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Isolation and identification of multi-drug resistant *Klebsiella pneumoniae* from patients of some hospitals in Dakahlia Governorate, Egypt

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Abstract: *Klebsiella pneumoniae* poses a significant risk to public health as it is a crucial human pathogen responsible for opportunistic nosocomial and community-acquired infections. One of the major concerns is the rising prevalence of multidrug-resistant strains. 102 clinical samples of urine, blood and wound swabs were collected from diabetic, urinary and respiratory tract infections patients who were admitted to Talkha, Nabrouh Central Hospitals and Sandoub Health Insurance Hospital in Dakahlia Governorate. 25 of 102 isolates resembling *Klebsiella pneumoniae* were identified by conventional biochemical methods. An antibiotic sensitivity test was conducted on isolates of *Klebsiella pneumoniae* using 9 antibiotic disks. The results revealed that 92% of the isolates exhibited resistance to Piperacillin, while 84% demonstrated resistance to both Ceftriaxone and Ceftazidime. Additionally, a majority of the isolates, accounting for 60%, displayed resistance to Ampicillin-Sulbactam and Cefepime. 16s rRNA analysis was performed to isolate KP26 that which was the most resistant to all antibiotics. It was similar to *Klebsiella pneumoniae* strain PF-4 according to NCBI-BLAST and its alignment in the neighbor-joining tree results. The study findings emphasized a notable threat to human health, as the preponderance of isolates exhibited resistance to multiple antibiotics.

Keywords: *Klebsiella pneumoniae*, antibiotic sensitivity, 16s rRNA.

1. Introduction

Klebsiella pneumoniae, a member of the Enterobacteriaceae family, is a gram-negative nosocomial pathogen, non-motile bacterium with a capsule [1]. It is known for its ability to ferment lactose and is a facultative anaerobe, often associated with pneumonia [2]**.** *Klebsiella* species are widely distributed in nature and can be commonly encountered in various settings like water, soil, and dirt with the ability to colonize the nasopharynx and gastrointestinal tract, enabling them to function as opportunistic pathogens in humans [3, 4, 5]. *Klebsiella pneumoniae*, a clinically considerable member of the *Klebsiella* genus, is reported to account for approximately 86% of human infections caused by *Klebsiella*, establishing itself as the most prominent pathogen within this genus [6]. *Klebsiella* spp. play an important role among hospital-acquired pathogens, as they are responsible for a wide range of infections, including those affecting the respiratory, pyogenic liver abscess and urinary tracts, as well as soft tissues, wounds, sepsis, and septicaemia [6]. Following *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* has been identified as the third leading cause of Hospital-Acquired Infections (HAIs) in Egypt [7]. Over the past few decades, the World Health Organization (WHO) has highlighted antimicrobial resistance (AMR) as a top global public health issue [8]. The rise of strains resistant to almost all traditional antimicrobials has raised significant worldwide concerns [9]. The growing resistance of pathogens to multiple antibiotics is a leading cause of increased morbidity and mortality [10]. *Klebsiella pneumoniae*, in particular, has been observed to exhibit resistance to aminoglycosides, fluoroquinolones and beta-

lactam antibiotics. The resistance of *Klebsiella* species to a wide range of antibiotics is attributed to their production of beta-lactamase enzymes [11]. *K. pneumoniae's* resistance to various antibiotics is a result of multiple mechanisms, including alterations in metabolic pathways, modifications to antibiotic target sites, changes in permeation of membrane, releasing antimicrobial-inactivating enzymes and stimulation efflux mechanisms are encoded either substantially or by acquired resistance genes [12, 13]. Efflux pump systems and enzymatic degradation have substantial roles in the development of multidrug resistance among these mechanisms [14, 15]. Many enzymes can degrade β-lactam antibiotics produced by *Klebsiella pneumoniae* like oxacillinases, carbapenemases, extended-spectrum and metallo-β–lactamases [16]. The prevalence, along with the incidence rates of multidrug resistant *Klebsiella pneumoniae* fluctuate based on geographical regions and can exhibit variations even within the same country due to disparities in adherence to infection control strategies and antibiotic policies [17]. Finally, detecting multidrug-resistant *K. pneumoniae* becomes a challenge due to the frequent presence of a broad spectrum of resistance determinants, leaving limited available treatment options [17]. The aim of this study was to isolate and manage antimicrobial resistance profile of *Klebsiella pneumoniae* isolates and identify the most multidrug resistant *Klebsiella pneumoniae* from patients who admitted to some Dakahlia Governorate hospitals, Egypt.

