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Sub-Inhibitory Exposure to Meropenem Upregulates Virulence Gene Expression in Carbapenem Resistant *Klebsiella oxytoca*

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ABSTRACT: ***Background:*** *Klebsiella oxytoca (K. oxytoca) is an opportunistic Gram-negative pathogen that is becoming linked to healthcare-associated infections. The development of carbapenem-resistant strains is a major cause for concern in the clinical setting. Sub-inhibitory concentrations (sub-MICs) of antibiotics have been shown to affect the virulence and the formation of biofilms for bacteria.* ***Objective:*** *This study was designed to assess the effect of sub-MIC levels of meropenem on the expression of some virulence genes in carbapenem-resistant K. oxytoca.* ***Methods:*** *Ten clinical isolates of carbapenem-resistant K. oxytoca were isolated from patients diagnosed with hemorrhagic colitis. Isolates were determined by phenotypic, biochemical, and molecular techniques. Antibiotic susceptibility testing and minimum inhibitory concentration (MIC) determination were done based on the CLSI guidelines. Biofilm formation was measured by using the microtiter plate assay. The expression of virulence-associated genes (mrkA, fimA, pilQ, matB, and npsB) was studied by means of quantitative real-time PCR (RT-qPCR) under planktonic and biofilm growth conditions and after exposure to sub-MIC concentrations of the antibiotic meropenem.* ***Results:*** *All isolates were resistant to multiple drugs but sensitive to colistin, fosfomycin, and nitrofurantoin. Biofilm formation assays showed that all the isolates were moderate producers of biofilm. Gene expression analysis showed that virulence genes were significantly more expressed in biofilm than in planktonic condition, i.e., mrkA (2.6-fold), fimA (2.2-fold), pilQ (1.6-fold), matB (1.4-fold), and npsB (1.2-fold). Exposure to sub-MIC meropenem led to further expression of mrkA (1.8-fold) and fimA (1.6-fold).* ***Conclusions:*** *The results suggest that the expression of virulence-associated genes in carbapenem-resistant K. oxytoca can be increased by biofilm growth and sub-MIC antibiotic exposure. These results call on the potential function of sub-therapeutic levels of antibiotics in modulating the pathogenicity of bacterial pathogens.*

KEYWORDS: *Klebsiella oxytoca; Carbapenem resistance; Biofilm; Virulence genes; Meropenem*

INTRODUCTION

Klebsiella oxytoca (K. oxytoca) is an opportunistic, Gram-negative, family *Enterobacteriaceae* bacterial pathogen. It is increasingly known as an important nosocomial pathogen causing a variety of infections such as urinary tract infections, bacteremia, pneumonia, and antibiotic-associated hemorrhagic colitis [1]. Carbapenem-resistant *K. oxytoca* (CRKO or CR-*K. oxytoca*) is considered an important healthcare concern because of high rates of mortality and morbidity, similar to carbapenem-resistant *Klebsiella pneumoniae* [2], [3]. In recent years, the development of carbapenem-resistant strains of *Klebsiella* species has led to a significant public health concern globally because of the

lack of therapeutic options and the increase in mortality rates [4]. Carbapenem-resistant *Enterobacteriaceae*, including CRKO, are recognized as critical priority pathogens, underscoring the need for urgent research and development of novel antibiotics [5]–[7]. The pathogenic potential of *K. oxytoca* is attributed to a great extent to its capacity to adhere to host tissues and to form biofilms. Biofilms are organized populations of microorganisms that are encapsulated inside a self-produced, extracellular matrix material that promotes the survival of bacteria in the environment under stress conditions, including exposure to antibiotics. Adhesion factors such as fimbriae and pili are important in the process of initial attachment and biofilm development [8]. Several genes have been implicated in the adhesion and biofilm formation of *K. oxytoca*. For example, the *fimA* gene codes for type 1 fimbriae, which help bacteria to attach to epithelial cells. Similarly, *mrkA* encodes the major structural component of type 3 fimbriae that are strongly linked with biofilm formation on abiotic surfaces. Other genes such as *pilQ*, *matB*, and *npsB* are also involved in contributing to the virulence and pathogenicity of the bacteria [9]. The *npsB* gene in *K. oxytoca* encodes a toxin with a remarkable role in its pathogenesis. The presence of the *npsB* gene in *K. oxytoca* is associated with the synthesis of cytotoxins such as tilimycin and tilivalline that are contributed to the virulence of the bacteria [10]–[14]. The biofilm formation by *K. oxytoca* is associated with increased antibiotic resistance, particularly to extended-spectrum -lactamases (ESBLs) such as bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$, which are prevalent among drug-resistant isolates [15], [16]. This resistance complicates treatment and contributes to the chronicity and recurrence of infections [9].

