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RESISTANCE PATTERN IN *K.PNEUMONIA* ISOLATED FROM COVID 19 PATIENTS

Mustafa N. Hussein
Department of Micobiology, College of Science, Wasit University, Iraq

Prof Dr. Zainab N. Al-Saadi
Department of Micobiology, College of Science, Wasit University, Iraq

Dr. Jalal Abdulrazzaq Tofah Al-Azzawi
Department of Micobiology, College of Science, Wasit University, Iraq

Abstract

Background: Covid-19 cause acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) which leads to pulmonary failure. Secondary infections were diagnosed when patients showed clinical symptoms or signs of bacteremia, and had a positive culture of a new pathogen. In COVID-19 patients coinfected with bacteria in the ICU-death group, carbapenem resistant *Klepsiella pneumonia,* was isolated. This nosocomial, antibiotic-resistant pathogen is known to pose challenges in antibiotic therapy, and to increase the death-risk.

Materials and Methods: A total of three hundred eighty clinical samples were collected from covid19 patients, in Iraq from October 2021 to February 2021, in the bacteriology Unit. Patients samples were included upper respiratory tract, 190 (50%) from Nasopharyngeal (NP) and 190 (50%) from and Oropharyngeal (OP) specimen. The recovered isolates were subjected to different morphological and biochemical tests for identification to the species level and disk diffusion test, in addition to identification of resistance genes included *magA, K2A, entB, iutA, fyuA, kfu, bla Kpc2* and *carbapenemase* genes.

Result: Disc diffusion technique, was used to test the susceptibility of 50 isolates of *K. pneumoniae* using 10 different antibiotics, *K. pneumoniae* isolates were resistant to Ceftriaxone, Cefixime, Cloramphenicole, Augmentine, Aztreonam and Trimethoprime-sulphamethoxazole in percentage of 25 (50%), 19(38%), 29(58%), 20 (40%), 39 (78%) and 30(60%) respectively, while *K. pneumoniae* isolates has been showed sensitivity to Ciprofloxacillin, Amikacin, Gentamicin and Imipenem in a percent of 35 (70%), 36 (72%), 41 (82%) and 45 (90%).

entB, and fyuA of Klebsiella pneumoniae isolates showed that all isolates gave positive result for entB gene while only two lane gave positive result for fyuA gene. *intA* gene *of Klebsiella pneumoniae* isolates gave positive result for *intA gene*. Two gene *(bla kpc2 and kfu) of Klebsiella pneumonia* isolates using PCR showed that 9 isolates gave positive result for both genes. *MagA, K2A and Carbapenenmase* showed that the multiplex PCR product of all genes were negative.

Conclusion: Our results showed that *K. pneumonia* virulence genes was more correlated to covid-19 patients, especially *entB* gene.

Key words: Covid19, multiplex PCR , *K. pneumonia*.

1-Introduction The coronavirus disease (Covid-19), H5N1 influenza A, H1N1 2009 and Middle East respiratory syndrome coronavirus cause acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) which leads to pulmonary failure and result in fatality. These viruses were thought to infect only animals until the world witnessed a Covid 19 outbreak caused by Covid-19, 2002 in Guangdong, China (1). Only a decade later, another pathogenic coronavirus, known as Middle East respiratory syndrome coronavirus caused an endemic in Middle Eastern countries (2). Recently at the end of 2019, Wuhan an emerging business hub of China experienced an outbreak of a novel coronavirus that killed more than eighteen hundred and infected over seventy thousand individuals within the first fifty days of the epidemic (3). This virus was reported to be a member of the b group of coronaviruses. The novel virus was named as Wuhan coronavirus or 2019 novel coronavirus (2019-nCov) by the chinese researchers, the International Committee on Taxonomy of Viruses (ICTV) named the virus as Covid-19and the disease as COVID-19 (4). In the history, Covid-19(2003) infected 8098 individuals with mortality rate of 9%, across 26 contries in the world, on the other hand, novel corona virus (2019) infected 120,000 induviduals with mortality rate of 2.9%, across 109 countries, till date of this writing. It shows that the transmission rate of Covid-19is higher than the reason could be genetic recombination event at S protein in the RBD region of Covid-19may have enhanced its transmission ability. In this review article, some studies discuss the origination of human coronaviruses briefly, and further discuss the associated infectiousness and biological features of COVID-19 and MERS with a special focus on COVID-19 (5).

