Molecular Investigation of Virulence Genes of Klebsiella pneumoniae Isolated from Diabetic Foot Infections in Karbala Governorate

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Abstract

Diabetes mellitus is a chronic illness that continues to significantly enlarge. Its complications, including Diabetic foot infection (DFI), may be caused by pathogenic bacteria with virulence factors, which can hinder effective treatment. *Klebsiella pneumoniae is one of the most predominant isolates in diabetic foot infections.* The aim of this study is to determine the virulence of biofilm-forming Klebsiella pneumoniae that infect diabetic foot infections in Karbala city by isolating these species and identifying them molecularly. The current study, conducted at Imam Al-Hassan Center for Endocrinology and Diabetes in Karbala, Iraq, for 142 patients of diabetic foot of both genders and different ages begins from 35 years, 25 isolated Klebsiella strains from diabetic foot ulcers were identified. Susceptibility testing against 15 traditional antibiotics was performed, and quantification of biofilm formation using the microtiter plate method was conducted. Additionally, genotyping and screening for virulence using PCR techniques were applied to detect mrkD, Cps, K1, and K2A genes. Out of the 119 isolates obtained in the current study, 25 were identified as Klebsiella pneumoniae. All the isolated *K.pneumoniae* were highly resistant 100% toward Gentamycin and Levofloxacin followed by 96% resistance toward Ciprofloxacin and 92% resistance toward Amikacin while Pipracillin-Tazobactam, Cefotaxim, Ceftriaxone and Tetracyclin, resistance percent was 88% while Amoxicillin-Clavulanic acid, Cetazidime, and Cefepime were 84% resistant. Furthermore, *K.pneumoniae* has 76% resistance toward Imipenem and Meropenem and 68% toward Doxycycline and 56% with respect to Rifampin and according to the microtiter plate method, 60% of *K.pneumoniae* isolated from DFI in current study were strong producers of biofilm according to microtiter plate method while 40% of them were moderate producers . Finally, the PCR technique detected that 88% of K. pneumoniae isolates contained the mrkD gene, 100% contained the Cps gene, 12% contained the K1 gene, and 60% contained the K2A gene.

In Conclusions: Our current study displays that *Klebsiella pneumoniae* of diabetic foot ulcer are varied genetically, offer resistance to medically chief antibiotics and harbor for virulence factors. These characteristics suggest that Klebsiella can contribute to the persistence and severity of these infections, leading to treatment failures and the potential transmission of these traits to other microorganisms with similar characteristics.

Key Words: Diabetic foot, *Klebsiella pneumoniae*, PCR, Virulence genes, AST

التحري الجزيئي عن جينات ضراوة بكتريا الكلبسيلا الرئوية المعزولة من إصابات القدم السكري في محافظة كربلاء

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يعد مرض السكري من الامراض المزمنة الذي يستمر بالتقدم مع العمر بشكل كبير وتعد التهابات القدم السكري واحدة من اهم مضاعفاته حيث انه من الممكن ان تكون مستعمرة من قبل البكتريا المرضية ذات عوامل الضراوة المختلفة والتي تساهم في تقليل استجابتها للعلاج وتعد بكتريا الكلبسيلا الرئوية أحد اهم العزلات البكتيرية السائدة في التهابات القدم السكري. الهدف من الدراسة هو تحديد خطورة الكلبسيلا الرئوية المكونة للغشاء الحيوي التي تصيب التهابات القدم السكري في محافظة كربلاء عبر عزل هذه الأنواع وتحديدها جزيئيا. تم اجراء الدراسة الحالية في مركز الامام الحسن ع للغدد الصم والسكري في محافظة كربلاء، العراق وتم الحصول على 25 عزلة من الكلبسيلا الرئوية من قرحة القدم السكري وتم اجراء فحص الحساسية لها ضد 15 مضاد حيوي تقليدي بعدا تم التقدير الكمي لقابلية هذه العزلات على انتاج الغشاء الحيوي

بطريقة المايكروتايتر وأخيرا تم التحري الجزيئي عن بعض جينات الضراوة بتقنية تفاعل البلمرة المتسلسل للكشف عن جينات *K1*, *Cps* , *mrk*D و *K2A* . اتضح ان من بين 119 عزلة بكتيرية تم الحصول عليها اثناء هذه الدراسة ان 25 منها كانت تعود لجنس الكلبسيلا الرئوية و تبين ان كل هذه العزلات كانت مقاومة بنسبة 100% لمضادي ال Gentamycin و Levofloxacin تليها مقاومتها لمضاد Ciprofloxacin بنسبة 96% و مضاد Amikacin بنسبة 92% بينما كانت هذه البكتريا مقاومة لمضادات Pipracillin-Tazobactam, Cefotaxim Ceftriaxone وTetracyclin بنسبة 88% بالإضافة الى ان مقاومتها كانت بنسبة 84% باتجاه مضادات Amoxicillin-Clavulanic acid Cetazidim وCefepime اما مضادي Imipenem و Meropenem فكانت مقاومتها لهما بنسبة 76% بنما كانت مقاومتها لمضادي Doxycycline و Rifampin بنسبة 68 % و 56% على التوالي. ووفق طريقة المايكروتايتر للكشف المظهري عن الغشاء الحيوي اتضح ان 60% من عزلات الكلبسيلا الرئوية كانت قوية الإنتاج للغشاء الحيوي بينما كانت 40% منها متوسطة الإنتاج للغشاء الحيوي وأخيرا فان تقنية تفاعل البلمرة المتسلسل كشفت عن وجود جين *mrk*D في العزلات المذكورة بنسبة 88% بينما كان وجود جين *Cps* بنسبة 100% و جين *K1* بنسبة 12% وجين *K2A* بنسبة 60%. نستنتج من ذلك ان بكتريا الكلبسيلا الرئوية المسببة لالتهاب القدم السكري مختلفة وراثيا وذات مقاومة عالية للمضادات الحياتية التقليدية وتمتلك العديد من عوامل الضراوة تلك الخصائص اثبتت ان هذه البكتريا لها مساهمة كبيرة في استمرار و حدة التهاب القدم السكري و تسبب فشل لعمل المضادات الحياتية وإمكانية انتقال هذه الخصائص للبكتريا الأخرى التي تمتلك نفس الطبيعة.

الكلمات المفتاحية: القدم السكري , الكلبسيلا الرئوية , جينات الضراوة , اختبار الحساسية للبكتريا

1. INTRODUCTION

DFI is one of the mutual long-standing complications of diabetes mellitus. The lifetime risk of developing a DFU in diabetic patients is expected to be (12% to 25%) [1] [2]. One of the primary contributing factors to DFU consequences is diabetic foot infection (DFI), which accounts for (40% to 60%) of all DFUs [3] [4]. DFI is associated with increased hospitalizations, worsened outcomes, and higher amputation rates [5] [6].

DFI is supposed to have two or more fundamental marks of inflammation (enlarged pain, erythema, purulent discharge, and high temperature) [5]. DFI can be categorized as (mild , moderate and severe) and are frequently poly-microbial, with numerous bacteria recognized in them [5] [7]. The variety of bacteria is deliberated an important provider to the chronicity of DFU [7] and the poly-microbial nature of DFI has been well investigated [5] [8] [9].

Klebsiella pneumoniae mainly affects patients with cooperated resistances to make some acute complications which is a precise problem for diabetic patients leading to diabetic foot infections [10]. When infection is proven, *Klebsiella* produces a biofilm that assists avoidance of the host's resistances [11] [12]. Furthermore, since Klebsiella possesses an outer polysaccharide capsule, a crucial factor in their pathogenicity, phagocytosis by polymorphonuclear granulocytes is significantly delayed [13].. Latest data show that *K. pneumoniae* is caused about 21.7% of diabetic foot infection cases [14]. If not efficiently cured. *Klebsiella* can avoid host defenses, and ultimately cause chronic osteomyelitis [15]. Therefore; we aimed to determine the severity of the predominant biofilm forming gram negative bacteria by isolation of these bacteria and identifying them molecularly.

