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Detection of some β-lactamase Genes in Klebsiella pneumoniae Isolated from some Baghdad Hospitals, Iraq

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ABSTRACT: ***Background:*** *Multi-Drug-ResistantKlebsiella pneumoniae (MDR K. pneumoniae) is considered as an important opportunistic pathogen, which causes life threatening infections. K. pneumoniae is known to causes life-threatening infections.* ***Objective:*** *The objective of this study is to detect some β-lactamase genes inKlebsiella pneumoniae.* ***Methods:*** *Thirty-five K. pneumoniae isolates were obtained from some hospitals in Baghdad City between October 2022 and February 2023. The identification of K. pneumoniae isolates was done phenotypically by the automated VITEK II system and genotypically by amplification of the rpob gene. The antibiotic susceptibility and detection of some β-lactamase genes were tested for all isolates.* ***Results:*** *The results showed that the K. pneumoniae isolates were resistant to most antibiotics used. A high percentage of K. pneumoniae isolates were resistant to cefixime, cefpodoxime, norfloxacin and doxycycline as 100% followed by ceftriaxone, ceftazidime, ticarcillin/clavulanate, ticarcillin, ceftazidime, tobramycin, and moxifloxacin (97.2%, 91.7%, 91.7%, 94.4%, 94.4%, and 94.4%, respectively). Furthermore, approximately 94.4% of the isolates were MDR. In addition, the molecular methods only detected the bla_{NDM} and bla_{TEM} genes as 33.3% and 75.6% of in the K. pneumoniae isolates, respectively, while the bla_{VIM}, bla_{CTX-M}, and bla_{KPC} genes were not observed in K. pneumoniae isolates.* ***Conclusions:*** *β-lactam antibiotics, including carbapenems, are widely used to treat bacterial infections; however, an increase in antibiotic resistance due to β-lactamases limits the effectiveness of these antibiotics. Therefore, alternative treatment methods are required to control these resistant isolates.*

KEYWORDS: *Klebsiella pneumoniae; MDR; β-lactamase; bla_{NDM}; bla_{TEM}*

INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) bacterium belongs to the Enterobacteriaceae family, which is Gram-negative, non-motile, encapsulated, and facultative anaerobic [1] . Klebsiella species are abundant in nature and usually occur in water, soil, and other surfaces. In addition, this bacterium can colonize various mucosal surfaces such as the gut and the upper respiratory tract in humans [2]. *K. pneumoniae* has many different virulence factors such as, capsule, which gives to Klebsiella mucoid appearance on culture medium, lipopolysaccharide, fimbriae type1 and type 3 , outer membrane protein, and many determinants for iron acquisition. Thus, *K. pneumoniae* uses these virulence factors to evade and survive the immune system during infections [3]. These bacteria are opportunistic pathogens, can enter the circulation and tissue and cause many infections including bacteremia, septicemia, urinary tract infections and pneumonia. Furthermore, *K. pneumoniae* is the main cause of nosocomial infections [4]. The transmission of these bacteria in hospitals occurs by different sources: person-to-person or contact occurs between patients and healthcare workers,

contaminated surfaces, and hospital instruments as a source of transmission [5]. Thus, *K. pneumoniae* is considered the main cause of hospital-acquired infections and the biggest challenge for global health.

The extensive misuse of antibiotics plays has a major role in emergence of antibiotic resistance. This leads to the prevalence of infections caused by MDR and XDR pathogens, especially those belonging to the Enterobacteriaceae family in hospitals. The infections caused by these bacteria are associated with high mortality rates and prolonged staying in hospitals [6]. Studies have shown an increase of antimicrobial resistance of *K. pneumoniae* to fluoroquinolones, aminoglycosides, cephalosporins and carbapenems [7], [8]. This is due to the intrinsic and acquired resistance of these bacteria to a broad spectrum of drugs, such as β-lactams [9]. These bacteria have developed many mechanisms to resist many groups of antibiotics such as producing β-lactamases. These enzymes can hydrolyze antibiotics that belong to β-lactam antibiotics which affect the usage of these antimicrobials [10]. Carbapenem is considered the last treatment option for infections caused by MDR Gram-negative bacteria. Many studies have reported an increase in carbapenem resistance worldwide [11]. The genes encoding these enzymes are commonly borne on mobile genetic components such as plasmids. In addition, these plasmids can carry genes that confer resistance to other classes of antibiotics such as fluoroquinolones, chloramphenicol, aminoglycosides, and trimethoprim /sulfamethoxazole [12]. This will lead to the spread of MDR strains and infections caused by these strains will be hard to eradicate leading to treatment failure and death. Thus, this study aimed to detect some β-lactamase genes in *Klebsiella pneumoniae* isolated from some Baghdad hospitals in Iraq.