Secondary bacterial infections associated with influenza pandemics are well described, with *Streptococcus pneumoniae, Haemophilus influenzae,* and *Staphylococcus aureus* being reported as the most common causes, and rates ranging between 11 and 35% of cases in a meta-analysis (6). Most deaths associated with influenza pandemic of 1918 were not caused by influenza virus alone, but by subsequent bacterial pneumonia, particularly caused by *S. pneumoniae*. More recently, secondary bacterial infections were also reported in the 2009 swine influenza pandemic and during the 2002 Covid 19 (2) and the Middle East respiratory syndrome (MERS), both caused by the coronavirus of zoonotic origin Covid-19and Covid 19, respectively. Secondary bacterial infections can be complications of viral respiratory diseases, and lead to increase in pneumonia severity, here its explored current literature on bacterial coinfections reported in the 2020 coronavirus pandemic (3).

Infections falling within the latter category are increasingly being attributed to Enterobacteriaceae, which are being reported with growing frequency in Italy and many other countries of the world, and the vast majority are caused by isolates producing the *Klebsiella pneumoniae* carbapenemase (7). However, there is a paucity of clinical evidence on the efficacy of treatments in this infections. In the phase 2 and 3 clinical trials conducted to support its marketing authorization in Europe and the United States, it was tested against carbapenems, which, prior to 2015, were considered the “best available therapy” for infections caused by ceftazidime-resistant *Klebsiella pneumoniae*. As

a result, individuals whose infections were caused by carbapenem-resistant *Klebsiella pneumoniae* isolates were excluded from enrollment in these trials (8).

2-Materials and methods:

2-1-Samples Collection

three hundred eighty samples were collected from COVID-19 Patients that has been diagnosed by PCR technique, from upper respiratory tract (Nasopharyngeal specimen (NP) and Oropharyngeal (OP) (throat)) according to Wasit University ethics committee (where all study subjects were informed about the aim and the details of the study) in AL-Kut hospital in Iraq from October 2021 to February 2021, in the bacteriology Unit. The patients were first assessed clinically by the doctors in the hospital and then referred for sample collection (9).

2-2 Isolation and identification of bacterial isolates

Each specimen was cultured on Blood agar, MacConkey agar, Mannitol Salt Agar and Chocolate Agar plates. The resultant colonies in these media were subcultured to be more purity. The recovered isolates were subjected to different morphological and biochemical tests for identification to the species level as described by Bergey's Manual for determinative Bacteriology. The isolates were identified on the basis of typical morphology by gram staining, coagulase test, Triple Sugar Iron Agar test (TSI) and the analytical profile index (API) system (10).

2-3 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed for all detected *K. pneumoniae* isolates using the Kirby-Bauer disc diffusion method based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (11). Accordingly, the antimicrobial resistance pattern of *K. pneumoniae* isolates was identified with respect to the following ten antibiotics: Ciprofloxacillin (CIP, 10 μg), Ceftriaxone (CRO, 10 μg), Amikacin (AK, 30 μg), Gentamicin (GN, 30 μg), Imipenem (IMI, 10 μg), Cefixime (CFM, 10 μg), Cloramphenicole (C, 30 μg), Augmentine (AUG, 30 μg), Aztreonam (AZ, 30 μg), and Trimethoprime-sulphamethoxazole (SXT, 10 μg). *K. pneumoniae* isolates which were resistant to three or more antimicrobial categories were considered as MDR (12).

2-4 Multiplex PCR

For epidemiologic typing, genomic DNA was extracted from bacterial cells through the method proposed by Purighalla et al. (13), and then, **Multiplex PCR** was performed for detect virulence genes among bacterial isolates using specific primers, table (1). PCR amplification was done using a mixture of 18 μl of sterile distilled water, 2.5 μl of 10× PCR buffer, 1 μl of 10 molar dNTP, 1 μl of each primer, 0.5 μl of Taq polymerase, and 1 μl of template DNA. PCR reaction consisted of an initial denaturation at 95°C for three minutes and then 35 thermal cycles consisting of denaturation at 94°C for one minute, annealing at 48°C for one minute, and final extension at 72°C for two minutes and at 72°C for five minutes.