2.MATERIALS AND METHODS:

Study design and Samples collection:

This study planned as a cross-sectional study. 142 swab samples were collected from diabetic foot patients with inclusion criteria included patient with type 2 diabetic foot ulcer, age of subjects was >35 years old of both genders who previously diagnosed by the clinical physician at Imam Al-Hasan center for Endocrinology and Diabetes during the period between October, 2022 until January, 2023. Samples were collected from 142 patients with various grades of ulcers. Samples obtained using a swab with transport media to keep bacteria survive until cultivation on suitable media. Diverse types of media used for sample cultivation such as MacConkey, blood purchased from (Himedia-India). Patients aged less than 35 years, Non-diabetic patients with foot ulcers, Type 1 Diabetic Mellitus and pregnant women were excluded from this study.

BACTERIAL ISOLATION:

The specimens were inoculated on to blood agar and MacConkey's agar purchased from(Himedia-India). Plates were incubated at 37°C for 24 hours. The bacteria were identified by Gram staining and the colony morphology and biochemical tests such a KOH test, catalase production, coagulase and oxidase. Additionally, the identification of K. pneumoniae using API 20E Kits purchased from BIOMERIEUX-France was performed.

ANTIBIOTIC SUSCEPTIBILITY:

The disc diffusion method was performed according to Hudzick (2009) [16] as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI) to assess the susceptibility of K. pneumoniae to traditional antibiotics, including Pipracillin/Tazobactam (100/10µg) and Amoxicillin-Clavulanic acid (20/10µg).Ceftazidime (30µg), Cefepime (30µg), Cefotaxime (30µg), Ceftriaxone (30µg),Imipenem (10µg), Meropenem (10µg), Rifampin (5µg), Ciprofloxacin (5µg), Levofloxacin (5µg), Amikacin (30µg), Gentamycin (10µg), Tetracycline (30µg) and Doxycycline (30µg) manufactured by Liofilchem, Italy.

INVESTIGATION OF BIOFILM FORMATION

QUANTIFICATION OF BIOFILM FORMATION USING THE MICROTITER PLATE METHOD

This method that described by Kırmusaoğlu (2019) [17] was achieved with some modification to explore the capability of bacteria to produce a biofilm whereas the young isolates were immunized into 5ml of a Brain-Heart broth and were incubated at 37°C for 24hours and the bacteria were diluted with the same media and compared with a 0.5 standard McFarland solution then two hundred microliter of diluted bacterial culture were transported into every well in micro titer plate in a 4 duplicates for each isolate. Uncultured Brain-Heart infusion broth was added as a control into the wells then the microplate was incubated at 37°C for 24 hours after closing it securely after that the culture was exhausted and the wells were rinsed 3 times by normal saline then the microplate was dried in oven at 60°C for 30 minutes and two hundred microliter of 0.5% crystal violet were added and were left for 15 minutes also the stain was shattered and the wells were washed 3 times until the dye was vanished then they were let to dry then two hundred microliter of 33% of glacial acetic acid were added. In addition, the optical density measured at a wave length of 630 nm by ELISA reader. Finally, the ability of isolates to produce biofilm was determined by comparing the optical density (OD) of the samples with that of the control as mentioned in table No. 1

Tab. No. 1: Evaluation method of biofilm formation

Mean OD value	Biofilm formation
OD ≤ ODc	None
OD ≥ ODc	Weak
OD ≥ 2 × ODc	Moderate
OD ≥ 4 × ODc	Strong

MOLECULAR DETECTION OF THE MRKD GENE BY PCR TECHNIQUE

DNA was extracted using DNA extraction kit purchased from Addbio, Korea. The virulence genes (*mrk*D, *Cps*, *K1* and *K2A*) genes were distinguished using the PCR technique by using specific primers illustrated in (Table No. 2). PCR reactions were performed in the PCR thermal cycler (Edison, NJ-USA) using PCR master mix (Microgen, South Korea). PCR reactions were performed in a total volume of 23 µL. The master mix contained 10 µL of reaction mixture with Taq DNA polymerase master mix, 1.5 µL of each of the forward and reverse primers in 10 µM/µL concentrations,3 µL of target DNA, and 7 µl of distilled water. The PCR products were visible by electrophoresis on a 1.5% agarose gel using 70 voltages for 50 minutes followed by consequent exposure to UV light in the presence of DNA load dye. The PCR mixtures were subjected to the PCR reaction conditions which included 1 cycle for initial denaturation of templet DNA for 15 minutes at 95°C for *mrkD* gene,5 minutes at 95°C for *Cps* gene and 15 minutes at 95°C for *K1 and K2A genes* while the second step included 30 cycles involving denaturation of template DNA for 30

seconds at 94°C for *mrkD* gene , 30 seconds at 95°C for *Cps* gene and 30 seconds at 95°C for *K1 and K2A genes genes* followed by annealing of primers with templet DNA for 90 seconds at 60°C for *mrkD, K1 and K2A* genes and 30 seconds at 60°C for *Cps gene* while the initial extension required 1 minute for *mrkD, K1* and *K2A* genes and 5 minutes for *Cps* gene at 72°C. Lastly, final extension of DNA strands required 1 cycle for 10 minutes' for *mrk*D, *K1* and *K2A* genes, and 7 minutes for Cps gene at 72°C. The products of PCR were exposed by electrophoresis using a 1.5% agarose gel using 70 voltages for 50 minutes followed by consequent exposure to UV light in the presence of DNA load dye.

Gene	Primers	Primer sequence 5'____3'	Amp. size (bp)	Annealing Tem.	References
***mrk*D**	*mek*D-F *mrk*D-R	AAGCTATCGCTGTACTTCCGGCA GGCGTTGGCGCTCAGATAGG	340	60	[18]
Cps	*Cps*-F *Cps*-R	TATTCATCAGAAGCACGAGCTGGGAGAAGCC GTCGGTAGCTGTTAAGCCAGGGGCGGTAGCG	418	60	[19]
K1	*K1*-F *K1*-R	GGTGCTCTTACATCATTGC GCAATGGCCATTTGCGTTAG	1283	60	[20]
K2A	*K2*A-F *K2*A-R	CAACCATGGTGGTCGATTAG TGGTAGCCATATCCCTTTGG	531	60	[21]

ETHICAL CONSIDERATION:

This study was accepted by Ethical Committee at College of Science/ University of Karbala. All subjects enrolled in this work were informed and verbal agreement obtained from each participant before the collection of sample.

Table No. 2 : Primer of *mrk*D gene with its sequences and amplicon sizes of *K.pneumoniae*

3.RESULTS:

Of the total 142 diabetic foot swab, 23 exhibited no growth while 119 were positive cultures, of these, *Klebsiella pneumoniae* has the greatest frequency as 25 isolates followed by *Pseudomonas aeruginosa, Proteus mirabilis, and Escherichia coli.* Susceptibility tests of *K.pneumoniae* were performed toward Tazobactam, Amoxicillin-calvulonic acid, Ceftazidime, Cefepime, Cefotaxime, Ceftriaxone, Imipenem, Meropenem, Amikacin, Gentamycin, Rifampin, Ciprofloxacin, Levofloxacin, Tetracycline and Doxycycline. It was shown that the highest resistance 100% was toward Gentamycin and Levofloxacin, followed by 96% resistance toward Ciprofloxacin and 92% resistance toward Amikacin. With respect to Pipracillin-Tazobactam, Cefotaxim, Ceftriaxone and Tetracyclin, resistance percent was 88% while Amoxicillin-Clavulanic acid, Cetazidime, and Cefepime were 84% resistant. Furthermore, *K.pneumoniae* has 76% resistance toward Imipenem and Meropenem and 68% toward Doxycycline and 56% with respect to Rifampin.

Investigation of biofilm according to the microtiter plate method revealed that 60% of *K.pneumoniae* isolated from DFI in current study were strong biofilm producers according to microtiter plate method while 40% of them were moderate producers.

PCR technique was used in current study to detect the incidence of the *mrkD* gene in *K.pneumoniae* which indicates that *K.pneumoniae* was biofilm producer. Figure No. 1 shows the electrophoresis of the PCR products, through which it is clear that the primer of the *mrkD* gene was successful in amplifying this gene through the appearance of a PCR product of 340 bp in size. PCR technique was also used to detect the presence of the *Cps* gene which is responsible of capsular polysaccharide. Figure No.2 shows that *Cps* primer was successful in amplifying this gene through the appearance of a PCR product of 418 bp in size in 100% of *K.pneumoniae* isolates. The presence of *K1* gene was detected. Figure No. 3 shows the *K1* gene was successfully amplified that has been indicated by the presence of a PCR product size of 1283 bp in *K.pneumonieae*. Finally, the current study included investigation

presence of *K2A* gene. The PCR technique in figure No.4 displays the successful amplification that has been distinguished by the appearance of bands related to *K2A* gene PCR product size of 531 bp in *K.pneumoniae* isolates.

