Article presented at 1st Int Conf Med Sci of North Technical University /Kirkuk, Iraq (1st ICMS 2023)

Original Article

Detection of Antibiotic Susceptibility Pattern and Biofilm Production in Multidrug Resistance *Klebsiella pneumoniae* Isolated from COVID-19 Patients in Kirkuk City

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Abstract

Background: The spread of multidrug-resistant pathogens is a serious problem and challenge for the whole medical community. Multidrug resistance *Klebsiella pneumoniae* (MDRKP) infections in COVID-19 patients have a severe course and may be fatal. Increasingly, these bacteria show resistance to more than one antibiotic category, which have been used to decrease the risk and severity of secondary bacterial infection caused by this pathogen. **Aim:** The study aimed to determine the prevalence of MDRKP among COVID-19 patients and antibiotic susceptibility pattern with biofilm formation of isolate. **Materials and Methods:** A cross-sectional study was done on 330 sputum sample collected from five hospitals and cultured. Antibiotic resistance pattern of *K. pneumoniae* isolates and their molecular characterization were studied using disk diffusion, vitek-2 AST-N222. The biofilm production was detected by microtiter plate method (MTP). **Results:** Of 49 isolates from 330 patients, 20 identified as *K. pneumoniae*; all *K. pneumoniae* isolates are MDR had varying levels of resistance to antibiotics 20 (100%) for ampicillin, 18 (90%) piperacillin, 16 (80%) for cefazolin, ceftazidime and cefepime, ceftriaxone, aztreonam, 7 (35%) for tetracycline, and 3 (15%), 2 (10%) for ciprofloxacin and levofloxacin, respectively. MTP method showed that eight (40%) of isolates were biofilm producers, and 60% of isolates were nonbiofilm producers and had more resistance than biofilm producers. **Conclusion:** MDRKP was prevalent among gram-negative bacteria isolated from COVID-19 patients and cause increased fatality rate.

Keywords: Antibiotic sensitivity test, biofilm, COVID-19, *K. pneumonia*, multidrug resistance

Introduction

Coronavirus disease-2019 (COVID-19) is a new emerging disease in human population. On March 11, 2020, the World Health Organization (WHO) declared COVID-19 to be a pandemic. A virus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the culprit behind the illness.[1,2] The alveolar epithelium's ciliated cells are frequently impacted by the SARS-CoV-2 virus, which inhibits their typical functions including clearing airways. Acute respiratory distress syndrome is caused by a subsequent, slow buildup of fluid, and leftovers in the lung. For intensive care and mechanical breathing, certain sick patients require admission in the intensive care unit (ICU).[3] Pulmonary infiltration and mechanical ventilation predispose patients with COVID-19 in ICU to secondary bacterial infections. Previous research found that 40%–86% of COVID-19 patients on mechanical ventilation developed ventilator-associated pneumonia (VAP). The frequency of VAP among hospitalized patients varies depending on their clinical circumstances, ICU admission regulations, and therapy kinds.[1,4] The most common gram-negative microorganisms are *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.[2] However, there is limited information about microorganisms

Access this article online

Quick Response Code:

Website: https://journals.lww.com/mjby

DOI: 10.4103/MJBL.MJBL_195_22

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Submission: 24-Aug-2022 **Accepted:** 03-Sep-2022 **Published:** 29-Mar-2024

How to cite this article: Sader AL-Deen DI, Obaid SS, Jasim WM. Detection of antibiotic susceptibility pattern and biofilm production in multidrug resistance *Klebsiella pneumoniae* isolated from COVID-19 patients in Kirkuk City. Med J Babylon 2023;20:S154-9.