Table (1) Primers Used in This Study

Gene	Sequence	Product size	Reference
magA	**ATATGGCCAGTCCGAAAGTG AACATTGCCGCTACTACAGGA**	**258 bp**	**Designed in this study**
K2A	**GGACATGATGTTGATTTGATCG TGGTAGCCATATCCCTTTGG**	**477 bp**	**Designed in this study**
entB	**GCGATGATGGAGAAAGTGGT ACCAGCACGGTATCGTCTTC**	**215 bp**	**Designed in this study**
iutA	**TTATTCCGGATAGCGACTGG CTGGCCCCAGATAGTGACAT**	**320 bp**	**Designed in this study**
fyuA	**GCGCTTCTCGCATGATAAAT ACCCGGTTACCGTGATACAA**	**498 bp**	**Designed in this study**
Kfu	**CTCGGTGCTGGCCTACTATC AGATCGGCATGTAGCCAGTT**	**543 bp**	**Designed in this study**
bla Kpc2	**GATAACACTGCGGCCAACTT TTGCCGGGAAGCTAGAGTAA**	**225 bp**	**Designed in this study**
carbapenemase	**CGGAACCATTCGCTAAACTC GTCCAGACGGAACGTGGTAT**	**398 bp**	**Designed in this study**

3-Results

3-1 Antibiotic sensitivity test for *K.pneumoniae*

The disc diffusion technique, as recommended by the National Committee for Clinical Laboratory Standards (NCCLs), was used to test the susceptibility of 50 isolates of *K. pneumoniae* using 10 different antibiotics, *K. pneumoniae* isolates were resistant to Ceftriaxone, Cefixime, Cloramphenicole, Augmentine, Aztreonam and Trimethoprime-sulphamethoxazole in percentage of 25 (50%), 19(38%), 29(58%), 20 (40%), 39 (78%) and 30(60%) respectively, while *K. pneumoniae* isolates has been showed sensitivity to Ciprofloxacillin, Amikacin, Gentamicin and Imipenem in a percent of 35 (70%), 36 (72%), 41 (82%) and 45 (90%) respectively, as showed in table (2).

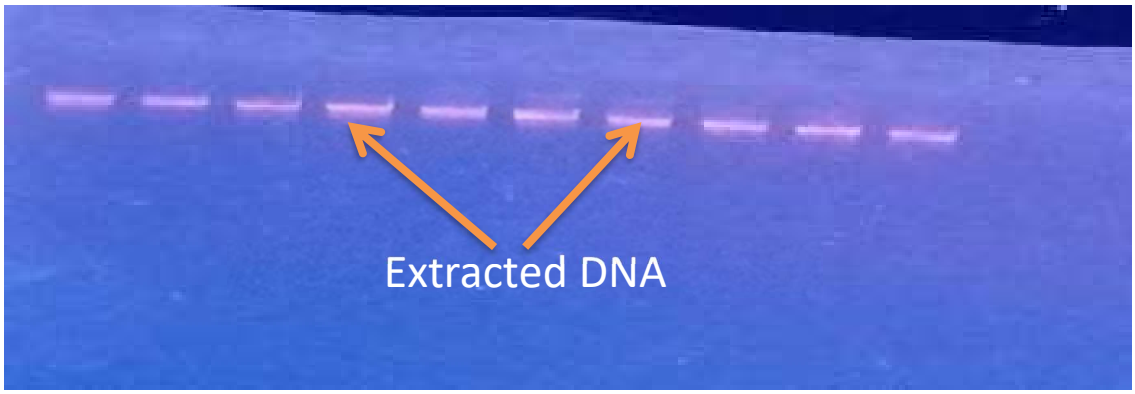
Table (2): Antibiotics resistance patterns of Klebsiella pneumoniae isolates

Antibiotics	Sensitivity		
	Resistance	Intermediate	Sensitive
Ciprofloxacillin	5 (10%)	10 (20%)	35 (70%)
Ceftriaxone	25 (50%)	2(4%)	23 (46%)

Amikacin	11(22%)	3(6%)	36 (72%)
Gentamicin	6 (12%)	3 (6%)	41 (82%)
Imipenem	5 (10%)	0(0%)	45 (90%)
Cefixime	19(38%)	15(30%)	16(32%)
Cloramphenicole	29(58%)	11(22%)	10 (20%)
Augmentine	20 (40%)	16 (32%)	14 (28%)
Aztreonam	39 (78%)	5(10%)	6 (12%)
Trimethoprime-sulphamethoxazole	30(60%)	10(20%)	10 (20%)
Means of results	17.9	7.5	23.6

3-2 Molecular study

3-2-1 Extraction of DNA



3-2-2 Determination of resistance genes in *Klebsiella pneumonia* using polymerase chain reaction

Agarose gel electrophoresis image that showed the multiplex PCR product of two gene *(entB, and fyuA) of Klebsiella pneumoniae* isolates at 215 and 498 bp PCR product size respectively, all isolates gave positive result for entB gene while only two lane gave positive result for fyuA gene, figure (2a) and (2b).