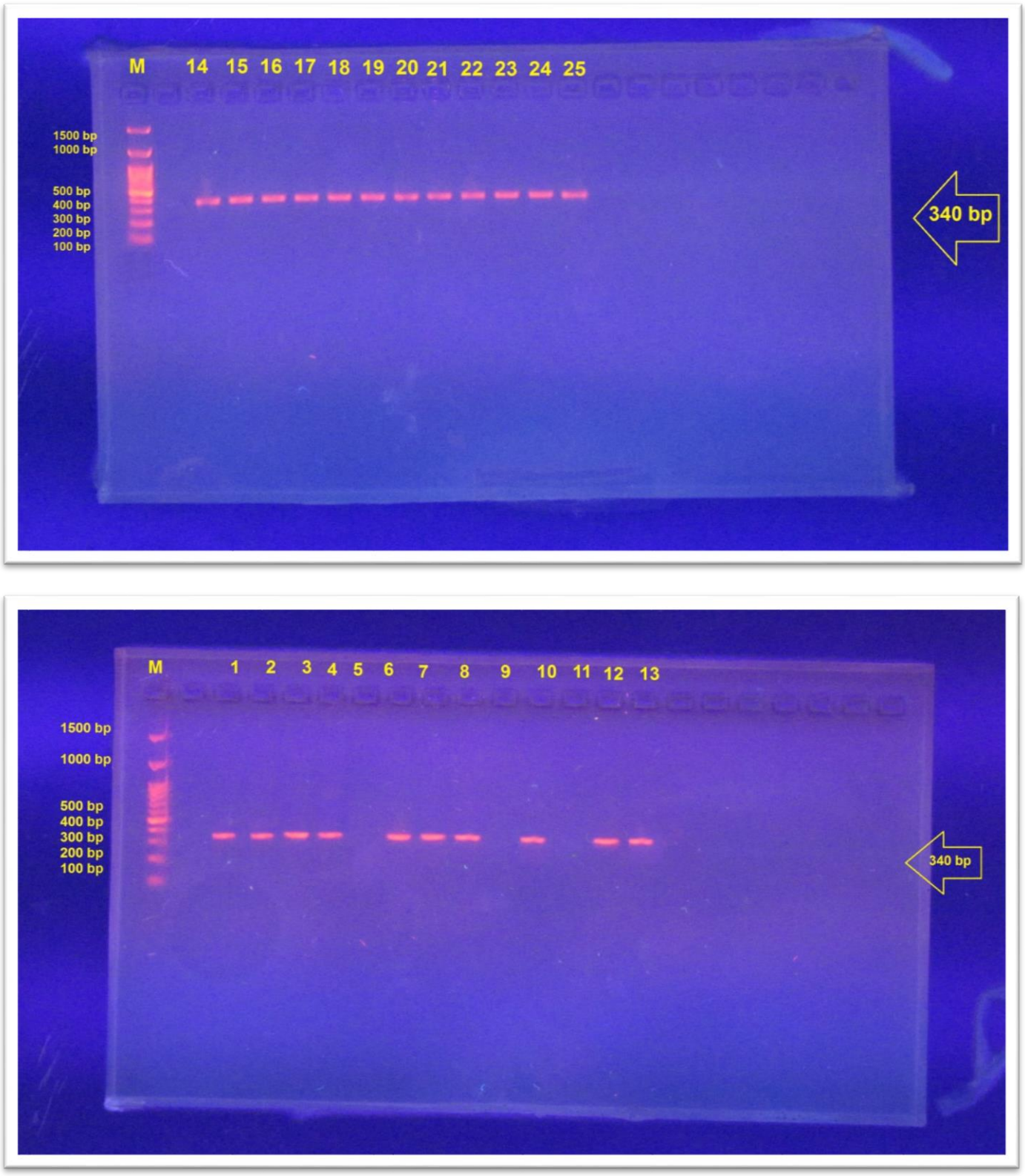

Fig. No. 1: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *mrk*D gene (340bp) using 1.5% Agarose gel, 70 voltages for 50 minutes

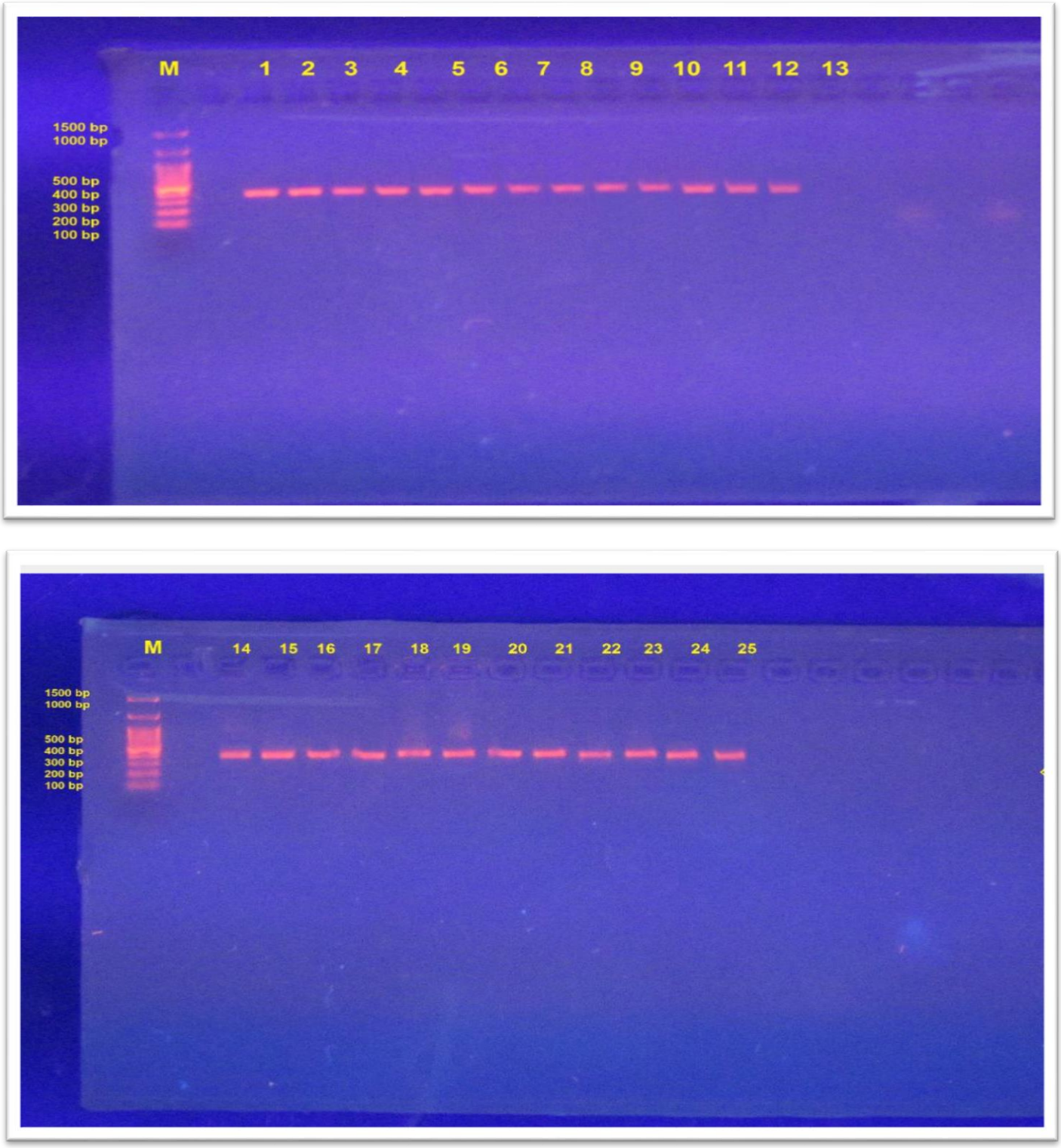

Fig. No. 2: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *Cps* gene (418bp) using 1.5% Agarose gel, 70 voltages for 50 minutes