that cause pneumonia among patients with COVID-19.[5] The bacteria *K. pneumoniae* are non-motile, facultative anaerobic bacilli and typically are 3–6 m long and up to 1.0 m wide. The *Klebsiella* strains that are encapsulated are known to create mucoid colonies.[6] Due to its potential to develop mechanisms of antimicrobial resistance leading to pandrug resistance and generate outbreaks that typically involve multiple medical facilities, it has lately emerged as a significant source of hospital-acquired infections.[7] *K. pneumoniae* has the capability to create a biofilm, an extracellular matrix made of proteins, exopolysaccharides, DNA, and lipopeptides that shields bacteria against antibiotics.[8,9] Biofilm promotes bacterial adhesion to both living and nonliving surfaces, hinders antibiotic penetration, and lessens antibiotic effects.[10] Due to their physical separation from blood circulation, their flexibility of adaptation to oxygen deprivation and pH changes, and the inability of antibiotics to completely penetrate the biofilm, these bacteria are largely resistant to antibiotics.[11] Furthermore, *K. pneumoniae* possesses virulence factors such as capsular polysaccharides, lipopolysaccharides, types 1 and 3 fimbriae, outer membrane proteins, and factors influencing iron acquisition and nitrogen supply, which it exploits for survival and evasion of the body's immune system.[12,13] Evaluation of bacterial genetic diversity aids in determining the most frequent bacterial strains in a specific setting, causes of illnesses, effective preventative strategies, and infection control plans for that setting.[14,15] The most major pathogen of this genus is *K. pneumoniae*, which accounts for approximately 86% of all *Klebsiella*-related human illnesses.[16] The study aimed to determine the prevalence of MDR *K. pneumoniae* among COVID-19 patients and antibiotic susceptibility pattern with biofilm formation of isolate.

Materials and Methods

Study design

A cross-sectional study was conducted on COVID-19 patients after receiving official agreements before establishing the study.

Study setting and period

The study was carried out in Kirkuk city on five main hospital (AL-Shifia 14 hospital, Kirkuk General Hospital, Azadi Teaching Hospital, AL-Naser Hospital, AND Tuberculosis Center). The study was conducted from 10 December 10, 2021 to March 31, 2022.

Study sample and sampling method

The sputum sample was collected among 330 COVID-19 patients according to following diagnostic criteria which had to be presented 48 h or more after admission:

1. Symptomatic patients (having one or more of the following; fever, sore throat, cough, and shortness of breath) with positive pulmonary computed tomography (CT) findings.
2. Patients had history of recent contact with confirmed COVID-19-infected patients and with positive pulmonary CT findings.
3. Available polymerase chain reaction (PCR) results for all patients whether the result is positive or negative.[17]

When three diagnostic criteria were met, sputum sample was taken to identify the etiological agent responsible for the possible superinfection. In the cultures, the isolation of a single or predominant bacterium was considered positive. the isolation was identified using the VITEK-2 GN card (bioMerieux 8.01).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the disk diffusion and Vitek-2 AST-N222 method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[18] The antibiotics tested included ampicillin (AM), amoxicillin-clavulanate (AX), cefazolin (CZ), ceftriaxone (CTX), Tetracycline (TE), and levofloxacin (LEV) (Liofilchem, Italy). Antibiotic sensitivity test number (AST-N222) cards included antimicrobial agents as follows: Ticarcillin (TIC), Piperacillin (PRL), Piperacillin/tazobactam (PTZ), Ceftazidime (CAZ), Cefepime (CPM), Aztreonam (AZT), Imipenem (IMI), Meropenem (MEM), Amikacin (AK), Gentamicin (GM), Tobramycin (TM), Ciprofloxacin (CIP), Minocycline (MI), and Trimethoprime/sulphamethoxazole (TS).

Biofilm detection

Sterile 96-well flatbottomed sterile polystyrene microplate well containing180 µL of Mueller–Hinton broth supplemented with 1% glucose were inoculated with 20 µL from suspended bacterium of 0.5–0.7 McFarland (1.108 cfu/mL). Microplates are incubated 24 h at 37°C. The liquid media was discarded, and the adherent cells were washed twice with phosphate buffered saline (PBS) and wells are dried at 60°C for 1 h or less. After that it was stained with 150 µL of 2% of crystal violet for 15 min. Then the crystal violet–stained wells of microplates were washed twice with PBS to discharge crystal violet stain. After air drying process of wells of microplate, dye of biofilms that lined the walls of the microplate is re-solubilized by 150 µL of 95% ethanol. After 5–10 min, microplate is measured spectrophotometrically at 570 nm by a microplate reader. The assay was done at least three times using fresh samples each time.[19] The optical density cut-of value (ODc) was established as three standard deviation (SD) above the mean of the optical density (OD) of the negative control as shown in the following formula: ODc = average OD of negative control + (3 × SD of negative control).