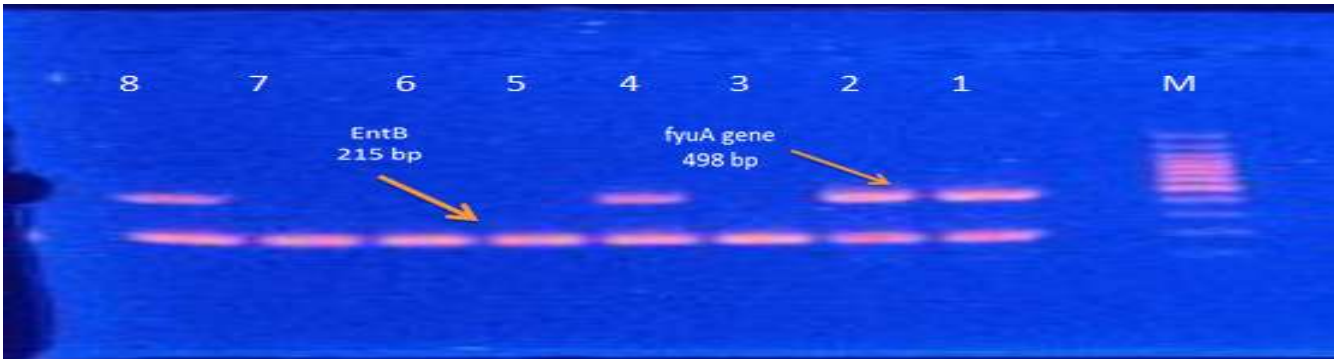
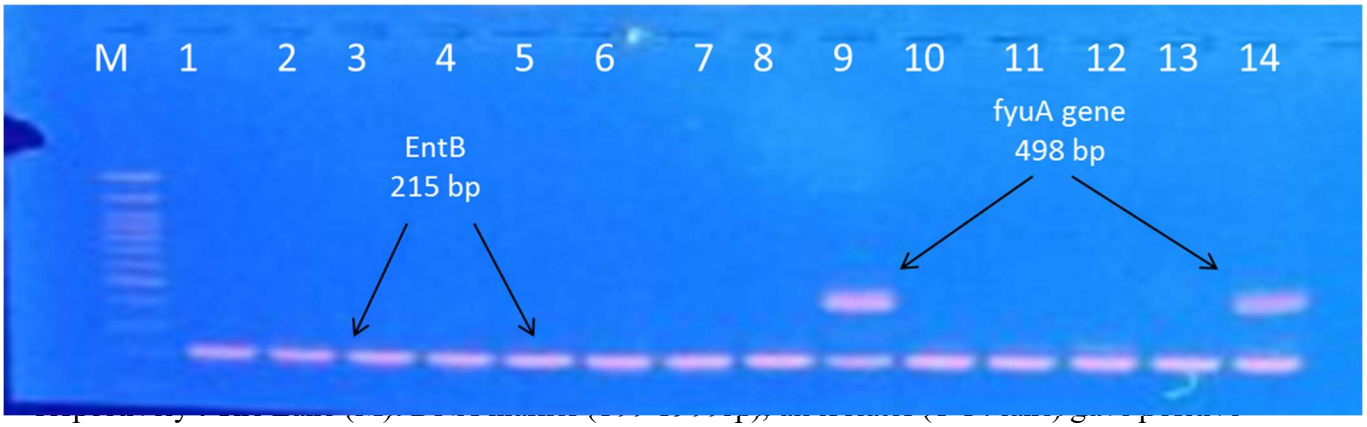


Figure (2a) Agarose gel electrophoresis image that showed the multiplex PCR product of two gene *(entB, and fyuA) of Klebsiella pneumoniae* isolates at 215 and 498 bp PCR product size respectively . The Lane (M): DNA marker (100-1500bp), all isolates (1-14 lane) gave positive result for entB gene while only two lane gave positive result for fyuA gene.



result for entB gene while only two lane gave positive result for fyuA gene.

3-2-3 Agarose gel electrophoresis of *intA gene* in *Klebsiella pneumoniae*

Agarose gel electrophoresis image that showed the monoplex PCR product of *intA* gene *of Klebsiella pneumoniae* isolates at 320 bp PCR product size, all isolates gave positive result for *intA gene*, figure (3).