MATERIALS AND METHODS

K. pneumoniae Isolation

Atotal of thirty five of *K. pneumoniae* isolates were obtained from some hospitals in Baghdad city as the following: 10 isolates from Al Karkh General Hospital, 8 isolates from Children's Hospital, 12 isolates from Teaching Laboratories of Medical city, and 5 isolates from Baghdad Teaching Hospital for the period from October 2022 to February 2023. The source of these isolates was divided into 16 isolates from urine, 4 from sputum, 4 from wound, 1 from Foley tip, 1 from pus of kidney, 3 from Endotracheal tube, and 6 from blood.

K. pneumoniae Identification

Primary isolation and phenotypic identification of the isolates were done using blood and MacConkey agar plates. VITEK II compact system (Biomerieux/France) was used to identify the isolates to species level . Finally, housekeeping gene β subunit of RNA polymerase (*rpob*) was used to confirm the identification of isolates using PCR-related techniques. The boiling method was used to isolate genomic DNA as following: For each isolate, ten single colonies were added to 400 µL ddH2O using Eppendorf tubes. For cell lysis, the samples were kept at 100ºC water bath for 10 min followed by cooling the tubes on ice immediately. Next, the tubes were frozen at -20°C for 20 min and left to thaw at room temperature. In the following step, the samples were homogenized by vortex for 10 seconds and centrifuged at 14000 rpm at 4°C for 15 min. Finally, the upper aqueous layer was transferred into new sterile Eppendorf tubes [13]. All DNA samples were stored at -20°C until been used. The PCR reaction was prepared at a total volume of 25 µL as the following: 12.5 µL Promega Master mix, 1 µL forward primer; 1 µL reverse primer, 5.5 µL nuclease-free distilled water and 5 µL DNA template.

Antibiotic Susceptibility Test

The antibiotic susceptibility test was performed by the Kirby-Bauer method for different antibiotics including Ticarcillin (75 µg), ticarcillin/clavulanate (75/10 µg) , piperacillin (30 µg), piperacillin / tazobactam (100/10 µg), cefixime (5 µg),cefpodoxime(10 µg), ceftazidime (30 µg),ceftriaxone (30 µg), cefepime (30 µg), aztreonam (15 µg),ertapenem (10 µg), imipenem (10 µg),meropenem (10 µg), tobramycin(10 µg), netilmicin (30 µg), levofloxacin (5 µg), moxifloxacin (5 µg), norfloxacin (10 µg), ofloxacin5 µg), pefloxacin(5 µg), doxycycline(30 µg), minocycline (30 µg), tetracycline(30 µg),colistin (10 µg),Trime-thoprim/sulfamethoxazole (1.25/23.75 µg), amikacin (30 µg), and gentamicin (10 µg)(Bioanalyse, LTD, Turkey). A few single colonies of each isolate were added to 5 ml of normal saline to prepare a bacterial suspension equal to 0.5 McFarland standards (1.5×10 CFU/ml) suspension. The swab was merged in this suspension and used to spread on Mueller Hinton agar medium. The plates were left for 10 min after which the antimicrobial discs were placed on the the agar and pushed firmly.

The plates were incubated at 37ºC for 24h. After incubation, the inhibition zone around each disc was measured in millimetres (mm) using a metric ruler and the results were interpreted according to Clinical Laboratories Standards Institute (CLSI, 2022).

Antibiotic Resistant Genes Detection by PCR

The PCR was used to detect antibiotic resistance genes (bla_{CTX-M}, bla_{KPC}, bla_{NDM}, bla_{TEM} and bla_{VIM}) in clinical isolates of *K. pneumoniae*. Uniplex and multiplex PCR were used to detect these genes. Table 1, Table 2 , and Table 3 show the PCR Mixture, PCR primers, and PCR steps, respectively. Gel(1%) electrophoresis was used to visualize DNA fragments using Invitrogen RedSafe dye (Thermo Fisher Scientific Inc. US) under a UV transilluminator documentation system. DNA bands were determined using DNA marker (100bp, Cleaver Scientific/UK).