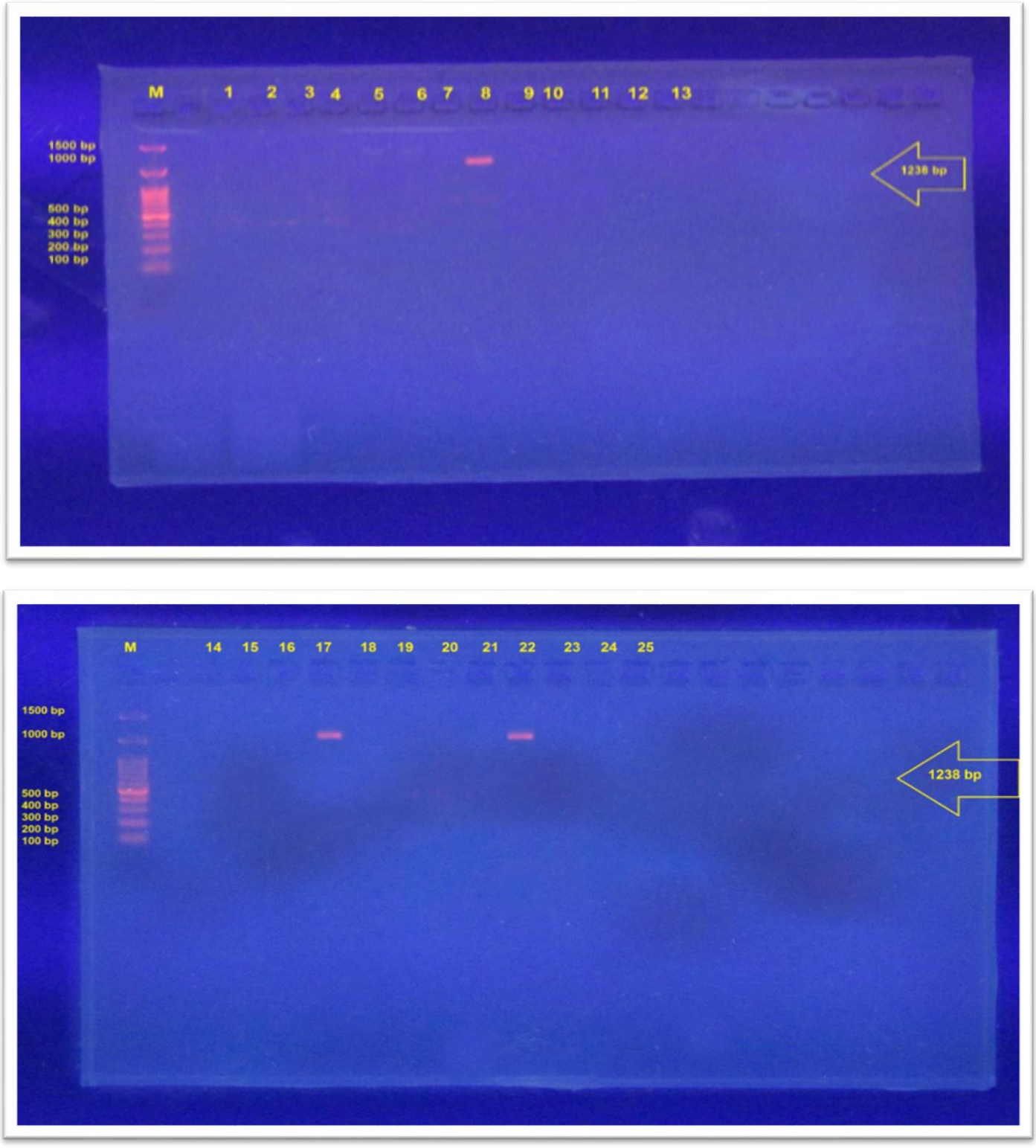

Fig. No. 3: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *K1* gene (1238bp) using 1.5% Agarose gel, 70 voltages for 75 minutes

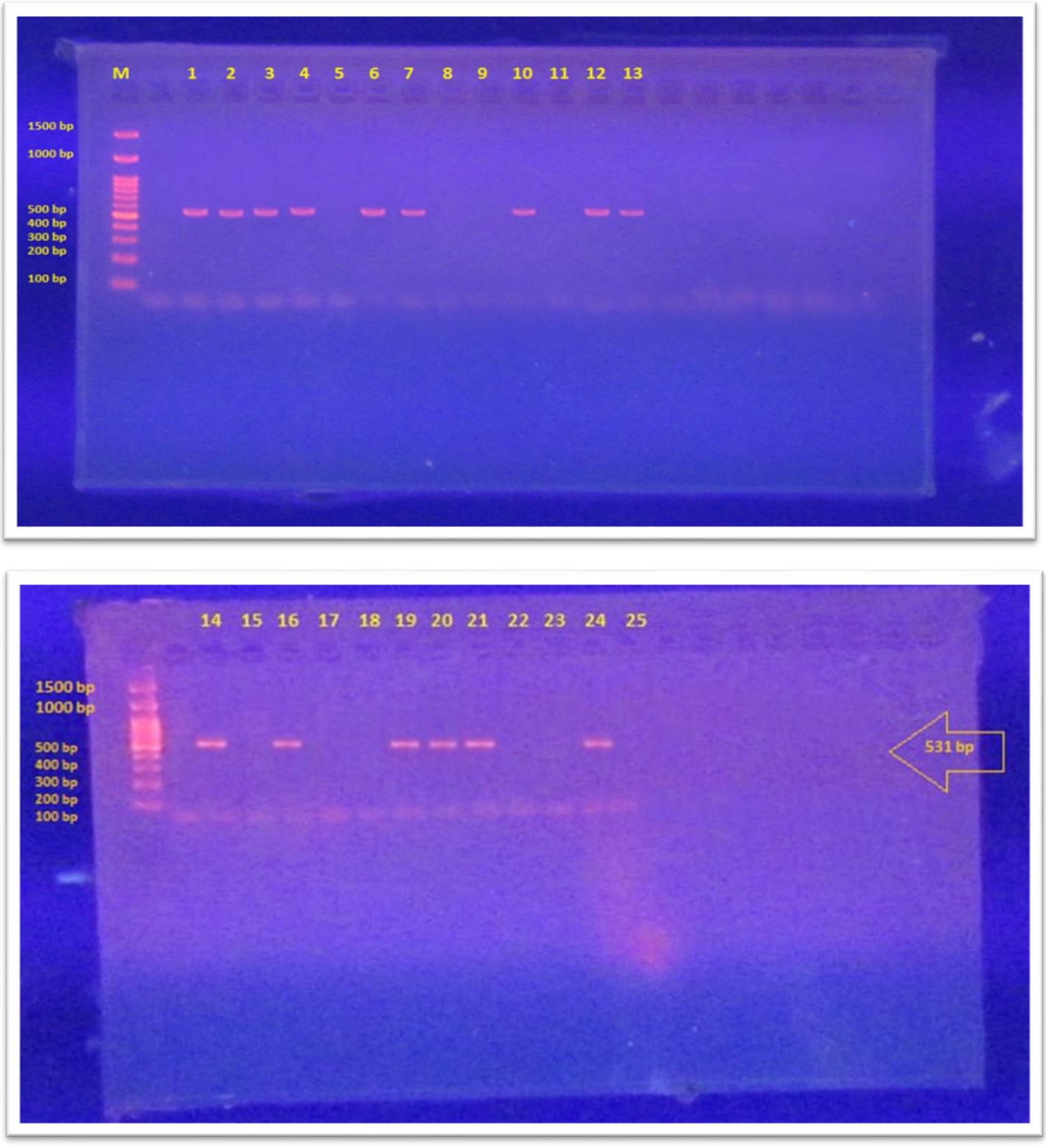

Fig. No. 4: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *K2A* gene (531bp) using 1.5% Agarose gel, 70 voltages for 50 minutes

4.DISCUSSION:

DFU is a main reason for diabetes interrelated illness and hospitalization and up to one-third of diabetic patients progress diabetic foot ulceration (DFU) during their lifespan and above 50% of these ulcers were infected as mentioned by Aleem *et al* (2021)

[22] in India. The foot ulcer is occupied by bacteria and when the overgrowth of the pathogens promotes tissue damage, it is named as a diabetic foot infection [23]. One hundred nineteen positive cultures were obtained during this study, of those, *Klebsiella pneumoniae* has the greatest frequency as 25 isolates. Our findings agreed with several studies, of those, Gram negative were the most prevalent bacteria [24] [25] [26] whereas Gram negative percentages were (59.2, 52.4 and 75.9) % in Nigeria, China and Bangladesh respectively.