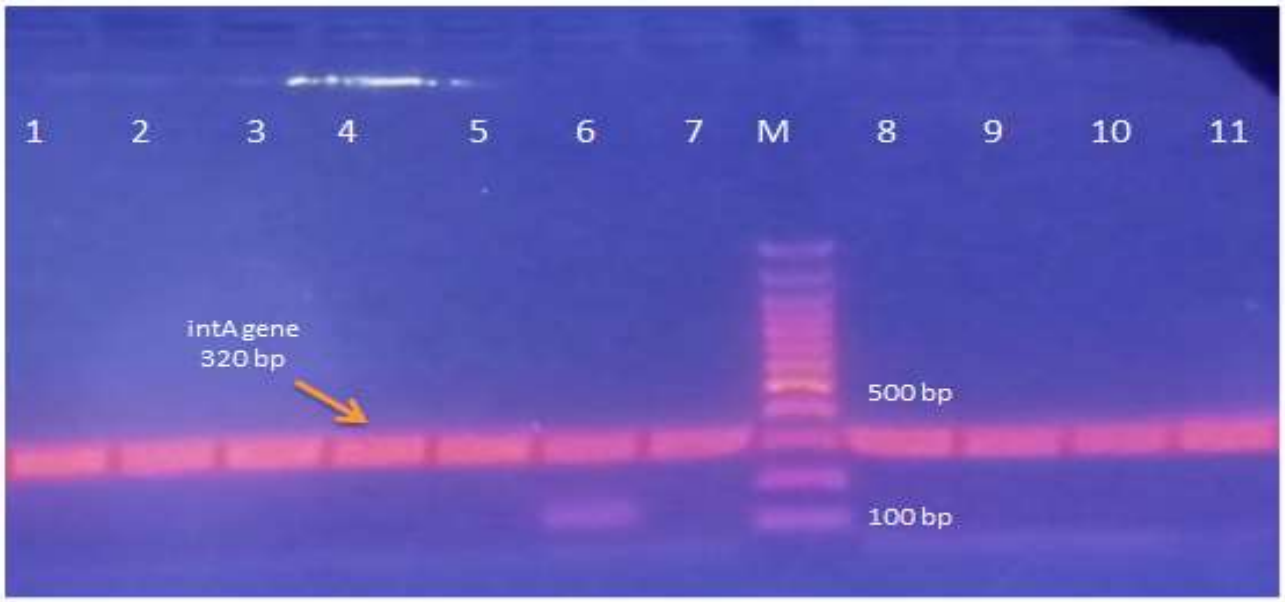


Figure (3) Agarose gel electrophoresis image that showed the monoplex PCR product of *intA* gene *of Klebsiella pneumoniae* isolates at 320 bp PCR product size . The Lane (M): DNA marker (100-1500bp), all isolates (1-11 lane) gave positive result for *intA gene* .

3-2-4 Agarose gel electrophoresis of *bla kpc2, and kfu genes* in *Klebsiella pneumoniae*

Agarose gel electrophoresis image that showed the multiplex PCR product of two gene *(bla kpc2, and kfu) of Klebsiella pneumoniae* isolates using PCR showed that isolates (1-3) gave positive result for only kfu gene ,4-6 lane positive only for bla kpc2 gene while 7-12 lane gave positive result for both genes. figure (4).

