Original Article

Study the Expression of Capsule Gene in Carbapenem-Resistant *Klebsiella pneumoniae*

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Abstract

Background: *Klebsiella pneumoniae* is widely recognized as a prominent opportunistic pathogen that is, commonly associated with both nosocomial and community-acquired illnesses. A notable proportion of *K. pneumoniae* isolates possess a significant polysaccharide capsule that encompasses the entire bacterial surface, which is widely recognized as a crucial factor contributing to its pathogenicity. **Objective:** The study was conducted to determine the effect of carbapenem antibiotics on *ycfm* gene expression a well-known capsule associated gene in carbapenem resistant *K. pneumoniae* isolates. **Materials and Methods:** A total of 150 samples obtained from various hospitals in Iraq were cultured on MacConkey agar. Subsequently, differential and selective media to obtain *K. pneumonia*. The Vitek-2 system was then used to diagnose the results, confirming that 50 isolates were indeed *K. pneumoniae*. The diagnosed isolates were further subjected to negative staining to validate the presence of a capsule, which was verified to be *Klebsiella* spp. through molecular analysis utilizing *16S rRNA*. The study employed sub-inhibitory quantities of carbapenem antibiotics, specifically meropenem and imipenem, to examine the increase in gene expression of the *ycfm* gene. Three isolates, including two carbapenem-resistant isolates and one sensitive isolate, were chosen for comparison before and after treatment. **Results:** From total of 150 clinical samples, 50 isolates were identified as belonging to the *K. pneumoniae* species. Out of the 50 verified isolates, 25 were confirmed to be producers of capsules. The *16S rRNA* identified the 25 isolates as *K. pneumoniae* using molecular techniques. The microbroth dilution approach yielded a result of 3.9 for the resistant isolates and 1.9 for the sensitive isolate when using carbapenem antibiotics. The one-step quantitative reverse transcription polymerase chain reaction (RT-qPCR) technique identified a change in the gene expression of the capsule gene, with meropenem causing an upregulation of the *ycfm* gene expression in all isolates, whereas imipenem resulted in a downregulation of the *ycfm* gene expression in all isolates. **Conclusion:** The gene expression of the capsule-associated gene *ycfm* was influenced by carbapenem antibiotics in all isolates. The expression of the *ycfm* gene was decreased in all isolates when treated with imipenem, while it was increased in all isolates when treated with meropenem, regardless of the isolate's nature. This can be attributed to the fact that the *ycfm* gene is not responsible for acquiring resistance, but rather it is a stress response gene associated with capsule formation.

Keywords: Carbapenems resistant, gene expression, *Klebsiella pneumoniae*

Introduction

Klebsiella pneumoniae is a gram-negative member of the Enterobacteriaceae family. This microorganism colonizes several anatomical sites within the human body and constitutes an integral part of an individual's symbiotic microbial community. Within healthcare settings, the presence of this particular microorganism has the potential to induce severe infections among individuals who are critically ill, babies, those with impaired immune systems, or those who possess additional risk factors, despite its inherent role as a helpful constituent of the microbiome.[1] The use of antimicrobial agents is of great importance in the treatment of bacterial infections.[2] Antibiotics such as carbapenems are frequently used to treat infections, particularly those brought on by Enterobacteriaceae. Carbapenems possess

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broad-spectrum antibacterial activity and have a unique structure that is, defined by a carbapenem coupled to a β-lactam ring which confers protection against most β-lactamases.[3] The use or misuse of such antibiotics has contributed to the emergence of isolates resistant to carbapenems also known as carbapenem-resistant *Klebsiella pneumoniae* (CRKP).[4] CRKP has been recognized as a serious hazard to humanity in a number of papers.[5] *K. pneumoniae* causes a variety of illnesses, notably bacteremia, burns and wound infections, pneumonia, urinary tract infections, and pyogenic liver abscesses.[6] The presence of numerous virulence genes that encode virulence factors allow *K. pneumoniae* to invade the immune system of the body and cause a range of diseases and contributes to its pathogenicity. Examples of the aforementioned virulence factors include the formation of biofilm hypermucoviscosity, capsule production, adhesions, iron absorption, and lipopolysaccharides.[7] These virulence genes, particularly their number and mechanism of action have a significant effect on the clinical features of *K. pneumoniae* infections.[8] One of the primary factors contributing to the pathogenicity of *K. pneumoniae* is the presence of thin fibers, known as capsules which are approximately 160 nm thick. These fibers stretch perpendicularly from the outer membrane, effectively enveloping the bacterial cell from both sides.[9] The *ycfm* family is believed to have originated from a shared ancestor that played significant roles in attachment, colony formation through cell-to-cell contact, intercellular signaling, and self-identification, *ycfm* expression was observed to be approximately 12-fold higher in *K. pneumonia*.[10] This perspective article aims to study the expression of some capsule-associated genes in CRKP isolated from multiple sites.