Table 1. PCR components for multiplex and uniplex PCR

PCR reaction material	Amount per microliter	PCR reaction material	Amount per microliter	PCR reaction material	Amount per microliter
Master mix	12.5	Master mix	12.5	Master mix	12.5
DNA template	5	DNA template	5	DNA template	5
forward primer bla_{TEM}	1	forward primer bla_{VIM}	1	forward primer bla_{KPC}	1
reverse primer bla_{TEM}	1	reverse primer bla_{VIM}	1	reverse primer bla_{KPC}	1
forward primer bla_{CTX-M}	1	forward primer bla_{NDM}	1	——	—
reverse primer bla_{CTX-M}	1	reverse primer bla_{NDM}	1	——	—
Free nuclease distilled water	3.5	Free nuclease distilled water	3.5	Free nuclease distilled water	5.5
Total	25	Total	25	Total	25

Table 2. All the oligonucleotides used during this study

Gene	Primer sequence 5' —— 3'	PCR product(bp)	Annealing temperature	Reference
bla_{CTX-M}	F5'ACGCTGTTGTTAGGAAGTG 3' R5'TTGAGGCTGGGTGAAGT 3	759	94°C	[14]
bla_{KPC}	F5' CATTCAAGGGCTTTCTTGCTGC 3' R5'ACGACGGCATAGTCATTTGC3'	538	55 °C	[15]
bla_{NDM}	F5' GGTTTGGCGATCTGGTTTTC3' R5'CGGAATGGCTCATCACGATC3'	621	55 °C	[16]
bla_{TEM}	F5' CTCACCCAGAAACGCTGGTG3' R5'ATCCGCCTCCATCCAGTCTA3'	569	58 °C	[17]
bla_{VIM}	F5'GATGGTGTTTGGTCGCATA 3' R5' CGAATGCGCAGCACCAG3'	390	55 °C	[15]
Rpob	F5'GTTGGCGAAATGGCGGAAAAC 3' R5' ACGTCCATGTAGTCAACCTGG3'	599	57	[18]

Table 3. Stages of PCR used in this study

Stages	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	5 min	1
Denaturation	95	30s	30
Annealing	—[1]	30s	30
Extension	72	—[2]	30
Final extension	72	5 min	30
Hold	10	(∞)	

[1] Annealing temperature changes occasionally depending on TM of primers.
[2] Extension time depends on PCR product size; 1 min for 1 kb.

RESULTS AND DISCUSSION

K. pneumoniae Isolation and Identification

Thirty-five *K. pneumoniae* isolates were obtained in this study. Primary identification was performed on MacConkey agar, which *K. pneumoniae* colonies appeared as mucoid, large, and pink because of the ability of these isolates to ferment lactose present in this medium as shown in Figure 1 A. Furthermore, CHROM agar was used as a differential medium for *K. pneumoniae* isolates, and the isolate colonies were blue on this medium, Figure 1 B.

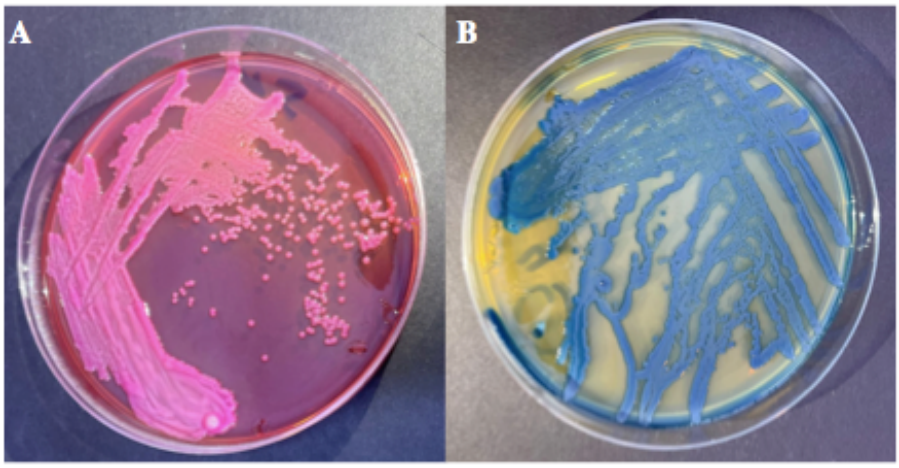

Figure 1. Phenotypic identification of *K. pneumoniae* isolates on Macckoncy and Chrom agar plates. (A) *K. pneumoniae* isolates showed pink colonies on MacConkey agar (B) *K. pneumoniae* colonies appeared as blue on CHROM agar