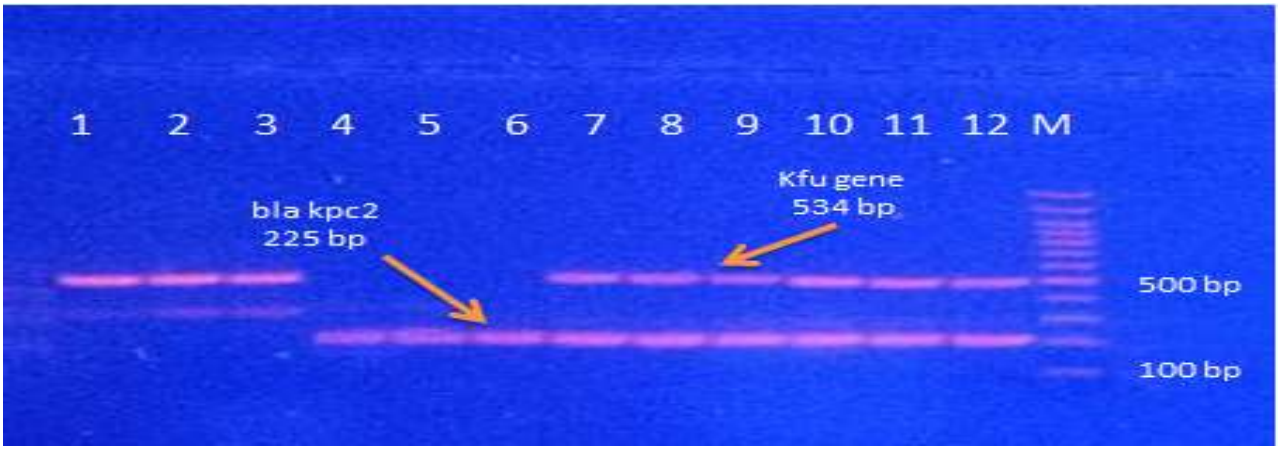


Figure (4) Agarose gel electrophoresis image that showed the multiplex PCR product of two gene *(bla kpc2, and kfu) of Klebsiella pneumoniae* isolates at 225 and 534 bp PCR product size respectively . The Lane (M): DNA marker (100-1500bp), isolates (1-3 lane) gave positive result for only kfu gene ,4-6 lane positive only for bla kpc2 gene while 7-12 lane gave positive result for both genes.

3-2-5 Agarose gel electrophoresis of *MagA, K2A and Carbapenenmase genes* in *Klebsiella pneumoniae*

Agarose gel electrophoresis image of *MagA, K2A and Carbapenenmase* showed that the multiplex PCR product of all genes were negative, figure (5).

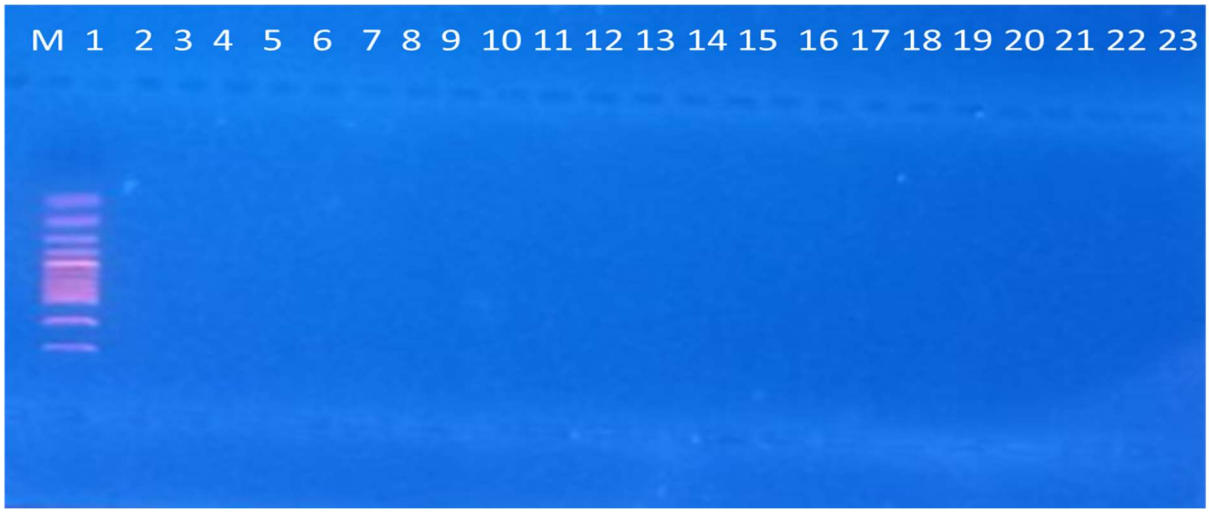


Figure (5) Agarose gel electrophoresis image of *MagA, K2A and Carbapenenmase* genes, where all genes were negative.

4-Discussion

In this study patients age ranged between (41-50) and (51-60) were more frequent among all age groups, these information were in consistence with (Dergaa et al. (2022) (14) compared of vital signs between the symptomatic and asymptomatic groups among eight age groups. Statistical significant differences were noted values for the age groups [21–30], [31–40], [41–50], and [51–60] year old) higher number of patients were in the age groups [41–50] and [51–60] years, in covid-19 infection rate among elderly patients and this may belongs to that various factors including immunosenescens, comorbid chronic diseases, and alterations in normal physiological organ functions may modify the frequency and severity of infections in elderly patients. Normal body reactions to ensuing infection, such as increased body temperature, may be blunted in those patients causing difficulties in differential diagnosis between infection and other diseases. In severe infections the respiratory and urinary tracts are the most frequently involved systems which may be accompanied by severe sepsis. Bacteremia and sepsis are also associated with indwelling vascular catheters in the elderly who are admitted to the intensive care unit (ICU) (15, 16). Literature review presents evidence for significantly lower susceptibility to infection for children aged under 10 years compared to adults given the same exposure, for elevated susceptibility to infection in adults aged over 60y compared to younger/middle aged adults, possibly due to age-related differences, generally in covid-19 there is uncertainty as to the role of different age subgroups of children in the spread of SARS-CoV-2, including how susceptibility to infection varies in different age groups of children, and how it compares to susceptibility to infection in different age groups of adults (17, 18). While present outcomes were not agreed with other mentioned that it was does not suggest that the oldest individuals necessarily play the leading role in the spread of SARS-CoV-2 in the community, suggest that younger adults, particularly those aged under 35y often experience the highest cumulative rates of infection (19, 20). The differences

in results among studies may belongs to long or shortness in a period of data collection in each study.