One of the critical virulence factors in *K. oxytoca* is its capsule, which plays a significant role in bacterial attachment, biofilm formation, and resistance to host immune responses [17], [18]. The capsule enables the bacterium to evade phagocytosis and opsonization, thereby enhancing its ability to cause systemic infections. In a previous study using PCR analysis and other epidemiological analyses, various serotypes of *K. oxytoca* were identified, but not the frequent serotypes K1 and K2 found in *Klebsiella pneumoniae* [19], [20]. This diversity in capsular types suggests a complex role of the capsule in the pathogenesis of *K. oxytoca*. *K. oxytoca* produces cytotoxins such as tilivalline and kleboxymycin, which induce cell apoptosis and are implicated in antibiotic-associated hemorrhagic colitis (AAHC). Notably, cytotoxin production is influenced by environmental conditions, with microaerobic and aerobic conditions promoting higher toxin levels [1], [21]. *K. oxytoca* synthesizes and secretes siderophores, which are high-affinity iron-chelating compounds essential for bacterial metabolism and virulence [22]–[24]. Siderophore production is crucial for iron acquisition in the host environment, where free iron is limited. Aerobactin, a specific type of siderophore, is occasionally produced by *Klebsiella* strains and is associated with increased virulence [25], [26]. Sub-inhibitory concentrations (sub-MICs) of antibiotics have the potential to modulate the expression of bacterial virulence factors, thereby influencing bacterial pathogenesis. For example, sub-MICs of imipenem have been shown to increase the formation of biofilms and alginate production by Pseudomonas aeruginosa through modifications in the expression of genes associated with alginate biosynthesis [27]. Sub-inhibitory concentrations (sub-MICs) of antibiotics are frequently found in the clinical and environmental environment due to inappropriate antibiotic use, individual differences in their pharmacokinetics, or incomplete treatment courses [28].

Previous research has shown that when bacteria are exposed to sub-MIC levels of antibiotics, they may change the way their genes are expressed and increase the production of virulence factors [29]. Understanding how bacteria are affected by sub-MIC concentrations is therefore important to understand how to best optimize antibiotic therapy and prevent unwanted stimulation of pathogenic traits. Accordingly, the present study aimed to investigate the effect of sub-MIC meropenem on the expression of selected virulence genes in carbapenem-resistant *K. oxytoca* isolates. The aim of this study was to determine the effect of sub-MIC concentrations of meropenem on the expression of *mrkA*, *fimA*, *pilQ*, *matB*, and *npsB* genes in CR- *K. oxytoca*.

MATERIALS AND METHODS

Bacterial Isolates

A total of ten clinical isolates from patients diagnosed with hemorrhagic colitis were collected from patients in the Medical City Hospital, Baghdad, between March and September 2024, and they were carbapenem-resistant *K. oxytoca*. Bacterial identification was done by standard phenotypic and biochemical tests and subsequently confirmed by PCR amplification of *pehX* gene. positive Simmons citrate test, and amplification of the *pehX* gene via polymerase chain reaction (PCR), as shown in Table 1 [30], [31].

Table 1. The sequences and primers utilized for PCR and RT-qPCR in this research

Primer	Sequence 5 to 3	Product size (bp)	Annealing (°C)	Reference
pehX	F: GATACGGAGTATGCCTTTACGGTG R: TAGCCTTTATCAAGCGGATACTGG	344	55	[32]
16s rRNA	F: AGAGTTTGATCCTGGCTCAG R: GGTTACCTTGTTACGACTT	300	52	[33]
mrkA	F: CTGGCCGGCGCTACTGCTAAG R: CACCCGGGATGATTTTGTTGG	127	60	[11]
fimA	F: GCACCGCGATTGACAGC R: CGAAGGTTGCGCCATCCAG	132	61	[11]
matB	F: GTACTGGGCGGCAACCTTAG R: GTGCCGCTGATGATGGAGAA	98	61	[11]
pilQ	F: TCCGCCAGGCTCCACTTC R: GCTCGCGGGCATCTGAC	194	61	[11]
npsB	F: GGGCAGCGTTTTCCAGATGTAG R: CGCTCCCCACTTCGCTCAA	424	60	[34]

Extraction of DNA

Using a PCR technique, the reaction was initiated using an initial total reaction volume of 20 µL; this included 5 µL of the PCR master mix, 1 µL of the specific forward and reverse primers, 9 µL of double-distilled water (ddH_2O), and 4 µL of the template DNA source. The thermal cycling conditions were as follows: denature at 94 °C for 3 minutes, then perform 30 denaturation cycles at 94 °C for 30 seconds, anneal at 61 °C for 30 seconds, and extend at 72 °C for 30 seconds. The cycle was then completed with a 10-minute single extension at 72 °C. The outcomes of the PCR product are checked using agarose gel electrophoresis on a 1% (w/v) agarose gel at 70 volts for 60 min.