2. Materials and methods

Collection of samples

A total of 102 clinical samples were collected from urine, blood and wound swabs of diabetic, urinary and respiratory tract infections patients who were admitted to Talkha, Nabrouh Central Hospitals and Sandoub Health Insurance Hospital in Dakahlia Governorate, Egypt during from November 2020 till June 2021. The samples were promptly brought to the Laboratory of Microbiology under aseptic conditions and then underwent bacterial culturing and testing.

Pathogen Isolation *(Klebsiella pneumoniae)*

Under aseptic conditions, the collected samples were injected into the pre-prepared sterilized medium of MacConkey agar, then, they were incubated for 24 hours at 37°C. Colonies that were mucoid, circular, and capable of fermenting lactose were subcultured onto Eosin Methylene Blue agar (EMB) to confirm and differentiate with *Escherichia coli* [18].

Morphological and biochemical characterization

Isolates that were presumptively selected underwent Gram staining and characterization of morphology like size, color and form were described by microscopy studies. The form and the colony type were observed on nutrient agar, cysteine lactose electrolyte deficient agar and blood agar [19, 20]. Isolates were subsequently identified through the application of conventional biochemical tests such as catalase, oxidase, urease, citrate utilization, lactose and glucose fermentation, motility, growth at 10°C, sulfur, indole, gas produnction, Ornithine decarboxylase, lysine deaminase and lysine decarboxylase [18, 20, 21].

Antibiotic sensitivity assay

The antibiotic sensitivity assay for the isolates of *Klebsiella pneumoniae* was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA) [22]. Sterile cotton swabs were employed to evenly distribute the target bacteria across the surface of sterile Mueller-Hinton agar (MHA) plates. nine Antibiotics discs; Piperacillin (100µg), Ceftriaxone (30µg), Ampicillin-Sulbactam (10µg), Cefepime (30µg), Ceftazidime (30µg), Meropenem (10µg), Amikacin (30µg), Levofloxacin (5µg) and Trimethoprim/sulfamethoxazole (25µg) were placed over the surface of the bacterial lawn and incubated at 37°C for 24 hours. In millimeters (mm), the inhibition zones for each antibiotic were measured and matched against the Clinical and Laboratory Standard Institute (CLSI) standard values specific to each antibiotic [23].

16S rRNA analysis

It was carried out for the most resistant isolates to antibiotics. The total genomic DNA of selected bacterial isolates was extracted using PrepMan® Ultra Sample Preparation Reagent Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's

instructions. Amplifications were performed using thermal cycler and with a temperature profile standardized for 16S rRNA gene amplification. The PCR amplification was carried out in microcentrifuge PCR tubes with 15 µl of PCR master mix. Conditions of the PCR reaction were initial denaturation for 10 min at 95°C, 30 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec. A final extension for 10 min at 72°C was done. The resulting product was observed on agarose gel (1%) under light of UV following staining with ethidium bromide. [24, 25, 26]. Sequencing of purified PCR product was performed by MicroSeq® 500 16S rRNA Bacterial Identification Sequencing Kit for forward and reverse directions and developed by Sanger sequencing. The sequences acquired were utilized in comparisons with the database of NCBI via BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were aligned with Clustal W, and a phylogenetic tree was constructed from the evolutionary distances by the neighbor-joining method with the software MEGA [27, 28].

Results

Klebsiella spp Collection and Isolation from clinical samples

The clinical samples screening, including wounds, blood, and urine gathered from hospital patients, revealed that out of the 102 samples, 25 tested positive for *Klebsiella* spp that gave pink or purple, mucoid colonies on MacConkey agar plates, as shown in **Table (1)**. Among these samples, the highest occurrence of *Klebsiella* spp isolates were observed in urine samples (44.0%), followed by wound samples (32.0%). In contrast, the lowest percentage of these bacteria was found in blood samples (24.0%), as depicted in **Figure (1**).