Agarose gel electrophoresis image that showed the multiplex PCR product of two gene *(entB, and fyuA) of Klebsiella pneumoniae* isolates, all isolates gave positive result for entB gene while only two lane gave positive result for fyuA gene, the present information were the same findings of Eghbalpoor et al. (2019) (21) as the antibiotic resistance rate of *K. pneumoniae* isolates ranged from 12.1% for meropenem to 100% for amoxicillin. The prevalence of virulence genes ranged from 1.4% for *cnf-1* to 100% for *mrkD*, *fimH*, *kpn*, and *entB* genes. Weak association was observed between the presence of *traT*, *fyuA,* or *cnf*-1 genes with antibiotic resistance. Additionally multiplex PCR revealed that K. pneumoniae harbored virulence genes for adhesins (mrkD, ycfM, and kpn) and enterobactin (entB) and, in one case, while (ybtS, irp1, irp2, and fyuA) were less numerous (22). All Klebsiella isolates carried the *ycfM*, *entB*, and *wabG* genes. The *fimD*, *fimH*, *mrkC*, and *mrkD* genes were almost ubiquitous among the strains (98.43%). The prevalence of the other virulence genes was as follows: *uge* (73.23%), *irp2* (41.73%), *ybtS* (40.94%), *fyuA* (40.16%), *iucA* (11.02%), *rmpA* (7.09%), *iroN* (5.51%), *clbA* (1.57%), and *clbQ* (1.57%) (23). On the other hand few studies indicated the prevalence of *fyuA* gene in *Klebsiella pneumonia*e such as that by Kumar et al., (2020) as the potential of recombinant FyuA of *K. pneumoniae* has been evaluated against lung infection. Immunization generated both humoral and cell mediated response which conferred protection against the lethal dose of bacteria These results indicate the protective role of FyuA which can be a potential vaccine candidate (24).

The current data were agreed to studies revealed that multidrug-resistant and hypervirulent *Klebsiella pneumoniae* (hvKP) poses a significant risk to public health. To better understand the molecular characteristics of multidrug-resistant *K. pneumoniae* of animal origin, fifteen *K. pneumoniae* strains from the liver, all *K. pneumoniae* isolates were subjected to antimicrobial susceptibility testing, string test, multi-locus sequence typing and whole genome sequencing. *K. pneumoniae* isolates were found carrying the *intA* gene (25, 26). On the other hand the isolates of *Klebsiella* in another prominent pathogen that may be involved in diseases and life-threatening infections, generally encoded the siderophore gene entB and not *intA*, but were negative to most of the other virulence genes (27, 28).

Agarose gel electrophoresis image that showed the multiplex PCR product of two gene *(bla kpc2, and kfu) of Klebsiella pneumoniae* isolates using PCR showed that isolates (1-3) gave positive result for only kfu gene ,4-6 lane positive only for bla kpc2 gene while 7-12 lane gave positive result for both genes. *kpc2, and kfu* has emerged as one of the most challenging pathogens in the latest years (29). *kpc2, and kfu* showed resistant to almost all available antibiotics and was related to limited treatment options and high mortality rates. *kpc2, and kfu* has been listed as a “critical priority” by the World Health Organization (WHO). For pathogen survival, the acquisition of virulent traits is necessary (30), and some reports suggest that the virulence of carbapenem-resistant *Klebsiella pneumoniae* is enhanced (31).