Automated VITEK II system was used to confirm the identification of these isolates. Furthermore, amplification of the *rpob* gene was used as molecular identification of the isolates . The *rpob* gene was suggested as an excellent tool for identification of *K. pneumoniae* isolates, which encodes a β-subunit of RNA polymerase and [19] . Thus, PCR products (599bp) of this gene confirmed the identification of the isolates in this study as in Figure 2.

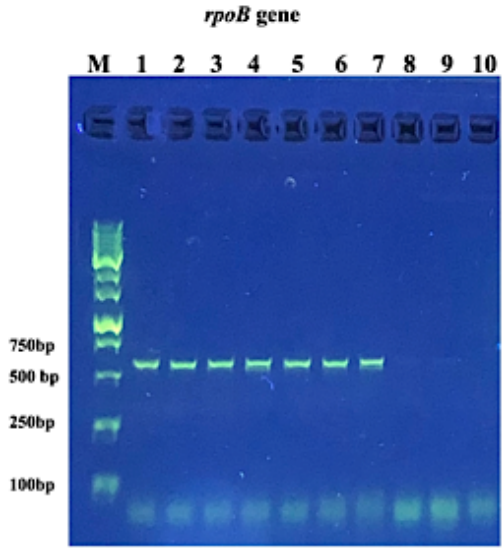

Figure 2. PCR for *K. pneumoniae* isolate identification. *rpob* gene (599bp) was amplified to identify these isolates. The electrophoresis using 1% agarose was done at 100 V for 45min and the DNA bands were visualized under UV light using a gel documentation system. M: 100bp DNA marker, 1-7: *K. pneumoniae* isolates, and 8 : negative control

Antibiotic Resistance Profile

In this study, the isolates of *K. pneumoniae* showed resistance to the most antibiotics used. A high resistance rate of the isolates was shown for cefixime, cefpodoxime, norfloxacin and doxycycline as 100% followed by ceftriaxone, ceftazidime, ticarcillin/clavulanate, ticarcillin, ceftazidime, tobramycin, and moxifloxacin as 97.2%, 91.7, 91.7%, 94.4%, 94.4%, and 94.4%, respectively. In addition, a variable resistance against other antibiotics of these isolates was observed as shown in Table 3 .

Table 4. percentage of antibiotics resistance of *K. pneumoniae* isolates

Antibiotics name	Percentage of resistance %	Antibiotics name	Percentage of resistance %
Ticarcillin	94.4	Tobramycin	94.4
Ticarcillin/clavulanate	91.7	Netilmicin	0
Piperacillin	77.8	Levofloxacin	77.8
Piperacillin/tazobactam	80.6	Moxifloxacin	94.4
Cefixime	100.0	Norfloxacin	100.0
Cefpodoxime	100.0	Ofloxacin	0.0
Ceftazidime	91.7	Pefloxacin	0.0
Ceftriaxone	97.2	Doxycycline	100.0
Cefepime	0.0	Minocycline	72.2
Aztreonam	88.9	Tetracycline	0.0
Ertapenem	0.0	Colistin	0.0
Imipenem	52.8	Trimethoprim /sulfamethoxazole	80.6
Meropenem	77.8	Amikacin	55.6
		Gentamicin	75.0

The isolates of *K. pneumoniae* were (94.4%) MDR in this study, as these isolates were found resistant to most β-lactam antibiotics, carbapenem, cotrimoxazole, tetracycline and aminoglycosides. The spread of MDR isolates is responsible for serious infections and increasing morbidity and mortality. MDR K. pneumoniae is recognized as an urgent threat to public health by the World Health Organization (WHO), the Public Health England (PHE) and the US Centers for Disease Control and Prevention (CDC) [20] . Carbapenems are widely used to treat infections caused by multidrug resistant (MDR) Enterobacteriaceae [21] , including *K. pneumoniae*. However, the resistance against these antibiotic has emerged [22] . In this study, the isolates showed resistance against imipenem and meropenem as 52.8%, 77.8%, respectively and none of the isolates were resistant to ertapenem.