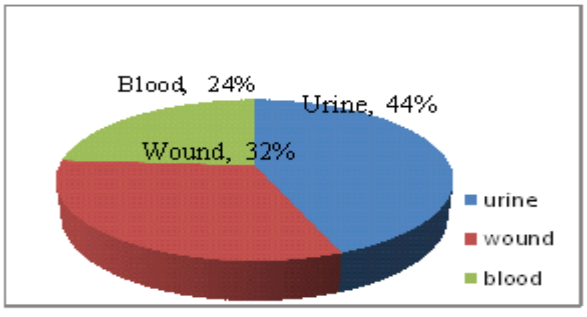

Figure (1): Distribution of *Klebsiella* spp isolates according to sample type

Table (1): Isolates of *Klebsiella* spp recovered from clinical samples on the medium of MacConkey agar.

Origin of isolates	Sample	Code of isolate	Number of isolate n=25
Sandoub Health Insurance Hospital (SHIH)	urine	KP5,KP11,KP13	3
	wound	KP16,KP20, KP21	3
	blood	KP26,KP31	2
Nabrouh Central Hospital (NCH)	urine	KP57, KP60, KP61,KP66, KP67	5
	wound	KP48, KP51,KP54	3
	blood	KP37,KP38, KP44	3
Talkha Central Hospital (TCH)	urine	KP95,KP97, KP101	3
	wound	KP73,KP76	2
	Blood	KP86	1

Characterization of *Klebsiella* spp isolates

Gram's staining was performed on the isolates to assess their morphological characteristics, revealing that *Klebsiella* spp are non-motile Gram-negative, capsulated, bacilloid bacteria. Subsequently, various biochemical tests were conducted on all 25 isolates to determine their biochemical characteristics, and the results are shown in **Table (2**).

Antibiotic sensitivity

The sensitivity of 25 isolates from *klebsiella* was tested against 9 antibiotic disks. The findings are outlined in **Table (3)** showed a high level of antibiotic resistance among the majority of isolates. 23 isolates exhibited resistance to Piperacillin at a rate of 92%, 21 isolates showed resistance to Ceftriaxone and Ceftazidime with 84% while 15 *klebsiella* isolates exhibited Ampicillin-Sulbactam and Cefepime resistance at 60% followed by 13 isolates exhibited Trimethoprim/sulfamethoxazole resistance at 52 percent. Meanwhile, 15 isolates exhibited Meropenem sensitivity at a rate of 60% Subsequently 14 isolates exhibited susceptibility to Amikacin with 56%.

Table (2): Morphological and Biochemical tests identification of *Klebsiella* spp isolates

Microscopy & Cultural Characters & Biochemical tests	Results
Gram stain	Negative
Shape of cell	Short rods
Growth on macconky's agar	Mucoid pink colonies
Growth on nutrient agar	Creamy white and raised circular colonies
Growth at 10° C	-ve (no growth)
Growth on Cystine Lactose Electrolyte Deficient (CLED) agar	Mucoid yellow colonies
Haemolysis on blood agar medium	γ haemolysis
Glucose fermentation	+ve
Lactose fermentation	+ve
Gas production	+ve
H2S production	-ve
Citrate test	+ve
Catalase test	+ve
Urease test	+ve
Lysine decarboxylase	+ve
Lysine deaminase	-ve
Motility	Non motile
Indole test	-ve
Ornithine decarboxylase	-ve
Oxidase test	-ve

Table (3): Presenting the percentages of sensitivity, intermediate, and resistance among 25 *Klebsiella* spp isolates to different antimicrobial agents.

Antibiotic disk	*Klebsiella* spp isolates (n = 25)					
	Sensitivity rate		Intermediate rate		Resistant rate	
	No	%	No	%	No	%
Piperacillin (PRL)	0	0	2	8	23	92
Ceftriaxone (CRO)	4	16	0	0	21	84
Ampicillin-Sulbactam (SAM)	5	20	5	20	15	60
Cefepime (FEP)	2	8	8	32	15	60
Ceftazidime (CAZ)	0	0	4	16	21	84
Trimethoprim/ sulfamethoxazole (SXT)	8	32	4	16	13	52
Meropenem (MEM)	15	60	4	16	6	24
Amikacin (AK)	14	56	7	28	4	16
Levofloxacin (LEV)	9	36	9	36	7	28

