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Research Article
Molecular-diversity, Prevalence and Antibiotic Susceptibility of Pathogenic *Klebsiella pneumoniae* under Saudi Condition

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Abstract

Background and Objective: *Klebsiella pneumoniae* is an important multi-drug-resistant Gram-negative pathogen, associated with nosocomially acquired infections. This study aimed to determine the genotypic and phenotypic characterization of *Klebsiella pneumoniae* isolates and to correlate the antibiotic resistance with the presence of virulence genes revealed by molecular genotypic testing in Riyadh, Saudi Arabia. **Materials and Methods:** About 23 *Klebsiella pneumoniae* isolates were collected from various specimen types. Identification of the organisms was carried out. Antimicrobial susceptibility performed against 12 antibiotics. The DNA was isolated and purified then genotypic confirmation was done through polymerase chain reactions (PCR) to detect TEM, SHV, CTX-M, IMP and KPC genes. PCR products were sequenced and aligned with GenBank sequences. **Results:** Out of 23 isolates of *K. pneumoniae*, the majority (43.5%) was from tracheal aspirate. The percentage of females (65.2%) was more than males (34.8%). The highest isolates prevalence was found in the age group of $\geq$58 (39.1%). About 100% of isolates were resistant to cefotaxime, ceftriaxone, ceftazidime, cefepime and ampicillin and 91.3% were sensitive to amikacin and Imipenem. Most isolates were SHV-9 gene positive (52.2%). It was found that tested isolates had 99-100% similarity when compared to GenBank sequences. **Conclusion:** There was a preponderance of SHV-9 gene which suggests dissemination of the gene in the tested isolates.

Key words: *Klebsiella pneumoniae*, extended-spectrum beta-lactamase, anti-biotic resistance, virulence genes, DNA sequencing, GenBank sequences

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anti-biotic resistance is a natural phenomenon of bacteria found naturally in the surrounding environment or pathogens of humans and animals. As newly produced antibiotics push them to develop biochemical mechanisms for resistance through a large number of responsible genes. As a result of which, worldwide problems are being arrived with serious consequences on the treatment of infectious diseases[1]. These pathogenic bacteria are frequently reported as multi-drug-resistant bacteria (MDR) and may produce extended-spectrum β-lactamases (ESBL). The incidence and type of ESBL producer strains vary according to different geographical locations[2]. Infections with MDR organisms often lead to increased mortality, longer hospital stays and inflated healthcare costs. Hence, MDR bacterial pathogens are important health challenge worldwide[3].

The highest incidence of bacterial resistance usually observed at intensive care units (ICU) due to excessive use of antibiotic agents. And so, understanding the resistance profile of hospital micro-biota against antibiotics is fundamental for the prevention and control of nosocomial infections[4]. Another risk factor is the international travel which forms a major mode of spread of MDR Gram-negative bacteria. So Gulf Cooperation Council countries (GCC) (Saudi Arabia, United Arab Emirates [UAE], Oman, Kuwait, Qatar and Bahrain) suffers this significant issue as large numbers of expats seek medical care in specialized centers hand in hand with millions visit the region annually for the Hajj and other religious events[5].

Out of particular concern is the spread of MDR strains of *Klebsiella pneumoniae*, a Gram-negative bacterium that causes nosocomial infections. *Klebsiella* accounts for 3-7% of all nosocomial bacterial infections in the United States, placing them among the eight most important infectious pathogens in hospitals. The *K. pneumoniae* can harbor both extended-spectrum-lactamases (ESBL) and carbapenemases capable of hydrolyzing newer carbapenem drugs. Frequently associated with ESBL-producing *K. pneumoniae* is resistance to other antibiotics, including fluoroquinolones, amino glycosides, trimethoprim and sulfamethoxazoles[6,7]. The prevalence of ESBL in *Klebsiella pneumoniae* is increasing worldwide. Global studies showed that the frequency of ESBL-producing *K. pneumoniae* was 44% in South America, 33% in Europe, 22% in Asia and 12% in the United States[4]. The ESBL are enzymes produced by many bacterial species as a means for defense against β-lactam drugs with the genes encoding for those enzymes being mainly located on mobile genetic elements[8].

The *K. pneumoniae* has recently gained popularity as an infectious agent due to a rise in the number of severe infections. These pathogens showed much resistant in response to treatment of urinary tract, intra-abdominal, skin infections and pneumonia, among neonates, elderly and immuno-suppressed individuals within the healthcare-associated settings[9,10]. Treatment of these infections depends heavily on effective antimicrobial therapy and delaying treatment may lead to a higher mortality rate. Therefore, the presence of MDR in the infecting pathogen would adversely affect the treatment outcome[11].