Detection of β-Lactamase Genes

In this study, we investigated the occurrence of some β-lactamase genes including (bla_{VIM}, bla_{NDM}, bla_{CTX-M}, bla_{TEM}, and bla_{KPC}) in *K. pneumoniae* isolates. Multiplex and uniplex PCR were used to detect these genes. The isolates showed that 33.3% and 75.6% had bla_{NDM} and bla_{TEM}, respectively, while non of these isolates showed any PCR products for bla_{VIM}, bla_{CTX-M} and bla_{KPC} as shown in Figure 3, 4, 5, Table S1.

Resistance phenotype-genotypic correlation was showed that all isolates with bla_{CTX-M}, and bla_{TEM} genes were non susceptible to most β-lactam. All isolates positive for bla_{NDM} were non-susceptible to all β-lactam except 2 isolates, which were sensitive to piperacillin. Furthermore, these isolates were non-susceptible to imipenem and meropenem. The extensive and misuse of β-lactams led to the emergence of resistance against these antibiotics. Many mechanisms are responsible for this resistance including target site modification (mutation or expression of alternative PBPs), and down regulation of porin, which results in a reduction in cell, efflux pump and modifying enzymes [23].

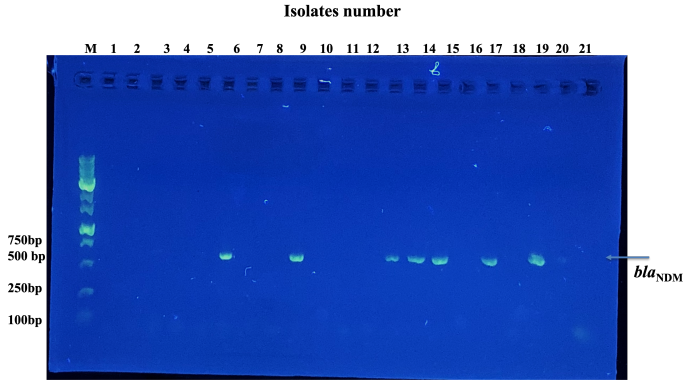

Figure 3. Multiplex PCR to detect bla_{VIM} (390bp) and bla_{NDM} (621bp) in clinical isolates of *K. pneumoniae*. The electrophoresis using 1% agarose was done at 100 V for 45 min and the bands were visualized under UV light using a gel documentation system. M: 100bpDNA marker, 1-21: *K. pneumoniae* isolates

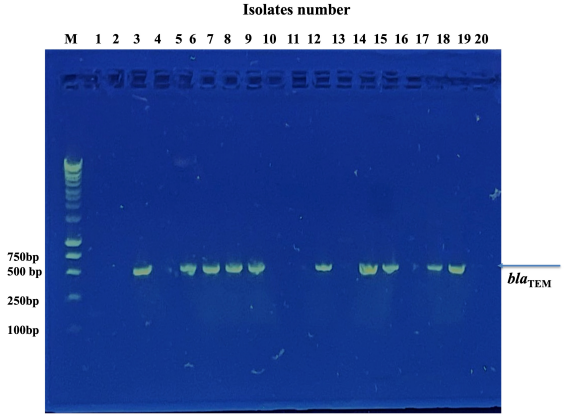

Figure 4. Multiplex PCR to detect bla_{CTX-M} and bla_{TEM} genes in *K. pneumoniae* isolates . PCR was used to amplify bla_{CTX-M} (759bp) and bla_{TEM} (569bp). The electrophoresis using 1% agarose was done at 100 V for 45 min and the bands were visualized under UV light using a gel documentation system. M: 100bpDNA marker, 1-20: *K. pneumoniae* isolates