The results were divided into four categories according to their optical densities as follows:

1. strong biofilm producer (4 × ODc< OD)
2. medium biofilm producer (2 × ODc< OD ≤ 4 × ODc)
3. weak biofilm producer (ODc< OD ≤ 2 × ODc)
4. nonbiofilm producer (OD ≤ ODc)

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 4245 (including the number and the date in 9/12/2021) to get this approval.

Table 1: Distribution of bacterial superinfection among patients with COVID-19

Bacterial superinfection	Number (n = 49)	%
Klebsiella pneumoniae	20	41
Staphylococcus aureus	13	27
Escherichia coli	11	22
Acinetobacter baumannii	3	6
Enterobacter cloacae	1	2
Serratia marcescens	1	2
Total	49	100

Table 2: Distribution of COVID-19 patients infected with *Klebsiella pneumoniae* according to sex and age

Sex and age		Number	%
Sex	Male	15	75
	Female	5	25
Age	≥60	16	80
	<60	4	20

Results

From a total of 330 COVID-19 patients, the prevalence of bacterial were 49 (14.8%), identifying six different species of bacteria, with *Klebsiella pneumoniae* and *Staphylococcus aureus* being the most frequent (41%, 27%), respectively [Table 1].

Distribution of COVID-19 patients infected with *K. pneumoniae* according to age and sex

The study revealed that 75% of COVID-19 patients infected with *K. pneumoniae* were males and 25% females. In the current study, it was found that 80% of *K. pneumoniae* were isolated from patients their age ≥ 60 and only 20% from patients younger than 60 as described in Table 2.

Antibiotic Susceptibility Testing (AST)

The result obtained from the antibiotic sensitivity test (by both Disk diffusion method and Vitek-2 system AST-N222) of *K. pneumoniae* isolates in our study showed varied levels of resistance rate to antibiotic, with a highest resistance rate reached to 20 (100%) for ampicillin, 18 (90%) piperacillin, 16 (80%) for cefazolin, ceftazidime and cefepime, ceftriaxone, aztreonam, 15 (75%) for gentamicin, 14 (70%) for minocycline, ticarcillin and tobramycin, 13(65%) for amoxicillin /clavulanicacid, 12 (60%) for amikacin, piperacillin/tazobactam and imipenem, 11 (55%) for trimethoprim/sulfamethoxazole and meropenem, then moderate resistance rate reached to 8 (40%) for tetracycline, and recorded lowest resistance to ciprofloxacin and levofloxacin reached to 3 (15%), and 2(10%), respectively. The percentage resistance toward each antibiotic is shown in Table 3.

Classification of resistance among *K. pneumoniae* isolates

The study shows that 55% of isolate were MDR, 10% were MDR possible XDR, 20% were XDR, 20% were XDR possible PDR, and finally 5% of isolates were PDR, as shown in Table 4 .

Table 3: Antimicrobial sensitivity test results of *Klebsiella pneumoniae* isolates

Antimicrobial agent	Resistance (%) (n = 20)	Sensitive (%) (n = 20)	Antimicrobial agents	Resistance (%) (n = 20)	Sensitive (%) (n = 20)
Ampicillin	20(100)	0	Tobramycin	14(70)	6(30)
Piperacillin	18(90)	2(10)	Amoxicillin /clavulanic acid	13(65)	7(35)
Cefazolin	16(80)	4(20)	Amikacin	12(60)	8(40)
Ceftriaxone.	16(80)	4(20)	Piperacillin/ tazobactam	12(60)	8(40)
Ceftazidime	16(80)	4(20)	Imipenem	12(60)	8(40)
Cefepime	16(80)	4(20)	Trimethoprime/sulphamethoxazole	11(55)	9(45)
Aztreonam	16(80)	4(20)	Meropenem	11(55)	9(45)
Gentamicin	15(75)	5(25)	Tetracycline	8(40)	12(60)
Minocycline	14(70)	6(30)	Ciprofloxacin	3(15)	17(85)
Ticarcillin	14(70)	6(30)	Levofloxacin	2(10)	18(90)

Table 4: Classification of 39 *Klebsiella pneumoniae* isolates into MDR, XDR, and PDR

Pattern	A	B	C	D	E	F	G	H	I	J	K	Classification
Kp1												MDR
Kp2												MDR
Kp3												MDR
Kp4												XDR
Kp5												MDR possible XDR
Kp6												MDR
Kp7												MDR
Kp8												XDR possible PDR
Kp9												XDR
Kp10												MDR
Kp11												MDR
Kp12												PDR
Kp13												MDR possible XDR
Kp14												MDR
Kp15												XDR
Kp16												XDR
Kp17												MDR
Kp18												XDR possible PDR
Kp19												MDR
Kp20												MDR
The isolates were sensitive to all agents listed in the category												
The isolates were resistant to some, but not all agents listed in the category												
The isolates were resistant to all agents listed in the category												