Our results disagreed Rahman *et al* (2021) in Bangladesh found that *Escherichia Coli* (51.9%)was the most prevalent bacteria followed by *Staphylococcus aureus* (24.1%), *Proteus* spp. (16.7%), *Pseudomonas aeruginosa* (5.6%) and *Klebsiella* spp (1.9%) [26]. This difference in bacteria obtained from patients with DFUs could be credited to dissimilarity in method of sample collection, geographic area, treatment remedy and cruelty of infection [27]. Banu *et al* (2015) reported that the bacterial variation of DFU infection is associated with the interval of the ulcer incidence and preceding antibiotic consumption [28].

In current study, *K.pneumoniae* isolates were 88% resistant toward Pipracillin-tazobactam which is not in agreement with Raheem *et al* ;(2021) [29] and Ali and Kamil; (2022) [30] who documented that *K.pneumoniae* isolated from clinical sources were 100% resistant to Pipracillin-tazobactam in a local study . *K.pneumoniae* were resistant to Cefipime, Ceftixime, Ceftriaxone and Ceftazidine in (84, 88, 88 and 84)%, respectively and that are not in agreement with Hamid *et al* (2020) [31] who reported that *K.pneumoniae* isolated from DFI were 100% resistant to the mentioned antibiotics in Sudan. In current study, *K.pneumoniae* isolates were 84% resistant to Amoxicillin-clavulanic acid while (Hamid *et al*;2020) [31] documented that *K.pneumoniae* were 90.1% resistant to the mentioned antibiotic also it approaches to Aiswariya *et al* (2018) [32] findings whereas *K.pneumoniae* resistance toward Amoxicillin-clavulanic acid was 81.25% in India. There are numerous mechanisms of resistance against β-lactams including modifications in the target site of drug, reduced membrane penetrability, and the action of efflux pump, but β-lactamases are the most public features. This may be credited to the extreme use of antibiotics or to the capacity of the bacteria to develop as biofilms, or affected by genetic revenues, containing mutations and the transmission of resistance genes [33]. *K.pneumoniae* in this study were 76% resistant toward Imipenem and Meropenem. This conclusion is not astonishing with the fact *K.pneumoniae* is not only the chief reason of hospital-acquired infections but also a well-known "accumulator" of multidrug resistance plasmids and result in reduced susceptibility to carbapenems [34]. *K.pneumoniae* were 100% resistant to Gentamycin in our study and this result agreed with Rahman *et al* (2021) in Bangladesh who documented that *K.pneumoniae* isolated from DFI were 100% resistant to Gentamycin [28]. Additionally, *K.pneumoniae* were 92% resistant toward Amikacin while Ali and Kamil (2022) documented that *K.pneumoniae* isolated from DFI were 100% resistant toward Amikacin in a local study [30]. The best resistance mechanism to aminoglycosides comprises aminoglycoside-modifying enzymes contain acetyltransferases, nucleotidyltransferases and phosphotransferases which vary in their capacity to alter aminoglycosides [35]. Besides, the efflux pump upregulation (Poole;2004) and lessened consumption of antibiotics into the bacteria [36]. *K.pneumoniae* were 100% resistant toward Levofloxacin and that result agreed with Liu *et al* (2022) whereas *K.pneumoniae* isolated from Diabetic foot ulcer were 100% resistant toward Levofloxacin in China [37]. Also 96% resistance toward Ciprofloxacin showed by *K.pneumoniae* which are close to Ali and Kamil (2022) findings who reported that *K.pneumoniae* obtained from DFI were 100% resistant toward Ciprofloxacin in a local study [30]. Fluoroquinolone resistance is facilitated by numerous mechanisms including the mutation at DNA gyrases (*gyr*A and *gyr*B genes) and topoisomerase IV (*par*C and *par*E genes) [38]. In current study, *K.pneumoniae* isolates were 88% resistant to tetracycline and this result is close related to Liu *et al* (2022) findings in China whereas they documented that *K.pneumoniae* isolated from DFI were 87.5 % resistant to tetracycline [37]. Additionally, *K.pneumoniae* isolates were 68% resistant toward Doxycycline in current study whereas resistance rate was 85% toward Doxycycline with respect to *K.pneumoniae* isolated from DFI in India [39]. Tetracycline resistance is resulted from three mechanisms. First, proteins which guard ribosomes (S30 and S16) from tetracycline, modify the structure of these proteins, producing resistance to doxycycline and minocycline. Second, overexpression of efflux pumps which decreases the cell's penetrability to antibiotics [40]. Third, enzymatic alterations in antibiotics also result in resistance. The *tet*X gene result in antibiotic resistance due to tetracycline enzyme inhibition [41]. *K.pneumoniae* isolates were 56% resistant toward Rifampin in current study and that result is agreed with HA *et al* (2016) in Egypt who reported that generally all *K.pneumoniae* isolates were resistant toward the mentioned antibiotic [42]. Rifampin is an appreciated antibiotic for the treatment of mycobacterial

and other infections. The mechanism of resistance is mutation within the *rpo*B gene, which encodes the β-subunit of bacterial RNA or RNA polymerase which are the target of Rifampin [43].

Sixty percentage of *K.pneumoniae* isolated from DFI in current study were strong producers of biofilm according to microtiter plate method while 40% of them were moderate producers. However, 62.5% of *K.pneumoniae* isolated from DFI in a local study were strong producers of Biofilm and 37.5% were moderate producers as reported by Mahmood and Abdullah (2015) [44]. In another study performed in Indonesia, among biofilm producers, there were 26.95% isolates as strong, 28.74% isolates as moderate, and 29.94% *K.pneumoniae* isolates identified as weak biofilm producers [45]. On the other hand, the biofilm analysis indicates that 32.5% *K. pneumoniae* isolates formed biofilm weakly, 21.6% isolates created moderately, and 20.4% isolates were strong producers of biofilms whereas 25.5% were non producers of Biofilm in a study performed in Iran [46]. Biofilm production is a vital feature in *Klebsiella pneumoniae* pathogenesis, sponsoring increased resistance against environmental stressors and giving a reservoir for spreading and further gene exchange linked with antimicrobial resistance [47]. Numerous studies revealed that *mrk*D genes are essential in biofilm production of *K. pneumoniae*. Therefore, the *mrk*D gene may play main roles in biofilom formation [48].