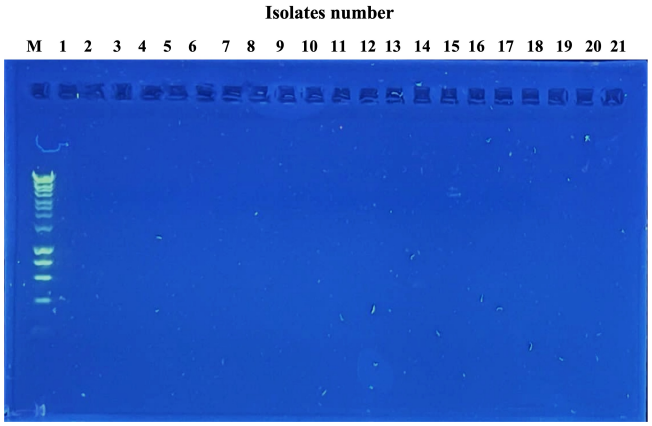

Figure 5. PCR to detect bla_{KPC} gene in of *K. pneumoniae* isolates. The electrophoresis using 1% agarose was done at 100 V for 45 min and the bands were visualized under UV light using a gel documentation system. M: 100bp DNA marker, 1-21: *K. pneumoniae* isolates

β- lactamases assist in enzyme-mediated resistance, which are produced by both Gram-positive and Gram-negative bacteria. Furthermore, these enzymes are encoded chromosomally or on extra-chromosomal elements, which degrade the β-lactam ring .TEM, SHV, and CTX β-lactamase genes presence in the bacteria results in β- lactam resistance (penicillins and cephalosporins). However, the presence of carbapenem resistance genes in these bacteria is crucial. Enterobacteriaceae producing

these enzymes cause serious infections and are resistant to antimicrobial treatment. In the US, KPC (Klebsiella pneumoniae Carbapenemase) is the most common and important resistance mechanism to carbapenem in Enterobacteriaceae. However, we could not find any isolate carrying bla_{KPC}, this may be because of the small number of samples, which limit our study. Thus, a large study scale is required to investigate these genes. Metallo-β-Lactamases: such as VIM, IMP and NDM are more common in Asia [11] as in this study, we found 33.3% and 75.6% of the isolates had bla_{NDM} and bla_{TEM}. VIM, IMP and NDM-1 belong to class B of lactamases. These enzymes can hydrolyze penicillins, cephalosporins, monobactams and carbapenems except aztreonam [24]. *Klebsiella pneumoniae* can carry multiple β-lactamase genes in the same strain, which assist in surviving this bacterium [25]. Out of the 35 isolates, 13 were found to contain both bla_{NDM} and bla_{TEM} in this study. Furthermore, one study reported combinations of all types of bla genes found in this bacterium. This may belong to an antibiotic resistance plasmid carrying an array of antibiotic resistance genes or due to acquisition of transposons containing different bla genes on the same plasmid [25].

CONCLUSION

Antibiotic resistance is considered as one of the biggest threats to public health. Different local studies suggest the rapid and uncontrolled spread of antibiotic resistant bacteria among Iraqi patients. These bacteria have developed many mechanisms to resist many groups of antibiotics, such as producingβ lactamases. These enzymes can hydrolyze antibiotics that belong to β-lactam antibiotics, which affect the usage of these antibiotics. The β-lactamase-producing *K. pneumoniae* can resist a wide range ofβ lactams including penicillins, cephalosporins and carbapenems. Thus, in our study, most of the isolates were MDR and carried two types ofβ lactamase enzymes. Metallo-β-Lactamases: such as VIM, IMP and NDM are more common in Asia .Thus, bla_{NDM} and bla_{TEM} genes are dominant in this study. Large scale local study is needed to get actual disturbance of these genes. Screening for MDR isolates should provide an actual image of the increase in antibiotic resistance and the spread of β-lactamases in Iraq. Thus, there is an urgent need to discover new treatments, such as phage therapy, for resistant isolates.

SUPPLEMENTARY MATERIAL

The following supporting information can be downloaded at: https://mjs.uomustansiriyah.edu.iq/index.php/MJS/article/view/1472/768.

AUTHOR CONTRIBUTIONS

Ali Y. Hussein and Nadal A. Al-Saryi performed the experiments. All authors interpreted the results. Ban O. Abdulsattar, Nadal A. Al-Saryi and Wadhah H. Edrees wrote the manuscript.

FUNDING

None.

DATA AVAILABILITY STATEMENT

None.

ETHICAL APPROVAL

This study did not involve direct contact with humans, and all clinical isolates were obtained from hospital laboratories. Therefore, no ethical approval was needed.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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