In Gram-negative bacteria, MDR-ESBL mechanism of resistance may include a production of β-lactamases and these enzymes are usually acquired by gene transfer[12]. These strains have been isolated in several countries since the 1980s. Many outbreaks caused by such bacteria, especially *Klebsiella pneumoniae*. The predominant ESBLs/genes reported in Western and Asian countries are TEM- and SHV-derived ESBLs. These β-lactamases possess one or more amino acid substitutions that correspond to the narrow-spectrum prototype, TEM-1 and SHV-1 and these amino acid substitutions confer resistance to oxyimino β-lactam antibiotics[13]. About more than 390 different kinds of ESBLs are recognized around the world, among them blaSHV, blaTEM and blaCTX-M are more common[14].

Although few studies have been conducted on carbapenem-resistant *Klebsiella pneumoniae* isolates in the Arabian Peninsula, almost all countries in the Gulf Cooperation Council (GCC) share the same ESBL and carbapenemase-producing Enterobacteriaceae, most of which were recovered from nosocomial infections. Moreover, a recent review of β-lactamase producing Gram-negative bacilli from GCC states showed that β-lactamases genes such as OXA-48, CTX-M-15 and NDM-1 are the most common and widespread β-lactamases[15].

The developing regional surveillance of antibiotic resistance is urgent to detect multidrug-resistant (MDR) isolates. Clinical laboratories have to accurately screen isolates suspected of harboring virulence genes that are responsible for antibiotic resistance. For this reason, this study was conducted to determine the phenotypic and genotypic characterization of *Klebsiella pneumoniae* isolates and to correlate the antibiotic resistance with the presence of virulence genes revealed by molecular genotypic testing in Riyadh, Saudi Arabia.

MATERIALS AND METHODS

Bacterial Isolates: This study was carried out at King Khalid University Hospital, Riyadh, Saudi Arabia. The study included

23 non-duplicate, consecutive, phenotypically confirmed *K. pneumoniae* ESBL-producing isolates identified between June-December, 2016. These isolates were from various specimen types including blood, urine, wound swabs, sputum, tracheal aspirate, eye and groin, which were routinely cultured in the bacteriology laboratory. Identification of the organisms and susceptibility testing were carried out according to the laboratory policy by Vitek 2 (Biomerieux, Marcy l' Etoile). The isolates flagged as ESBL by Vitek 2 were confirmed using the E-test method as recommended by CLSI[16].

Anti-biotic susceptibility testing against *Klebsiella Pneumoniae* isolates: Anti-microbial susceptibility test by the disk diffusion method was performed to determine the resistance patterns of the isolates to 12 antibiotics: cefotaxime (CTX, 30 µg), ceftriaxone (CT, 30 µg), ceftazidime (CAZ, 30 µg), cefepime (Cefep 30 µg), gentamicin (GEN, 10 g), amikacin (AK, 30 µg), piperacillin/tazobactam (PIP/TAZ 100/10 µg), ciprofloxacin (CIP , 5 µg), tetracycline (TET, 30 µg), ampicillin (AMP, 10 µg), amoxicillin/clavulanic (AX/CLA, 30 µg), Imipenem (IMI, 10 g) [Oxoid, England, disc contents according to Clinical and Laboratory Standards Institute (CLSI) guidelines]. All antimicrobial testing was performed on Mueller-Hinton agar by the flooding technique and data interpreted according to the CLSI guidelines[16].

Extended-spectrum beta-lactamase E-tests (AB Biodisk, Solna, Sweden) were performed as confirmatory tests according to the CLSI guidelines[16].

Genotyping using PCR and sequence data analysis: DNA was isolated and purified using a Nucleospin kit (Macherey-Nagel), according to the manufacturer's instructions. Genotypic confirmation was done through polymerase chain reactions (PCR) to detect TEM, SHV, CTX-M, IMP and KPC genes[17].

The PCR products were sequenced by sending the products to GATC BIOTECH or by using Sanger sequencing in the lab. The DNA sequences were analyzed using Codon Code Aligner software and were assembled using Geneious 5.0.4. The nucleotide sequences of the genes of interest were aligned to the local database using a BLAST CLC Genomics Workbench 9.0 software package. Sequences obtained from the samples were aligned with GenBank sequences[18].

Statistical analysis: All data were stored in Microsoft Excel, Version 2016. Data management and statistical analyses were also performed in Excel. Descriptive statistics of the data and variables were presented in the form of frequencies and percentages.

Ethical statement: The institutional review board, college of medicine, King Khalid University Hospital, Riyadh, Saudi Arabia, has granted permission to conduct this study.