PCR was used in this study to detect the presence of the *mrk*D gene, which is one of the important genes used to diagnose *K. pneumonia*. Figure No. 1 shows the electrophoresis of PCR products, which can be seen thoroughly that the primer of the *mrk*D gene was successful amplifying this gene by producing a PCR product of 340bp in size involving 88% of *K. pneumoniae* isolates enrolled in this study. Our results were similar to Rastegar *et al* (2021) [49] and Anis *et al* (2021) [18] who obtained a successful amplifying of *mrk*D gene of the same product size in Iran and Egypt respectively. Furthermore, Badger-Emeka and Emeka (2022) reported in their study performed in Saudi Arabia that 87% of *K.pneumoniae* isolates were included with *mrk*D gene [50]. The capability to form biofilm isolates results in enlarged resistance to antibiotics, as a result, treatment failure, increasing treatment costs and increasing mortality [51]. PCR also was performed to verify the presence of the *Cps* gene in the studied strains using primers designed for this gene under optimal temperature conditions. Figure No. 2 displays the successful amplification of the *Cps* gene through the appearance of PCR product of 418bp in size in 100% of *K. pneumonia* isolates enrolled in current study. Abdul-Razzaq *et al* (2014) [20] and Akbari *et al* (2017) [52] success in obtaining the same amplicon size of *Cps* gene in their study performed in Iraq and Iran, respectively. The presence of *Cps* genes in most isolated bacteria indicate that all these isolates can contain the genes of *Cps* biosynthesis as that stated by Lin *et al* (2011) in Taiwan [53]. The synthesis of capsule in *Klebsiella* is encoded by a gene called capsule polysaccharides (CPS) that enable the formation of the capsule [54]. CPS has a vital role in the linking of bacteria to epithelial and mucosal surfaces. It also guards the bacteria from phagocytosis, thus hiding the bacteria from the host immune system. On the other hand, the capsule offers defense against hostile environmental conditions and decreases the permeability of antibiotics into the bacteria [55] [56]. Molecular technique particularly PCR was used in current study to verify the presence of *K1* gene in *K. pneumoniae* isolates under study. It has been found that only 12% of *K. pneumoniae* isolates possess K1 gene of 1238bp in size as mentioned in Figure No. 3. Abdul-Razzaq *et al* (2014) [20] and Qassim and Khalid (2022) [57] in a local study and Siu *et al* (2011) in Taiwan success to amplify the same amplicon size of *K1* gene [58]. Similar percent obtained by Qassim and Khalid (2022) whereas they obtained only 15.8% of *K1* gene from the *K. pneumoniae* isolates [57]. Fang *et al* (2010) in Taiwan termed the *mag*A gene firstly in 2004 and exposed that the aggressive *K. pneumoniae* strains possessed high levels of hyper-mucoviscosity and *mag*A, whereas mutant variants missing *mag*A [59]. A cluster of capsular serotype *K1* and *K2A* gene of capsule gene of *K. pneumoniae* could be used as a very particular technique to distinguish the capsule *K2* serotype [60]. The absences of mannose recurrences on the capsule, evading it from detection by macrophages. *mag*A is only found in the *K1*capsule gene cluster, but *K2A* is found in the K2 serotype [61] [62]. *Mag*A is a chromosomal gene that serves a crucial role in *Klebsiella* infections and is accompanying with formation of a mucoviscous layer that makes *Klebseilla* resistant to phagocytosis [63]. In the present study, the molecular identification of serotype *K2A* was performed. Figure No. 4 shows that *K2A* gene was successfully amplified through the appearance of a PCR product of 531bp in size in 60% of *K. pneumoniae* isolates. Remya *et al* (2020) [22] and Anis *et al* (2021) [18] obtained the same results and success to amplify the amplicon of the same size of *K2A* gene in India and Egypt respectively while Alyassari *et al* (2019) in a local study obtained 72.72% of *K2A* gene of *K. pneumoniae* from clinical sources [64]. The chromosomal K2 capsule related gene A *(k2A*) for the *K2* serotype [61] [62] which isolates with capsule serotypes *K1* and *K2* are

more resistant to phagocytosis than Non-*K1/K2* strains [65]. The *k2A* gene of *K. pneumoniae* might be used as a specific diagnostic technique to classify the *Cps* of *K. pneumoniae* capsule K2 serotype, which matches to the *magA* region in the capsules gene clusters of *K1* isolate [60]. **Conclusions**: *Klabsiella* spp. Isolated from diabetic foot ulcer are genomically varied, display resistance to commonly used antibiotics and harbor for virulence determinants. These features propose that *Klebsiella* can contribute to perseverance and severity of these infections, leading to treatment failing and to the opportunity of transferring these features to other microorganisms having the same function.

ACKNOWLEDGEMENTS

Author is thankful to Imam al Hassan center for endocrinology and diabetes staff and everyone who helped in this research.

REFERENCES

[1] Ramsey SD, Newton K, Blough D, McCulloch DK, Sandhu N, Reiber GE, et al: Incidence, outcomes, and cost of foot ulcers in patients with diabetes. Diabetes Care 1999, 22:382–387. https://doi.org/10. 2337/diacare.22.3.382 PMID: 10097914

[2] Lavery LA, Armstrong DG, Wunderlich RP, Tredwell J, Boulton AJ: Diabetic foot syndrome: evaluating the prevalence and incidence of foot pathology in Mexican Americans and non-Hispanic whites from a diabetes disease management cohort. Diabetes Care 2003, 26:1435–1438. https://doi.org/10.2337/ diacare.26.5.1435 PMID: 12716801

[3] Prompers L, Huijberts M, Apelqvist J, Jude E, Piaggesi A, Bakker K, et al: High prevalence of ischaemia, infection and serious comorbidity in patients with diabetic foot disease in Europe. Baseline results from the Eurodiale study. Diabetologia 2007, 50:18–25. https://doi.org/10.1007/s00125-006-0491-1 PMID: 17093942

[4] Jia L, Parker CN, Parker TJ, Kinnear EM, Derhy PH, Alvarado AM, et al: Incidence and risk factors for developing infection in patients presenting with uninfected diabetic foot ulcers. PLoS One 2017, 12: e0177916. https://doi.org/10.1371/journal.pone.0177916 PMID: 28545120

[5] Lipsky BA, Berendt AR, Cornia PB, Pile JC, Peters EJ, Armstrong DG, et al: 2012 Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections. Clin Infect Dis 2012, 54:e132–173. https://doi.org/10.1093/cid/cis346 PMID: 22619242

[6] Hicks CW, Selvarajah S, Mathioudakis N, Sherman RE, Hines KF, Black JH 3rd, et al: Burden of Infected Diabetic Foot Ulcers on Hospital Admissions and Costs. Ann Vasc Surg 2016, 33:149–158. https://doi.org/10.1016/j.avsg.2015.11.025 PMID: 26907372

[7] Sugandhi P, Prasanth DA: Bacteriological Profile of Diabetic Foot. International Journal of Innovative Research in Science 2014, 3:14688–14692.

[8] Abdulrazak A, Bitar ZI, Al-Shamali AA, Mobasher LA: Bacteriological study of diabetic foot infections. J Diabetes Complications 2005, 19:138–141. https://doi.org/10.1016/j.jdiacomp.2004.06.001 PMID: 15866058

[9] Al Benwan K, Al Mulla A, Rotimi VO: A study of the microbiology of diabetic foot infections in a teaching hospital in Kuwait. J Infect Public Health 2012, 5:1–8. https://doi.org/10.1016/j.jiph.2011.07.004 PMID: 22341838

[10] Podschun, R., & Ullmann, U. (1998). *Klebsiella spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors* (Vol. 11, Issue 4).

[11] Akers, K. S., Mende, K., Cheatle, K. A., Zera, W. C., Yu, X., Beckius, M. L.,Aggarwal, D., Li, P., Sanchez, C. J., Wenke, J. C., Weintrob, A. C., Tribble, D. R., & Murray, C. K. (2014). Biofilms and persistent wound infections in United States military trauma patients: A case-control analysis. *BMC Infectious Diseases*, *14*(1). https://doi.org/10.1186/1471-2334-14-190

[12] Gupta, P., Sarkar, S., Das, B., Bhattacharjee, S., and Tribedi, P. (2016). Biofilm, pathogenesis and prevention–a journey to break the wall: a review. *Arch. Microbiol.* 198, 1–15. doi: 10.1007/s00203-015-1148-6

[13] Diago-Navarro, E., Chen, L., Passet, V., Burack, S., Ulacia-Hernando, A., Kodiyanplakkal, R. P., Levi, M. H., Brisse, S., Kreiswirth, B. N., & Fries, B. C. (2014). Carbapenem-resistant klebsiella pneumoniae exhibit variability in capsular polysaccharide and capsule associated virulence traits. *Journal of Infectious Diseases*, *210*(5), 803–813. https://doi.org/10.1093/infdis/jiu157

[14] Mukkunnath, S., Manjunath, R., and Desai, M. (2015). A study of the bacteriological profile of diabetic foot ulcer and antibiotic sensitivity pattern. *J. Evol. Med. Dental Sci.* 4, 6832–6840. doi: 10.14260/jemds/2015/991

[15] Calhoun, J. H., and Manring, M. M. (2005). Adult osteomyelitis. *Infect. Dis. Clin. North Am.* 19, 765–786. doi: 10.1016/j.idc.2005.07.009

[16] Hudzicki. J. (2009). Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. American Society for Microbiology. Downloaded from www.asmscience.org by IP:66.208.62.130 On: Wed, 29 Aug 2018 12:55:47

[17] Kırmusaoğlu S (ed.) (2019) Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods. IntechOpen. DOI: 10.5772/intechopen.78751

[18] Anis, R., Ahmed, S., & Esmaeel, N. (2021). Virulence determinants associated with biofilm formation by Klebsiella pneumoniae causing hospital-acquired bloodstream infection. *Microbes and Infectious Diseases*, *0*(0), 0–0. https://doi.org/10.21608/mid.2021.62223.1117

[19] Booq, R. Y., Abutarboush, M. H., Alolayan, M. A., Huraysi, A. A., Alotaibi, A. N., Alturki, M. I., Alshammari, M. K., Bakr, A. A., Alquait, A. A., Tawfik, E. A., Alsaleh, N. B., Bahwerth, F. S., Alarawi, M. S., Alyamani, E. J., & Sendy, B. K. (2022).