16S rRNA analysis

It was carried out for the most resistant isolate KP26 to all antibiotics. Isolate KP26 (PP464225) isolated from blood possessed 522 base pair nucleotide sequence **Figure (2)**. The results of the NCBI-BLAST search indicated the closest sequence resemblance to *Klebsiella pneumoniae strain* PF-4 belonging to the family Enterobacteriaceae, and the accession number was KT751178.1. Consequently, the Genbank BLAST search for the isolate yielded the percentage of resemblance between the tested microorganism and those identified in GenBank, as illustrated in **Table (4**). Moreover, a neighbor-joining tree was constructed based on 16S rRNA gene sequences to demonstrate the relationship between isolate KP26 (PP464225) and 16 other related genera **Figure (3)**. This result showed that isolate KP26 (PP464225) was similar to *Klebsiella pneumoniae strain* PF-4 with accession number KT751178.1.

TTGAAAAGTTGATCCTGCTCAGATAACGCGGCGGC AGGCCTACACATGCAAGTCGAGCGG TAGCACAGAGAGCTGCTCTCGGTGACGAGCGGAG GACGGGTGAGTAATGTCTGGGAAACT GCCTGATGGAGGGGGATAACTACTGGAAACGGTA GCTAATACCGCATAACGTCGCAAGAC CAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGA TGTGCCCAGATGGGATTAGCTAGTA GGTGGGGTAACGGCTCACCTAGGCGACGATCCCTA GCTGGTCTGAGAGGATGACCAGCCA CACTGGAACTGAGACACGGTCCAGACTCCTACGGG AGGCAGCAGTGGGGAATATTGCACA ATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT GTGAAGAAGGCCTTCGGGTTGTAAAG CACTTACAGCGGGGAGGAAGGCGATAAGGTTAATA ACCTTGTCGATTGACGTTACCCGCA GAAGAGCACCGGCTAACTCCGTGCCAGCAGCCGC GTTAAATA

Figure (2). The obtained KP26 (PP464225) 16S rRNA sequence comprised 522 bases.

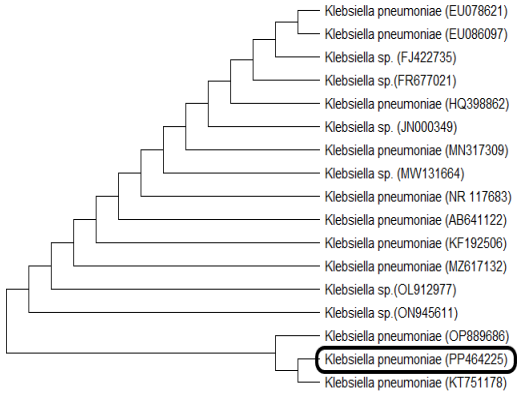

Figure (3). A neighbor-joining tree, based on sequences of 16S rRNA with a length of 522

bases, depicts the connection between the unidentified isolate KP26 (PP464225) and other genera closely related *Klebsiella pneumoniae* and it was closed to *Klebsiella pneumoniae* strain PF-4 (KT751178.1)

Table (4): The most significant similarities for 16S rRNA isolate KP26 (PP464225) gene with some other related genera resulted by BLAST research alignment.

No	Description	Query cover	Per. Ident	Accession
1	*Klebsiella pneumoniae* strain PF-4	97%	98%	KT751178.1
2	*Klebsiella pneumoniae* strain E1	97%	98%	OP889686.1
3	*Klebsiella pneumoniae* subsp. pneumoniae strain ZSST3	97%	98%	MZ617132.1
4	*Klebsiella pneumoniae* gene for 16S rRNA, strain:SW	97%	98%	AB641122.1
5	*Klebsiella pneumoniae* strain DSM 30104	97%	98%	NR_117683.1
6	*Klebsiella pneumoniae* strain MCB19	97%	98%	MN317309.1
7	*Klebsiella pneumoniae* strain PB12	97%	98%	KF192506.1
8	*Klebsiella pneumoniae* strain L13	97%	98%	HQ398862.1
9	*Klebsiella pneumoniae* strain HR16	97%	98%	EU078621.1
10	*Klebsiella pneumoniae* strain HR9	97%	98%	EU086097.1
11	*Klebsiella sp.* strain RGUDBI01	97%	98%	ON945611.1
12	*Klebsiella sp.* strain ZXY-9	97%	98%	OL912977.1
13	*Klebsiella sp.* strain SR-4	97%	98%	MW131664.1
14	*Klebsiella sp.* U25C	97%	98%	JN000349.1
15	*Klebsiella sp.* MB45	97%	98%	FR677021.1
16	*Klebsiella sp.* 2N3	97%	98%	FJ422735.1