Materials and Methods

Identification of bacterial strains

A total of 150 bacterial samples were collected from clinical specimens, specifically bronchial, urea, catheter, burns, tracheal, and wound infections. These samples were taken from five distinct hospitals located in Baghdad, during the period from October 2022 to January 2023. All of these samples were included in the present research. Isolates were isolated by streaking the sample on MacConkey agar plates and then purified by taking a single colony and re-culturing it on the surface of a sterilized MacConkey agar plate. Isolates were then identified with the use of an automatized system (Vitek-2 System, bioMerrieux, France), and an additional molecular technique known as *16S rRNA* was used to confirm the Vitek-2 system findings.

Detection of the capsule using a phenotypic method

The capsule presence was determined using a nigrosine stain (negative staining). A bacterial colony that had been growing overnight was placed on a clean, dry slide, carefully mixed with nigrosine, and then let air dry before being washed with some water. After 2 min of methylene blue staining and 2 min of air drying, the slide was carefully rinsed with water. The nigrosine stain provides a black background with a light microscope, whereas the methylene blue stain will stain the cell capsule.[11]

Determination of the minimal inhibitory concentration of carbapenems

K. pneumonia's minimum inhibitory concentration (MIC) value was calculated using a microtiter plate technique specified by the National Committee for Clinical Laboratory Standards.[12] Imipenem and meropenem were serially diluted in Mueller Hinton Broth (MHB) at concentrations of 500, 250, 125, 62.5, 31.25, 15.6, 7.81, 3.9, and 1.9 μg/mL on a 96-well round-bottom plate. *K. pneumoniae* subculture in MHB was used to create the bacterial inoculum. The added bacterial suspension was diluted to 1×10^8 colony forming units (CFU)/mL to achieve turbidity that was comparable to 0.5 on the McFarland scale. Thereafter a dilution in MHB at a ratio of 1:200 was carried out to get an ideal concentration of 5×10^5 CFU/mL. The 96-well plate with the serially diluted peptides was supplied with the diluted bacterial suspension of 100 μL of the chemical-based compound and 100 μL of diluted bacteria solution made up the 200 μL total volume per well. The bacterial suspension without additives was added to the 11th well and for the 12th well it MHB broth was added to the bacterial suspension in order to conduct a positive and negative controls. Resazurin (0.015%) was distributed to all wells (20 μL per well) after incubation for 24 h at 37°C, and additional incubation for 2–4 h was performed to observe any color change. Upon the end of the incubation period, columns exhibiting no shift in color (the color of the blue resazurin stayed constant) were given a score above the MIC value. The lowest concentration of compounds at which no bacterial growth was noticed was deemed to be the MIC.

Molecular study

The primers utilized in this investigation can be found in Table 1.

Genomic DNA extraction

A whole DNA (plasmid and chromosome) was extracted from an overnight grown bacterial culture for all 25 isolates that were found to have a capsule by following the instructions of the Easypure genomic DNA extraction kit.