Identification and Characterization of Plasmids and Genes from Carbapenemase-Producing Klebsiella pneumoniae in Makkah Province, Saudi Arabia. *Antibiotics*, *11*(11). https://doi.org/10.3390/antibiotics11111627

[20] Abdul-Razzaq, M. S., Al-Khafaji, J. K. T., Khair, E. H., & Al-Maamory, -Allah. (2014). Molecular characterization of capsular polysaccharide genes of Klebsiella pneumoniae in Iraq. In *Int.J.Curr.Microbiol.App.Sci* (Vol. 3, Issue 7). http://www.ijcmas.com

[21] Remya, P., Shanthi, M., & Sekar, U. (2018). Occurrence and characterization of hyperviscous K1 and K2 serotype in Klebsiella pneumoniael. Journal of Laboratory Physicians, 10(03), 283–288. https://doi.org/10.4103/jlp.jlp_48_18

[22] Aleem, S., Multani, H., & Bashir, H. (2021). Bacteriological profile and antimicrobial sensitivity pattern of isolates from diabetic foot of patients attending a teaching hospital in Northern India. Asian Journal of Medical Sciences, 12(5), 83–87. https://doi.org/10.3126/ajms.v12i5.34415

[23] Spichler, A., Hurwitz, B. L., Armstrong, D. G., & Lipsky, B. A. (2015). Microbiology of diabetic foot infections: From Louis Pasteur to "crime scene investigation." *BMC Medicine*, *13*(1). https://doi.org/10.1186/s12916-014-0232-0

[24] Adeyemo, A. T., Kolawole, B. A., Rotimi, V. O., & Aboderin, A. O. (n.d.). *MULTICENTER STUDY OF THE BURDEN OF MULTIDRUG-RESISTANT BACTERIA IN THE ETIOLOGY OF INFECTED DIABETIC FOOT ULCERS*. https://doi.org/10.1101/625012

[25] Du, F., Ma, J., Gong, H., Bista, R., Zha, P., Ren, Y., Gao, Y., Chen, D., Ran, X., & Wang, C. (2022a). Microbial Infection and Antibiotic Susceptibility of Diabetic Foot Ulcer in China: Literature Review. *Frontiers in Endocrinology*, *13*. https://doi.org/10.3389/fendo.2022.881659

[26] Rahman, M. S., Hossain, K. S., Das, S., Kundu, S., Adegoke, E. O., Rahman, M. A., Hannan, M. A., Uddin, M. J., & Pang, M. G. (2021). Role of insulin in health and disease: An update. In *International Journal of Molecular Sciences* (Vol. 22, Issue 12). MDPI. https://doi.org/10.3390/ijms22126403

[27] Al-Rubeaan, K., Al Derwish, M., Ouizi, S., Youssef, A. M., Subhani, S. N., Ibrahim, H. M., & Alamri, B. N. (2015). Diabetic foot complications and their risk factors from a large retrospective cohort study. *PLoS ONE*, *10*(5). https://doi.org/10.1371/journal.pone.0124446

[28] Banu, A., Noorul Hassan, M. M., Rajkumar, J., & Srinivasa, S. (2015). Spectrum of bacteria associated with diabetic foot ulcer and biofilm formation: A prospective study. *Australasian Medical Journal*, *8*(9), 280–285. https://doi.org/10.4066/AMJ.2015.2422

[29] Raheem, H. Q., Hussein, E. F., Ghosh, S., & Alkafaas, S. S. (2021). Resistance of Klebsiella pneumoniae from Different Clinical Samples to Penicillin, Cephalosporin, Carbapenem and Fluoroquinolone (Vol. 44, Issue 06). http://atlas.ecdc.europa.eu/public/index.aspx?Instance

[30] Ali, S. Q., & Kamil, Y. M. (2022). Identifying the Resistant Bacterial Pattern in Patients with Diabetic Foot Ulcer. Journal for Research in Applied Sciences and Biotechnology, 1(4), 151–158. https://doi.org/10.55544/jrasb.1.4.20

[31] Hamid, M. H., Arbab, A. H., & Yousef, B. A. (2020). Bacteriological profile and antibiotic susceptibility of diabetic Foot infections at Ribat University hospital; a retrospective study from Sudan. https://doi.org/10.1007/s40200-020-00660-8/Published

[32] Aiswariya, A., Pavani, K., & Rajendra, B. S. (2018). Bacteriology of diabetic foot infections and their antibacterial susceptibility. International Journal of Research in Medical Sciences, 6(10), 3276. https://doi.org/10.18203/2320-6012.ijrms20184032

[33] Albu, S., Voidazan, S., Bilca, D., Badiu, M., Truta, A., Ciorea, M., Ichim, A., Luca, D., & Moldovan, G. (2018). Bacteriuria and asymptomatic infection in chronic patients with indwelling urinary catheter the incidence of ESBL bacteria. Medicine (United States), 97(33). https://doi.org/10.1097/MD.0000000000011796

[34] Tzouvelekis, L. S., Markogiannakis, A., Psichogiou, M., Tassios, P. T., & Daikos, G. L. (2012). Carbapenemases in Klebsiella pneumoniae and other Enterobacteriaceae: An evolving crisis of global dimensions. *Clinical Microbiology Reviews*, *25*(4), 682–707. https://doi.org/10.1128/CMR.05035-11

[35] Miró E, Grünbaum F, Gómez L, Rivera A, Mirelis B, Coll P, Navarro F. Characterization of aminoglycoside-modifying enzymes in enterobacteriaceae clinical strains and characterization of the plasmids implicated in their diffusion. Microb Drug Resist. 2013 Apr;19(2):94-9. doi: 10.1089/mdr.2012.0125. Epub 2012 Dec 3. PMID: 23206280.

[36] Nakamatsu, E. H., Fujihira, E., Ferreira, R. C. C., Balan, A., Costa, S. O. P., & Ferreira, L. C. S. (2007). Oligopeptide uptake and aminoglycoside resistance in Escherichia coli K12. *FEMS Microbiology Letters*, *269*(2), 229–233. https://doi.org/10.1111/j.1574-6968.2007.00634.x]

[37] Liu, X., Ren, Q., Zhai, Y., Kong, Y., Chen, D., & Chang, B. (2022). Risk Factors for Multidrug-Resistant Organisms Infection in Diabetic Foot Ulcer. *Infection and Drug Resistance*, *15*, 1627–1635. https://doi.org/10.2147/IDR.S359157

[38] Strahilevitz, J., Jacoby, G. A., Hooper, D. C., & Robicsek, A. (2009). Plasmid-mediated quinolone resistance: A multifaceted threat. In *Clinical Microbiology Reviews* (Vol. 22, Issue 4, pp. 664–689). https://doi.org/10.1128/CMR.00016-09

[39] Wasnik, R. N., Marupuru, S., Mohammed, Z. A., Rodrigues, G. S., & Miraj, S. S. (2019). Evaluation of antimicrobial therapy and patient adherence in diabetic foot infections. *Clinical Epidemiology and Global Health*, *7*(3), 283–287. https://doi.org/10.1016/j.cegh.2018.10.005

[40] García-Álvarez, L., G Holden, M. T., Lindsay, H., Webb, C. R., J Brown, D. F., … Maskell, D. J. (2011). Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. *Articles Lancet Infect Dis*, *11*, 595–603. https://doi.org/10.1016/S1473

[41] Taylor, D. E., & Chau, A. (1996a). MINIREVIEW Tetracycline Resistance Mediated by Ribosomal Protection. In *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY* (Vol. 40, Issue 1).

