, Mulecular investigation of Biofilim and Quarum sensing genes in Klebsiella pneumoniae isolated from different clinical cases.University of Diyala, Collage of Science .Department of Biology, (2022).
16. D. L. Sumitra, B. S. Rautela, R. Singh, G. Shyam Sunder, C. Anita, C. Debasish, International journal of applied & basic medical research, 10(3), 156-163(2020)
17. S. M. Masoud, R. M. Abd El-Baky, S. A. Aly, R. A. Ibrahem, Antibiotics, 10(7), 835, (2021)
18. P. Samatha, K. V. Parveen, Journals of bioscience and technology, 24, 353-357(2011)
19. I. Q. Mohamed, Molecular investigation of biofilm and quorum sensing genes in Klebsiella pneumoniae isolated from different clinical cases, University of Diyala, College of Science, Department of Biology.
20. M. T. Moghadam, A. Shariati, S. Mirkalantari, A. Karmostaji, new microbes and new infections, 36, 100693(2020)
21. C. Ejikeugwu, O. Nworie, M. Saki, H. O. Al-Dahmoshi, N. S. Al-Khafaji, C. Ezeador, M. U. Adikwu, BMC microbiology, 21, 1-9(2021)
22. E. Rodríguez-Guerrero, J. C. Callejas-Rodelas, J. M. Navarro-Marí, J. Gutiérrez Fernández, Microorganisms, 10(3), 611(2022)
23. W. Liu, J. W. Tang, J. W. Lyu, J. J. Wang, Y. C. Pan, X. Y. Shi, L. Wang, Microbiology spectrum, 10(1), (2022)
24. T. Chérif, M. Saidani, D. Decré, I. Boutiba-Ben Boubaker, G. Arlet, Antimicrobial Agents and Chemotherapy, 60(1), 44-51(2016)
25. X. Huang, X. Li, H. An, J. Wang, M. Ding, L. Wang, J. R. Zhang, PLoS Pathogens, 18(8), (2022)
26. C. Nunez, X. Kostoulias, A. Y. Peleg, F. Short, Y. Qu, Biofilm, 5, 100105(2023)
27. A. G. Aboulela, I. El-Soudany, Microbes and Infectious Diseases, 4(1), 97-107(2023)
28. P. Sabbagh, M. Rajabnia, A. Maali, E. Ferdosi-Shahandashti, Iranian Journal of Basic Medical Sciences, 24(2), 136, (2021)
29. C. N. B. J. R. Laibi, Turkish Journal of Physiotherapy and Rehabilitation, 32(3), 10564-10569, (2021)
30. M. A. Yakout, G. H. Ali, Current Microbiology, 77, 3897-3902(2020)
31. A. Singh, M. Shahid, G. P. Singh, H. M. Khan, Singapore: Springer Nature Singapore, 141-152 (2022)
32. H. R. Noel, J. R. Petrey, L. D. Palmer, Annals of the New York Academy of Sciences, 1518(1), 166-182(2022)