Molecular identification of *K. pneumoniae* via 16S *rRNA*

All the extracted DNA samples have undergone polymerase chain reactions after being diagnosed by

Table 1: The primers employed in the course of this investigation

Primers	Sequence	Annealing temperature (ºC)	Product size	Reference
16S rRNA-F	(CGGTCTCAAGTCGGATGT)	55	172	This study
16S rRNA-R	(AGCGTCAGTCTTCGTCCAGG)			
ycfm-F	(CGATATTCCTCGCAGGCTGTG)	55	163	This study
ycfm-R	(TGATCCTGATGCTCAATCGGC)			

Table 2: Thermocycling conditions for the identification of *K. pneumoniae*

Steps	Temperature (°C)	min	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:30	
Annealing	55	00:30	30
Extension	72	01:00	
Final extension	72	07:00	1
Hold	10	10:00	

Vitek-2. Primers used in this test are listed in Table 1 as well as the listing of the thermocycling conditions for the reaction shown in Table 2.

RT-PCR technique to determine the gene expression

Three isolates of *K. pneumoniae* were used in the experiment; two of which were resistant to imipenem and carbapenem and one of which was sensitive to those mentioned antibiotics and all had the *ycfm* gene. The expression of the gene in both resistant and sensitive isolates was measured before and after the treatment with imipenem and meropenem. The concentrations of those antibiotics belonging to the carbapenem family ranged from 1.9 to 3.9 µg/mL. The levels used in the procedure were lower than the MIC value to enable bacterial growth and resistance development to investigate how the antibiotic's sub-inhibitory doses affect the expression of the capsule-associated gene.

RNA extraction

The RNA was obtained using TRIzol™ reagent (Invitrogen, Waltham, Massachusetts, USA) following the guidelines provided by the manufacturer. Subsequently, the concentration and purity of the extracted RNA were determined using the Qubit 4 instrument (ThermoFisher®). The synthesis of cDNA was performed using the ProtoScript® First Strand cDNA Synthesis Kit (NEB, Ipswic, Massachusetts, USA). The cDNA that was produced was measured for its quantity using the Qubit™ dsDNA HS Assay Kit (ThermoFisher®).The quantitative detection method relied on the measurement of SyberGreen's fluorescence intensity. The composition of the reaction mixture consisted of the following elements: 10 µL of Luna Universal qPCR Master Mix, 1 µL of Forward primer (10 µM), 1 µL of Reverse primer (10 µM), 5 µL of template DNA, and 3 µL of nuclease-free water, the thermocycling conditions consisted of 4 main cycles, first cycle being the initial denaturation at 95ºC, second cycle denaturation at 95ºC third cycle is extension at 60ºC and the fourth cycle being the melting curve at 60–90ºC. Depending on the fluorescence power of SyberGreen the quantity was detected. To assess relative quantification, the delta Ct (ΔΔCt) technique entails selecting a calibrator sample to conduct a direct analogy of the (cycle threshold) Ct values for the target gene with the reference gene. The calibrator can be any sample that acts as a standard versus whereby unidentified samples may be evaluated. The difference in Ct between the target gene and the reference gene is determined for every isolate (unidentified sample and calibrator sample).[13] First step by applying the following equation

$$\Delta \text{Ct} = \text{Ct target gene} - \text{Ct reference gene}$$

Then, the difference between the ΔCt of the calibrator and ΔCt of the unknown sample is calculated resulting in the value of the Δ ΔCt

$$\Delta\Delta \text{Ct} = (\text{Ct target} - \text{Ct reference})\ \text{sample} - (\text{Ct target} - \text{Ct reference})\ \text{control}$$

The normalized target quantity in the sample, therefore, becomes equal to $2^{-\Delta\Delta Ct}$, and this value may be utilized for comparing the expression levels in samples. CT technique ($2^{-\Delta\Delta Ct}$) was used to determine the relative fluctuation in the mRNA expression levels. Livak formula was used to calculate and analyze the results.[14]

Ethical approval

The Ethics Committee of College of Science, University of Baghdad approved this work (Ref. CSEC/1022/0154) dated October 5, 2022. All participants agreed to provide the investigator with the specimens. An informed consent according to the Declaration of Helsinki was obtained from all participants.

Results

Fifty isolates were identified as *K. pneumoniae* out of the 150 isolates by using selective and differential media to isolate *K. pneumoniae*. The Vitek-2 system was utilized to confirm bacterial species diagnosis of those 50 isolates.