3. Discussion

Klebsiella is the second most common bacterium causing nosocomial infections, following *Escherichia coli*, among the Enterobacteriaceae family [29]. In the past, *Pseudomonas* was the primary bacterium demonstrating significant resistance to commonly prescribed antibiotics [29]. However, presently, In addition to *Acinetobacter*, species of *Klebsiella* are increasingly acquiring resistance to multiple medications, with *Klebsiella pneumoniae* being responsible for the majority of *Klebsiella* infections. [29]. *Klebsiella pneumoniae* is a significant human pathogen, known to induce pneumonia, nosocomial infections, as well as urinary and respiratory tract infections, septicaemia, posing a potential threat to human life [30]. In our study, 25 *Klebsiella pneumoniae* isolates were obtained from 102 clinical specimens of patients with (24.5%) of total samples. *Klebsiella pneumoniae* samples were mainly isolated from urine samples (44.0%) followed by wound samples (32.0%) and blood samples (24.0%). This aligns with the findings of a study conducted by Morsi and Rabie and Abdallah [31, 32] at Zagazig University Hospitals in Egypt. Their research indicated that samples of urine were the predominant isolated *K. pneumoniae* source**.** The biochemical characteristics in bacteria are an important tool in their identification. In our current study, all *Klebsiella* spp isolates were initially distinguished using standard biochemical tests, which yielded similar results to those reported in previous studies for *K. pneumoniae*. Further differentiation from *K. oxytoca* was achieved through an indole test relying on the tryptophan metabolism to indole through tryptophanase [33].

The Kirby Bauer disc diffusion method was employed to conduct an antimicrobial susceptibility test on *Klebsiella* isolates. The results revealed a notable prevalence of resistance across diverse antibiotic classes in clinical samples, likely attributed to the frequent administration of these antibiotics in patient treatments. These findings align with the research of Rawy et al and Shawkey et al. [18, 34]**.** The multidrug resistant *K. pneumoniae* isolates (MDR) had evolved resistance against four distinct antibiotic classes, namely aminoglycosides, cephalosporins, carbapenems and fluoroquinolones [10]. Resistance of isolates to Piperacillin and ampicillin-sulbactam as a β-lactam/β-lactamase inhibitor is similar to study of Al-Baz et al [35] that reported β-lactam Resistance of *Klebsiella* isolates with a range. On the other hand, Nirwati et al [36] demonstrated high sensitivity to β-lactam such as pipracillin-tazobactam with only resistance rate of 10.5%. The results revealed that amikacin and meropenem owned the lowest resistance rates are highly compatible with the results of Nirwati et al [36] that reported close levels of resistance with much lower resistance rates with meropenem and amikacin. Unlike, a study by Aamir et al [37] detected the higher resistance rate with amikacin. The variability in resistance levels to meropenem could be attributed to the careful selection of cases for

carbapenem treatment, thus maintaining the efficacy and sensitivity of these drugs [35].

Numerous searches have been undertaken to assess the effectiveness of various techniques for the identification of species of *Klebsiella*. Test of PCR has been documented as demonstrating greater accuracy in *Klebsiella* species identification when compared to other initial identification tests [38, 39, 40]. Accurate *Klebsiella* samples identification is crucial for molecular and taxonomic profiling; recently, techniques of molecular utilizing the 16S rRNA region amplification are now accessible to identify *Klebsiella* species [41, 42]**.** In this study, an analysis of the 16S rRNA was conducted to identify the isolate, which exhibited the highest level of antibiotic resistance. The analysis indicated its similarity to *Klebsiella pneumoniae* strain PF-4, as confirmed by NCBI-BLAST and alignment in the neighbor-joining tree results.

Conclusion

The majority of isolates in this study exhibited multi-drug resistance, showing resistance to more than one antibiotic. Notably, isolate KP26 demonstrated the highest resistance to all tested antibiotics. The increasing incidence and global dissemination of these clinical strains pose a significant threat to public health. Consequently, there is a pressing need for greater focus on rationalizing antibiotic usage and developing effective therapeutic alternatives to combat antibiotic resistance worldwide.

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