Antibiotic categories:- A: Aminoglycosides B: Antipseudomonal penicillins+*β*-lactamase inhibitors, C: Carbapenems, D: Non-extended spectrum cephalosporins; 1st and2nd generation cephalosporins, E: Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins, F: Fluoroquinolones, G: Folate pathway inhibitors, H: Monobactams, I:Penicillins, J: Penicillins+*β*-lactamase inhibitors K:Tetracyclines.

Table 5: Results of microtiter plate method method

Isolate	Biofilm former	Nonbiofilm former
Klebsiella pneumonia	8(40%)	12(60%)
Method	Weak biofilm	Moderate biofilm
Microtiter plate	4(20%)	4(20%)

Detection of biofilm formation of *Klebsiella pneumoniae* isolate by microtiter plate method

Biofilm formation in bacterial cells is complex with an adhesion capability to different surfaces, including plastics, metals, medical implant materials, and tissues. Biofilm formation enhances the survival of microorganisms, such as bacteria, and strengthens them against damage.[20]

The result shown that from all isolates (n = 20), 8 (40%) were biofilm former and 12 (60%) did not produce biofilm. Biofilm former strain of *Klebsiella pneumoniae* involves the following (which are shown in Table 5): of all *K. pneumoniae*, there is no strong biofilm former this study. However, the rate of moderate biofilm former is 4(20%) and 4(20%) was weak biofilm former.

Discussion

The prevalence of *K. pneumoniae* among gram-negative and gram-positive bacteria isolated from COVID-19 patients in our study is 41% and this is compatible with previous study,[21] in which the prevalence of infected *K. pneumoniae* in COVID-19 patients was 56%. *K. pneumoniae* is one of the most common pathogens contributing to VAP in ICU in the United States and Middle East countries like Iran.[22] All bacterium that infects the lung isolated from sputum causes ventilator-associated bacterial pneumonia.

In this study, it was found that the men appeared to be more infected than women; this result was compatible with result obtained byAli *et al.*,[23] in which the the male-to-female ratio was 74.3%:25.6%. But our finding is incompatible with the study by Ayatollahi *et al*.,[24] in which the results of study showed that 22 (29.3%) were males and 53 (70.7%) were females. Most of the *K. pneumoniae* isolates in this study were collected from patients whose age was over 60 years and this was in line with the study by Ghanizadeh *et al.*[25] which found that 71.4% of isolates were from the age group of over 60 years. This result was disagree with a study done by Khairy *et al.*[26] which found that all *K. pneumoniae* isolated from sputum sample obtain from patients age less than 57 years.

The results of antimicrobial susceptibility test of *K. pneumoniae* in this study were consistent with other results as the study done by,[27] which showed that the resistance rate of *K. pneumoniae* isolates toward the antimicrobials was 92.2% to piperacillin. A study done by[28] noticed that the highest resistance for *K. pneumoniae*

was 100% to ampicillin, ceftazidime, ceftriaxone, and cefepime followed by tobramycin (50%). A study done by[29] showed thatthe antimicrobial resistance patterns of isolates was 100% resistance for ampicillin, 87.6% for cefazolin, 85.71% for ceftazidime, cefepime, and ceftriaxone, 66.07% for gentamicin, and 57.14% for tobramycin. Low sensitivity toward amoxicillin/clavulanic acid, imipenem, and meropenem were reported as 65%, 55%, and 50%, which is compatible with study by[30] which recorded that resistance rate against amoxicillin/clavulanic acid, imipenem, and meropenem were 69%, 49%, and 40%, respectively. The resistance rate against ciprofloxacin in our study was 15% and this result was in line with[31] which revealed 24.3% of isolated *K. pneumonia* were resistance to ciprofloxacin.

K. pneumoniae strains are naturally resistant to ampicillin because of production of a chromosomal penicillinase.[32] Higher resistant to penicillin antibiotics may be due to mutation of gene-coding penicillin binding proteins (PBPs)[33] or due to production of β-lactamase such as ESBL mainly SHV-1 beta-lactamase besides OXA and TEM β-lactamase.