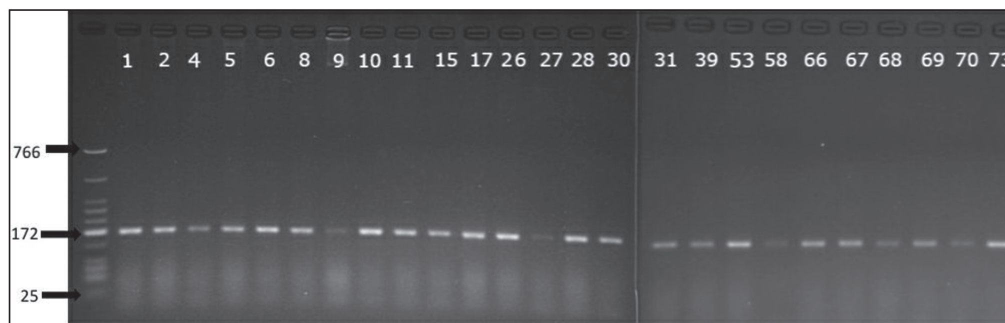

Figure 1: The positive results for the identification of Klebsiella spp. by utilizing the *16S rRNA* gene universal primers. Isolates 1, 2, 4, 5, 6, 8, 9, 10, 11, 15, 17, 26, 27, 28, 30, 31, 39, 53, 58, 66, 67, 68, 69, 70, 73 show a clear band on the gel electrophoresis on 2% agarose gel 80 V for 80 min

Detection of the presence of the capsule

A capsule detection test was done to confirm which isolate contained a capsule to undergo a polymerase chain reaction (PCR) for further confirmation before RT-PCR. Each bacterial sample was fixed on slide then examined by using light microscope. Out of the 50 bacterial isolates 25 tested positive for the presence of the capsule, and a positive result exhibited a clear halo surrounding the bacterium.

Molecular identification using 16S *rRNA*

All 25 (100%) isolates were confirmed to be *K. pneumoniae* using 16S RNA. As shown in Figure 1, *K. pneumoniae* yielded positive findings at the 172 bp domain for the *16S rRNA* gene by PCR using this molecular technique. Polymerase chain reaction products were examined on an agarose gel.

Sub inhibitory concentrations levels of carbapenems used on *K. pneumoniae*

The results of tests conducted on two resistant and one susceptible *K. pneumoniae* isolates showed that their MICs were 0.5 μg/mL for resistant isolates and 0.25 μg/mL for sensitive isolate [Table 3].

Table 3: Minimum inhibitory concentration of carbapenems used in this study

Number of isolates	subMIC (μg/mL) imipenem	subMIC (μg/mL) meropenem
K2	0.5	0.5
K6	0.5	0.5
K58	0.25	0.25

Quantitative reverse transcription polymerase chain reaction results for measuring the gene expression

Using the two-step RT-PCR approach, quantitative real-time PCR was carried out to ascertain the impact of sub-MIC carbapenems antibiotics on the expression of *ycfm* in *K. pneumoniae* isolates. The results demonstrated a change in the *ycfm* gene under the effect of both imipenem and meropenem for all three isolates an increase in the expression level (upregulation) of the *ycfm* gene in all three isolates treated with meropenem and a decrease in gene expression level (down-regulation) in all three isolates treated with imipenem using the Livak equation 2^ΔΔct which is a simple and direct method for measuring relative changes in gene expression in real-time quantitative PCR experiments. The result of qRT-PCR in this study revealed that the expression of the *ycfm* gene in the existence of carbapenems by firstly using imipenem for all three isolates was (0.067) for the first isolate, (0.050) for the second and (0.013) for the third. All resistant isolates had the same concentration for the subMIC which is 1.9 while the sensitive isolate had the subMIC of (1.9), while the expression of the *ycfm* gene for the second antibiotic meropenem the first sample was (4.08) the second isolate (8.1) as for the third isolate it was (60.8) and the concentration of the subMIC was (0.5) for both first and second isolates but was 0.25 for the third at a concentration compared to control samples as shown in Table 4.