RESULTS

Bacterial isolates: The study included a total number of 23 non-duplicate, consecutive, phenotypically confirmed *Klebsiella pneumoniae* ESBL-producing isolates from various body sites.

The majority of the isolates (n = 10, 43.5%) were isolated from tracheal aspirate samples and others as shown in Table 1. Male to female was 1:2 (Table 2). Table 3 shows that the highest isolates prevalence was mostly found in the age group of ≥58 (39.1%).

Anti-biotic susceptibility testing against *Klebsiella Pneumoniae* isolates: Antibiotic susceptibility patterns are shown in Table 4, 100% of the isolates were resistant to cefotaxime, ceftriaxone, ceftazidime, cefepime and ampicillin, while 91.3% of them were sensitive to amikacin and Imipenem.

Genotyping using PCR and sequence data analysis: Of the 23 clinical isolates of *Klebsiella pneumoniae* 19

Table 1: Prevalence of *Klebsiella pneumoniae* in various specimen types (n = 23)

Specimen types	Frequency	Percentage
Tracheal aspirate	10	43.5
Sputum	4	17.4
Blood	3	13.0
Urine	2	8.7
Eye	2	8.7
Wound swabs	1	4.3
Groin	1	4.3

Table 2: Prevalence of *Klebsiella pneumoniae* in different genders (n = 23)

Gender	Frequency	Percentage
Males	8	34.8
Females	15	65.2

Table 3: Prevalence of *Klebsiella pneumoniae* in different age groups (n = 23)

Age (years)	Frequency	Percentage
≤5	8	34.8
16-36	4	17.4
37-57	2	8.7
≥58	9	39.1

Table 4: Antibiotic susceptibility testing against *Klebsiella pneumoniae* isolates (n = 23)

	Resistant		Susceptible	
Antibiotics	Number	Percentage	Number	Percentage
Cefotaxime	23	100.0	0	0.0
Ceftriaxone	23	100.0	0	0.0
Ceftazidime	23	100.0	0	0.0
Cefepime	23	100.0	0	0.0
Gentamicin	8	34.8	15	65.2
Amikacin	2	8.7	21	91.3
Piperacillin/tazobactam	8	34.8	15	65.2
Ciprofloxacin	10	43.5	13	56.5
Tetracycline	13	56.5	10	43.5
Ampicillin	23	100.0	0	0.0
Amoxicillin/clavulanic	13	56.5	10	43.5
Imipenem	2	8.7	21	91.3

Table 5: Distribution of antibiotic resistance genes in *Klebsiella pneumoniae* isolates (n = 23)

			Gene found in isolates (%)				
Total isolates number	ESBL isolates number	Carbapenemase isolates number	SHV n (%)	CTX-M n (%)	TEM n (%)	KPC n (%)	IMP n (%)
23	19	4	12 (52.2)	8 (34.8)	6 (26.1)	3 (13)	2 (8.7)

Table 6: Distribution of single gene and gene combinations in *Klebsiella pneumoniae* isolates (n = 23)

Distribution of the single gene	Isolates number	Total (n = 23) (%)
SHV	8	34.0
CTX-M	2	8.7
TEM	1	4.3
KPC	3	13.0
IMP	1	4.3
Total No. of isolates with a single gene distribution of the gene combinations	15	65.0
TEM+CTX-M	3	13.0
SHV+CTX-M	3	13.0
TEM+SHV	2	8.7
Total No. of isolates with $\geq$2 genes	8	34.7

Table 7: Analysis of antibiotic resistance genes in tested *Klebsiella pneumoniae* isolates (n = 23)

Query	Strains No.	Identity (%)
TEM-1	2, 3, 6, 16, 17, 21	99.8
CTX-M-15	2, 6, 10, 12, 15, 19, 21, 23	100.0
IMP4	7, 22	100.0
KPC2	4	100.0
KPC3	9, 18	99.9
SHV-9	1, 5, 8, 11, 12, 13, 14, 15, 16, 17, 20, 23	99.0

ESBL-producing isolates comprising SHV, CTX-M and TEM genes and 4 carbapenemase isolates comprising KPC and IMP genes. For ESBL genes, distributions of SHV, CTX-M, TEM were demonstrated as 12 (52.2%), 8 (34.8%) and 6 (26.1%), respectively. On the other hand, carbapenemase genes were observed as 3 (13%) KPC and 2 (8.7%) IMP (Table 5). The distribution of single gene and gene combinations in *Klebsiella pneumoniae* isolates are shown in Table 6.