The present results were agreed with Du et al. (2021) (32) study aimed to characterize *kpc2, and kfu* co-harboring *bla* $_{KPC-2}$-carrying plasmid, the *bla* $_{KPC-2}$-carrying plasmids. Nineteen strains

(79.2%) had a 219-kb virulence plasmid possessed high similarity, two strains had a 224-kb virulence plasmid resembled plasmid pK2044 from *K. pneumoniae*. Moreover, three strains carried three different hybrid resistance- and virulence-encoding plasmids. Conjugation assays showed that both *bla* $_{KPC-2}$, of which three co-transferred *bla* $_{KPC-2}$ and *kfu* in large plasmids. Infection assays in the *Galleria mellonella* model demonstrated the virulence level of these isolates was found to be consistently higher than that of classic *Klebsiella pneumoniae*. In conclusion, *kpc2, and kfu bla* carrying plasmid enhanced virulence, and transferability, and should, therefore, be regarded as a real superbug that could pose a serious threat to public health. Hence, heightened efforts are urgently needed to avoid its co-transmission of the virulent plasmid (gene) and resistant plasmid (gene) in clinical isolates, also Xu et al. (2018) (33) has been mentioned the same outcomes. While outcomes of this study were non compatible with Su et al. (2020) (34) as sequencing results showed that the JmsCRE57 genome mainly consisted of a circular chromosome, three antimicrobial resistant plasmids and a virulent plasmid. The antimicrobial resistant plasmid expressing *bla*$_{KPC-2}$, *bla*$_{CTX-M-15}$, *aph(3")-Ib*, *aph(6)-Id*, *qnrB1*, *aac(3)-IIa*, *aac(6')-Ib-cr*, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$, *catB4*, *sul2*, *dfrA14* and *bla*$_{SHV-99}$ in approximately all isolates (90-100%). If large-scale horizontal transmission is possible, *kpc2, and kfu* strains can become highly resistant to antimicrobials. Isolates expressed the *bla*$_{CTX-M}$ gene, suggesting that this high carrier rate might facilitate horizontal transmission and lead to the formation of a highly resistant hvKP strain. Regardless, both mechanisms can result in a widespread, therefore, effective control measures are critical. The differences in virulence gene percentage may belongs to the site of infection in which sample was taken (35, 36).

Agarose gel electrophoresis image of *MagA, K2A* and *Carbapenenmase* showed that the multiplex PCR product of all genes were negative. It was reported that *magA* was characteristic of the K1 capsular operon, which was associated with the hypermucoviscosity phenotype of *K. pneumoniae* (37). Our data were not agreed with Al-Obadi (2014) (38) showed that K. pneumoniae isolates was diagnosed molecularly by using polymerase chain reaction using multiplex PCR. Results showed that all the isolates of K. pneumoniae gave a clear band with a molecular size 130 bp when PCR was performed with the primer that target the 16S rRNA. When using the primer specific for the capsule cluster gene magA and k2A, the result revealed that 23 (57.5 %) isolates belong to K1 serotype and 11 (27.5 %) isolates belong to K2 serotype. These results of Zedan et al., (2013) (39) suggested that magA and k2A genotype might be a useful marker to identify K1 and K2 serotypes of K. pneumoniae and these serotypes have been more prevalent than those that were neither K1 nor K2 (Non-K1/K2) isolates (15 %) in Iraq and this may belongs to methods of analysis in each study.

But our data were in agreement with Remya, Shanthi, and Sekar, (2019) indicated that in isolates of *K. pneumoniae* isolated from both hospitalised covid-19 patients and patients attending clinics using polymerase chain reaction (PCR) was carried out for the detection of various virulence genes such as mucoviscosity-associated gene A (*magA*), gene associated with allantoin metabolism (*allS*), Klebsiella ferric iron uptake(*Kfu*), capsule-associated gene A (*K2A*), regulator of mucoid phenotype A (*rmpA*), enterobactin (*entB*), yersiniabactin (*YbtS*), aerobactin, Fimbrial adhesin

(*FimH*) and uridine-diphosphate galacturonate 4-epimerase (*uge*), siderophore, *entB*, was present in most (90.5%) of the isolates, of the 370 isolates, 345 carried multiple virulence genes; 15 harboured single virulence genes and 10 did not harbour any of the studied virulence genes, the most common combination of occurrence was *entB* and *FimH*. A mortality rate of 12.75% (38/298) was observed among hospitalised patients, none of the virulence genes had any significant association with mortality (40). In agreement with their commensal behavior, none of the K. pneumoniae strains were positive for the two genes associated with invasive infections, including the mucoviscosity-associated gene magA and the regulator of mucoid phenotype rmpA (41-43). The negative results of magA may interrupted as that *magA* usually detected in a vast majority of *K. pneumoniae* liver abscess isolates not in oral or nasal swabs and often associated with hypermucoviscosity (HV) which is resistance to killing by human serum and phagocytosis, could be used as a diagnostic tool, it was believed that *magA* gene is exclusivly limited to liver abscess and HV positive phenotypes (44).

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