It was clear that tetracyclines, fluoroquinolones class still effective against isolates rather than other classes, this finding was also supported by others studies as the study done by[34] who obtained the resistance rate of *K. pneumoniae* to ciprofloxacin and tetracycline by (37.5%, and 31%), respectively.

The isolated strain of *K. pneumoniae* show various degree of resistance toward different category of antimicrobial agent. These resistant are classify as follow: MDRKp isolates mean isolate resist to at least one antimicrobial drug in three or more antimicrobial categories while extensive drug resistance (XDR) mean non-susceptibility of one bacteria species to all antimicrobial agents except in two or less antimicrobial categories. Within XDR, pandrug-resistant (PDR) is the non-susceptibility of bacteria to all antimicrobial agents in all antimicrobial categories. results in present study showed that about all 20 (100%) *K. pneumoniae* isolates were multidrug resistant isolates (MDR). Also, study done by[35] observed that ≥80% of *K. pneumoniae* isolates were resistant to third generation cephalosporin's and other antibiotics, making them multidrug resistant (MDR) strains. Consequently, it was necessary to classify these isolates into five categories including (MDR, MDR possible XDR, XDR, XDR possible PDR and PDR). This classification was applied in twenty different antibiotics from different category tested on strain from *Kp1* to *Kp20* (isolates showing intermediate levels of susceptibility were classified as resistant),[18] to define each isolate according to its resistance ability. Classification was done according to the criteria revealed by.[36] Consequently, all of 20 *K. pneumoniae* clinical isolates were classified into MDR in first place, but some of them were converted to XDR or even PDR depending on their resistance profile as presented in Table 4 . 11 isolates (55%) were classified as MDR since they were resistant to ≥3 out of all antimicrobial categories that match the criteria used, which including: *Kp1, Kp2, Kp3, Kp6, Kp7, Kp10, Kp11, Kp14, Kp17, Kp19, Kp20*. Two isolates (10%) were considered as MDR possible XDR since they were resistant to 9/11 antimicrobial categories, so there was a highly probability to be XDR, which included *Kp5*, and *Kp13* isolate. Four isolates (20%) of *K. pneumoniae* isolates were considered as XDR since they were susceptible to only 2 out of all antimicrobial categories that match the criteria used. Therefore, XDR defined as non-susceptibility to at least one agent in all but remain susceptible to only one or two antimicrobial categories, that include strain *Kp4, Kp9, Kp15, Kp16*. Two isolates 2(10%) of *K. pneumoniae* including *Kp8, Kp18* being resistant to all antimicrobial categories but sensitive to some antibiotic within one category or sensitive to all drug within one category, so classify as XDR possible PDR.

Finally, one isolates (5%) *K. pneumoniae* isolates, being resistant to all antimicrobial categories that match the criteria used were considered as PDR including *Kp12*. Thus, PDR was defined as non-susceptibility to all agents in all antimicrobial categories for *Enterobacteriaceae*. Study done by[37] revealed that from the total of 223 nonrepetitive patients who had *K. pneumoniae* isolates, among which, 137 (61.4%) were MDR isolates, 49 (22.0%) were XDR isolates, 4 (1.8%) were PDR isolates, and 33 (14.8%) were other types of isolates.

About the biofilm formation, tissue culture plate method (TCP) was found to be a method with good reproducibility and specificity which can be used routinely in the microbiology laboratory to detect biofilm formation.[38] The result obtain from our study is in line with previous study done by[39] that documented strong biofilm were not detected, 22% of isolates were characterized by average biofilm formation, 22% of isolates produced small biofilm, and 56% of isolates did not form biofilm. For comparison, the study by[40] encompassing a collection of 168 hospital isolates of *K. pneumoniae,* showed significant biofilm formation in 69% of isolates, weak biofilm production in 20.3% of isolates, whereas 10.1% of isolates did not produce biofilm at all. Non-biofilm producers were more resistant than biofilm producers indicating that resistance may be related to other mechanisms than biofilm such as efflux pumps, ESBL genes. and others. In conclusion MDR *K. pneumoniae* were prevalence among gram-negative bacteria isolated from COVID-19 patients and cause increased fatality rate.

Financial support and sponsorship
Not applicable.

Conflicts of interest
There are no conflicts of interest.

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