The SHV-9 gene was harbored by the majority of the isolates 12 (52.2%). However, about 8 isolates (34.8%) harbored the CTX-M-15 gene. Also, the TEM-1 gene was detected in 6 (26.1%) and 2 (8.7%) isolates were positive for IMP4. And for KPC gene 3(13%), it comprised of two groups, KPC2 (n = 1) and KPC3 (n = 2) (Table 7). On sequence similarity analysis, it was found that tested isolates had 99-100% similarity when compared to GenBank sequences (Table 7).

DISCUSSION

This result revealed that the majority of *K. pneumoniae* isolates were sensitive to amikacin and Imipenem. There was a preponderance of SHV-9 gene which suggested dissemination of the gene in the isolates. The majorities of the isolates were from tracheal aspirate samples and were mostly from females. The highest isolates prevalence was mostly found in the age group of 58, which is in agreement with Somily *et al.*[19], who studied 77 isolates from various body sites. They stated that isolates were mostly from females with predominance of adult patients[19] and in contrast to Podschun and Ullmann[20], who reported that nosocomial *Klebsiella* infections most commonly involve the urinary tract[20].

Klebsiella infections target immune-compromised patients, so serve as a paradigm of hospital-acquired infections[21] especially for patients who had underlying diseases and the predominance of females was noticed which might be due to previous higher antibiotic intake[22]. As risk factors associated with women are previous surgeries (cesarean, normal delivery) and diabetes[23]. High frequency in ($\geq$58) age group was observed which might be because of low immunity[2]. The current study confirmed by the results of previous studies that carbapenems and amikacin remain the most active agents against *K. pneumoniae,* while cefotaxime, ceftriaxone, ceftazidime, cefepime and ampicillin were the most resistant agents[21,24-27].

As noticed, many of the isolates were Multidrug-Resistant (MDR) strains and extracted from admitted immune compromised patients which confirm *Klebsiella pneumoniae* infection to be a serious nosocomial Hospital Acquired Infections (HAI)[21,23,28]. Major outbreaks were reported worldwide thus making them emerging pathogens. And Increasing drug resistance induce a real problem in therapeutic choices, especially with the rise of carbapenem resistance among isolates, which has emerged and been reported in many countries such as Saudi Arabia, Algeria, India, Ireland, Switzerland and Russia[26]. This multi-drug-resistant might be due to using antibiotics without restriction. In several studies, it has been shown that the high prescribing habits of the physicians and empirical use are the driving factors for the antibiotic resistance for this group of antibiotics[25,29]. Thus, it is highly recommended that practicing physicians should become aware of the antibiotic resistance problem and help in fighting this deadly threat by rational prescribing[29,30].

The majority of the isolates harbored SHV-9 gene followed by CTX-M-15 gene and then comes the TEM-1 gene. The fewer percentages of isolates for IMP4 gene and KPC gene were noticed which comprised of two groups (KPC2 and KPC3). On sequence similarity analysis, it was found that tested isolates had 99-100% similarity when compared to GenBank sequences, which were in agreement with Tawfik *et al.*[30], that characterized ESBL-producing *K. pneumoniae* in Al-Qassim area, Saudi Arabia. Their molecular study revealed that 89.1% of the isolates produced SHV, 70.9% produced TEM and 36.4% were CTX-M-producing strains. The prevalence of ESBL SHV-12 and SHV-5 was of 60% and 18.2%, respectively and various non-ESBL SHV, including SHV-1 (5.5%), -11 (3.6%) and -85 (1.8%) was detected. However, the prevalence of CTX-M-15 and CTX-M-14 was 34.5% and 1.8%, respectively[30].

Therefore, implementing genetic analysis is recommended as routine work up in future research and diagnostic modalities for MDR infections and this can open fields for researches in the production of target-specific antibiotics.

CONCLUSION

Molecular genotyping using PCR and sequence data analysis results revealed the preponderance of the SHV-9 gene which could be the virulence gene disseminate in those strains in our region. And the correlation between the presence of the virulence gene and antibiotic resistance in *Klebsiella pneumoniae* may help diagnose these resistant isolates and pick up the appropriate antibiotic. Imipenem and amikacin are still recommended as good choices for treating *Klebsiella pneumoniae* infections.

SIGNIFICANCE STATEMENT

This study may add a new theory in the diagnosis and treatment of multidrug-resistance *Klebsiella pneumoniae* strain as the predominance of the SHV-9 gene was discovered that can be beneficial for choosing the suitable antibiotic and for increasing the researcher's capabilities to uncover the critical areas of molecular genotyping aberration of *Klebsiella pneumonia* strain which extended chances for diagnosis and treatment of multi-drug-resistance *Klebsiella pneumoniae* in our region and worldwide.

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