[42] HA, F., Abd El Rehim, H., Hazaa M, M., & El Sayed SA, S. (2016). Prevalence of Pathogenic Bacterial Isolates Infecting Wounds and their Antibiotic Sensitivity. *Journal of Infectious Diseases and Treatment*, *02*(02). https://doi.org/10.21767/2472-1093.100020

[43] Goldstein, B. P. (2014). Resistance to rifampicin: A review. In *Journal of Antibiotics* (Vol. 67, Issue 9, pp. 625–630). Nature Publishing Group. https://doi.org/10.1038/ja.2014.107

[44] Mahmood, M. T., & Abdullah, B. A. (2015). The relationship between biofilm formation and presence of fimH and mrkD genes among E. coli and K. pneumoniae isolated from patients in Mosul. In *Mosul Nursing Journal* (Vol. 1, Issue 3).

[45] Nirwati, H., Sinanjung, K., Fahrunissa, F., Wijaya, F., Napitupulu, S., Hati, V. P., Hakim, M. S., Meliala, A., Aman, A. T., & Nuryastuti, T. (2019a). Biofilm formation and antibiotic resistance of Klebsiella pneumoniae isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. *BMC Proceedings*, *13*. https://doi.org/10.1186/s12919-019-0176-7

[46] Karimi, K., Zarei, O., Sedighi, P., Taheri, M., Doosti-Irani, A., & Shokoohizadeh, L. (2021). Investigation of Antibiotic Resistance and Biofilm Formation in Clinical Isolates of Klebsiella pneumoniae. *International Journal of Microbiology*, *2021*. https://doi.org/10.1155/2021/5573388

[47] Assoni, L., Girardello, R., Converso, T. R., & Darrieux, M. (2021). Current Stage in the Development of Klebsiella pneumoniae Vaccines. In *Infectious Diseases and Therapy* (Vol. 10, Issue 4, pp. 2157–2175). Adis. https://doi.org/10.1007/s40121-021-00533-4

[48] Jagnow, J., & Clegg, S. (2003). Klebsiella pneumoniae MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. *Microbiology*, *149*(9), 2397–2405. https://doi.org/10.1099/mic.0.26434-0

[49] Rastegar, S., Moradi, M., Kalantar-Neyestanaki, D., Dehdasht, A. G., & Hosseini-Nave, H. (2021). Virulence factors, capsular serotypes and antimicrobial resistance of hypervirulent klebsiella pneumoniae and classical klebsiella pneumoniae in Southeast Iran. *Infection and Chemotherapy*, *53*(1). https://doi.org/10.3947/IC.2019.0027

[50] Badger-Emeka, L. I., & Emeka, P. M. (2022). Genetic fingerprinting and profile analysis of virulence genes in XDR clinical isolates of Klebsiella Pneumoniae. *European Review for Medical & Pharmacological Sciences*, *26*(14).

[51] Moemen, D., & Masallat, D. T. (2017). Prevalence and characterization of carbapenem-resistant Klebsiella pneumoniae isolated from intensive care units of Mansoura University hospitals . *Egyptian Journal of Basic and Applied Sciences*, *4*(1), 37–. https://doi.org/10.1016/j.ejbas.2017.01.001

[52] Akbari, R., & Asadpour, L. (2015). This paper should be cited as: Akbari R, Asadpour L [Identification of Capsular Serotypes K1 and K2 in Clinical Isolates of Klebsiella Pneumoniae in North of Iran. In *Journal* (Vol. 11, Issue 1).

[53] Lin, C. T., Wu, C. C., Chen, Y. S., Lai, Y. C., Chi, C., Lin, J. C., Chen, Y., & Peng, H. L. (2011). Fur regulation of the capsular polysaccharide biosynthesis and iron-acquisition systems in Klebsiella pneumoniae CG43. *Microbiology*, *157*(2), 419–429. https://doi.org/10.1099/mic.0.044065-0

[54] Pan, Y. J., Fang, H. C., Yang, H. C., Lin, T. L., Hsieh, P. F., Tsai, F. C., Keynan, Y., & Wang, J. T. (2008). Capsular polysaccharide synthesis regions in Klebsiella pneumoniae serotype K57 and a new capsular serotype. *Journal of Clinical Microbiology*, *46*(7), 2231–2240. https://doi.org/10.1128/JCM.01716-07

[55] Burmølle, M., Bahl, M. I., Jensen, L. B., Sørensen, S. J., & Hansen, L. H. (2008). Type 3 fimbriae, encoded by the conjugative plasmid pOLA52, enhance biofilm formation and transfer frequencies in Enterobacteriaceae strains. *Microbiology*, *154*(1), 187–195. https://doi.org/10.1099/mic.0.2007/010454-0

[56] Struve, C., & Krogfelt, K. A. (2005). Role of capsule in Klebsiella pneumoniae virulence: lack of correlation between in vitro and in vivo studies . *FEMS Microbiology Letters*, *218*(1), 149–154. https://doi.org/10.1111/j.1574-6968.2003.tb11511.x

[57] Qasim, B. M., & Khalid, H. (2022a). Microbiological and Molecular Study of K1, K2 Genes among Klebsiella pneumoniae isolated from Urine Specimens in Duhok city, Iraq. *Journal of Life and Bio Sciences Research*, *3*(01), 12–16. https://doi.org/10.38094/jlbsr30157

[58] Siu LK, Fung CP, Chang FY, Lee N, Yeh KM, Koh TH, Ip M. Molecular typing and virulence analysis of serotype K1 Klebsiella pneumoniae strains isolated from liver abscess patients and stool samples from noninfectious subjects in Hong Kong, Singapore, and Taiwan. J Clin Microbiol. 2011 Nov;49(11):3761-5. doi: 10.1128/JCM.00977-11. Epub 2011 Sep 7. PMID: 21900521; PMCID: PMC3209116.

[59] Fang, C. T., Lai, S. Y., Yi, W. C., Hsueh, P. R., Liu, K. L., & Chang, S. C. (2007). Klebsiella pneumoniae genotype K1: An emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. Clinical Infectious Diseases, 45(3), 284–293. https://doi.org/10.1086/519262

[60] Chuang, Y., P., Fang, C., T., Lai,S., Y., Chang, S., C., and Wang,J., T. (2006).Genetic Determinants of Capsular Serotype K1 of *Klebsiella pneumoniae* Causing Primary Pyogenic Liver Abscess, *The Journal of Infectious Diseases*, Volume 193, Issue 5, ,Pages 645–654, https://doi.org/10.1086/499968

[61] Yu, W.-L., Ko, W.-C., Cheng, K.-C., Lee, H.-C., Ke, D.-S., Lee, C.-C., Fung, C.-P., & Chuang, Y.-C. (2006). *Association between rmpA and magA Genes and Clinical Syndromes Caused by Klebsiella pneumoniae in Taiwan*. https://academic.oup.com/cid/article/42/10/1351/277546

[62] Doo, R. C., Ha, R. L., Seung, S. L., Shin, W. K., Chang, H. H., Jung, S. I., Oh, M. D., Kwan, S. K., Kang, C. I., Kyong, R. P., & Song, J. H. (2008). Evidence for clonal dissemination of the serotype K1 Klebsiella pneumoniae strain causing invasive liver abscesses in Korea. *Journal of Clinical Microbiology*, *46*(12), 4061–4063. https://doi.org/10.1128/JCM.01577-08

[63] Guo, Y., Wang, S., Zhan, L., Jin, Y., Duan, J., Hao, Z., Lv, J., Qi, X., Chen, L., Kreiswirth, B. N., Wang, L., & Yu, F. (2017). Microbiological and clinical characteristics of hypermucoviscous Klebsiella pneumoniae isolates associated with invasive infections in China. *Frontiers in Cellular and Infection Microbiology*, *7*(FEB). https://doi.org/10.3389/fcimb.2017.00024

[64] Alyassari, A., Neamah, A. J., Meteab Alshammari, M. M., & Abdullah Al-Ibadi, I. N. (2019). Genotypic Characterization of Klebsiella pneumoniae Isolated from Human and Sheep in Al-Qadisiyah Province, Iraq. *Journal of Pure and Applied Microbiology*, *13*(3), 1783–1789. https://doi.org/10.22207/JPAM.13.3.54

[65] Wang, L., Gu, H., & Lu, X. (2012). *A rapid low-cost real-time PCR for the detection of klebsiella pneumonia carbapenemase genes*. www.ncbi.nlm.nih.gov.