Discussion

The current study revealed that out of the fifty confirmed isolates 25 (50%) isolate were confirmed to have the capsule using the forementioned phenotypic method. It is widely recognized that various strains of *K. pneumoniae* exhibit varying levels of capsular polysaccharide expression, and moreover, this phenomenon is associated with differences in virulence.[15]

Using the *16S rRNA* technique to confirm the finding because of its benefits Because of the influence of environmental factors such as temperature, pH, and growing conditions on phenotypic traits, genotypic characterization methods are frequently regarded as more accurate than phenotypic procedures[16] and in fact this technique confirmed that the 25 (100%) isolates were indeed *K. pneumonia*.

In the current study the microtiter plate results for *K. pneumoniae* were between 0.25 and 0.5, and it was

Table 4: Fold change in gene expression of *ycfm* gene in isolates before and after treatment with carbapenem antibiotics

Isolates	State of isolate	16S RNA	ycfm	ΔCT	ΔΔCT	Folding
K2	Treated with imipenem	8.06	32.95	24.89	3.88	0.067
K6	Treated with imipenem	7.21	31.16	23.95	4.3	0.050
K58 sensitive	Treated with imipenem	26.88	0	26.88	6.18	0.013
K2	Treated with meropenem	9.73	28.71	18.98	-2.03	4.08
K6	Treated with meropenem	22.68	35.99	13.31	-6.34	8.1
K58 sensitive	Treated with meropenem	24.53	35.98	11.45	-9.25	60.8
K2	Untreated	10.91	31.92	21.01	0	1
K6	Untreated	9.48	29.13	19.65	0	1
K58 sensitive	Untreated	10.48	31.18	20.7	0	1

discovered that meropenem was substantially more active than imipenem. There have been earlier reports of meropenem having greater activity against *K. pneumoniae in vitro* when compared to imipenem, the bacterial cell wall is affected by carbapenems, which also stop the synthesis of new bacterial cell walls, causing the bacterial cells to lyse.[17] The investigated bacteria varied in their intrinsic levels of antibiotic resistance, which might explain the reported variations in MIC values between isolates.

The experiment of the effect of carbapenem antibiotics namely meropenem and imipenem, the objective of this step was to estimate the *ycfm* gene expression by comparing the gene expression in the presence of carbapenem (imipenem, meropenem) (at subinhibitory concentration) and in the absence of carbapenem in order to determine the effect of carbapenem on the capsule gene in *K. pneumonia*.[18]

One of the most intriguing aspects of the *ycfm* gene is its role in stress response and tolerance. When bacteria encounter harsh environmental conditions, such as high temperatures, low pH, or exposure to toxins, the *ycfm* gene becomes upregulated. Studies suggest that the *ycfm* protein may help bacteria protect themselves from damage caused by various stressors, enhancing their chances of survival[19] and that explains the upregulation noticed in all isolates using meropenem. It is important to know how the more effective antibiotic against gram-negative bacilli is meropenem.[20]

It was also noticed that all isolates treated with sub-inhibitory concentrations of imipenem were downregulated and that could be due to adaptive response to antibiotic exposure. Bacteria can evolve to become resistant to antibiotics by reducing the expression of target genes or upregulating efflux pumps that remove the antibiotic from the cell. This downregulation of antibiotic-targeted genes can help the bacteria survive and grow in the presence of the drug.[21]

Although both antibiotics belong to the carbapenem class, the key structural distinctions between imipenem and meropenem are in the structures linked to positions on the C1 and C2 in the carbon chain and therefore it may show variation in how it affects the expression of the capsule-associated gene *ycfm*. However, it was noticed in this study that the mentioned effect was not limited to the type of isolate whether it was resistant or sensitive nonetheless the sensitive isolate showed extreme levels of change in expression even though all isolates were treated with the low level of subMIC, and that may be attributed to its sensitive nature.[22,23]

Conclusion

The gene expression of the capsule-associated gene *ycfm* was influenced by carbapenem antibiotics in all isolates. The expression of the *ycfm* gene was decreased in all isolates when treated with imipenem, whereas it was increased in all isolates when treated with meropenem, regardless of the isolate's nature. This can be attributed to the fact that the *ycfm* gene is not responsible for acquiring resistance, but rather it is a stress response gene associated with capsule formation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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