Assessment of Antibiotic Susceptibility

Antibiotic susceptibility testing was performed by the disk diffusion method on the Mueller-Hinton agar following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The antibiotics used for testing were tetracycline, amikacin, fosfomycin, nitrofurantoin, colistin, ciprofloxacin, gentamicin, piperacillin-tazobactam, cefoxitin, ceftazidime, meropenem, imipenem, cefotaxime, and cefepime. The isolates were evaluated in terms of antibiotic susceptibility using the disk diffusion technique, following the standards of CLSI (Clinical and Laboratory Standards Institute) in 2023. The antibiotics employed in this assessment included tetracycline (30 µg), amikacin (10 µg), fosfomycin (200 µg), nitrofurantoin (300 µg), colistin (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), piperacillin-tazobactam (100/10 µg), cefoxitin (30 µg), ceftazidime (30 µg), meropenem (10 µg), imipenem (10 µg), cefotaxime (30 µg), and cefepime (30 µg) (MAST Co. UK). A bacterial suspension was prepared for each isolate to achieve an opacity equivalent to a half McFarland standard, which was then spread onto Mueller-Hinton agar (MHA) (Rosco Company). Antibiotic disks were placed at standardized intervals, and the plates were incubated for 16-18 hours at 37 °C. Isolates exhibiting resistance to carbapenem were selected for further analysis [35].

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of meropenem (Sigma Co., USA) dilutions. was determined by the agar dilution method following the recommendations of CLSI. The MIC values ranged between 0.5 and 128 ug/mL.

Biofilm Formation Assay

The assay for biofilm formation was executed in accordance with previously documented methodologies [11]–[13]. Biofilm formation was determined by the microtiter plate assay method using crystal violet staining. Overnight cultures of bacteria were diluted and plated in 96-well plates with the media tryptic soy broth. Wells were then washed with phosphate buffered saline and stained with 0.1%

crystal violet after incubation. The bound dye was solubilized by ethanol, and the optical density was measured at the wavelength of 490 nm.

Gene Expression

1 RNA Extraction and cDNA Synthesis

Total RNA was extracted from bacterial cultures with TransZol UpPlus RNA extraction reagent as per the manufacturer's instructions. Extracted RNA was then reverse transcribed from cDNA using cDNA synthesis kit followed by a commercial cDNA synthesis kit.

2 The cDNA Synthesis

The cDNA template was obtained by reverse transcription (EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit) according to the manufacturer's instructions.

3 Gene Expression Analysis

Quantitative real-time PCR (RT-qPCR) was carried out to measure the level of expression of the virulence associated genes (*mrkA*, *fimA*, *pilQ*, *matB*, and *npsB*). The *gyrB* gene was used as an internal reference gene. Relative gene expression was calculated based on the 2-DDCt method.

Data Analysis

Statistical analyses were conducted using the software package of the statistical package system (SPSS, version 20). Differences between experimental groups were assessed with appropriate statistical tests, and a p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Antibiotic Susceptibility

All the isolates showed resistance to several antibiotics, such as cefepime, ceftazidime, cefotaxime, imipenem, ciprofloxacin, meropenem, piperacillin-tazobactam, and gentamicin. However, all isolates were still sensitive to colistin, fosfomycin, and nitrofurantoin.

Phenotypic Biofilm Formation

The microtiter plate assay indicated moderate biofilm-forming ability for all the isolates of *K. oxytoca* based on reading of optical density. All of the CR-*K. oxytoca* isolates were moderate-level biofilm producers, as shown in Table 2.

Analysis of Gene Expression

RT-qPCR analysis indicated that the virulence-related genes were significantly upregulated when grown in biofilm as compared to planktonic conditions. The fold increase in gene expression was as follows:

mrkA: 2.6-fold
fimA: 2.2-fold
pilQ: 1.6-fold
matB: 1.4-fold
npsB: 1.2-fold

Furthermore, exposure to sub-MIC concentrations of meropenem led to enhanced expression of some virulence genes, especially *mrkA* (1.8-fold) and *fimA* (1.6-fold). There was a significant increase in the expression of biofilm-related genes in biofilm mode compared to planktonic growth, including *mrkA* 2.6-fold ($p<0.0001$), *fimA* 2.2-fold ($p<0.0001$), *pilQ* 1.6-fold ($p<0.001$), *matB* 1.4-fold ($p<0.001$), and *npsB* 1.2-fold ($p=0.002$) genes. Moreover, meropenem at 8 µg/ml increased the expression of *mrkA* 1.8-fold ($p<0.0001$) and *fimA* 1.6-fold ($p<0.0001$) in planktonic growth compared to those untreated isolates. The gene expression profile is shown in Figure 1. In planktonic growth, the expression of biofilm-related virulence genes was lower.

Table 2. The biofilm formation by untreated bacterial isolates in this study

Isolate	Mean OD	Biofilm level
K1	0.250	Moderate
K2	0.261	
K3	0.249	
K4	0.291	
K5	0.254	
K6	0.268	
K7	0.257	
K8	0.291	
K9	0.284	
K10	0.257	

OD: Optical density

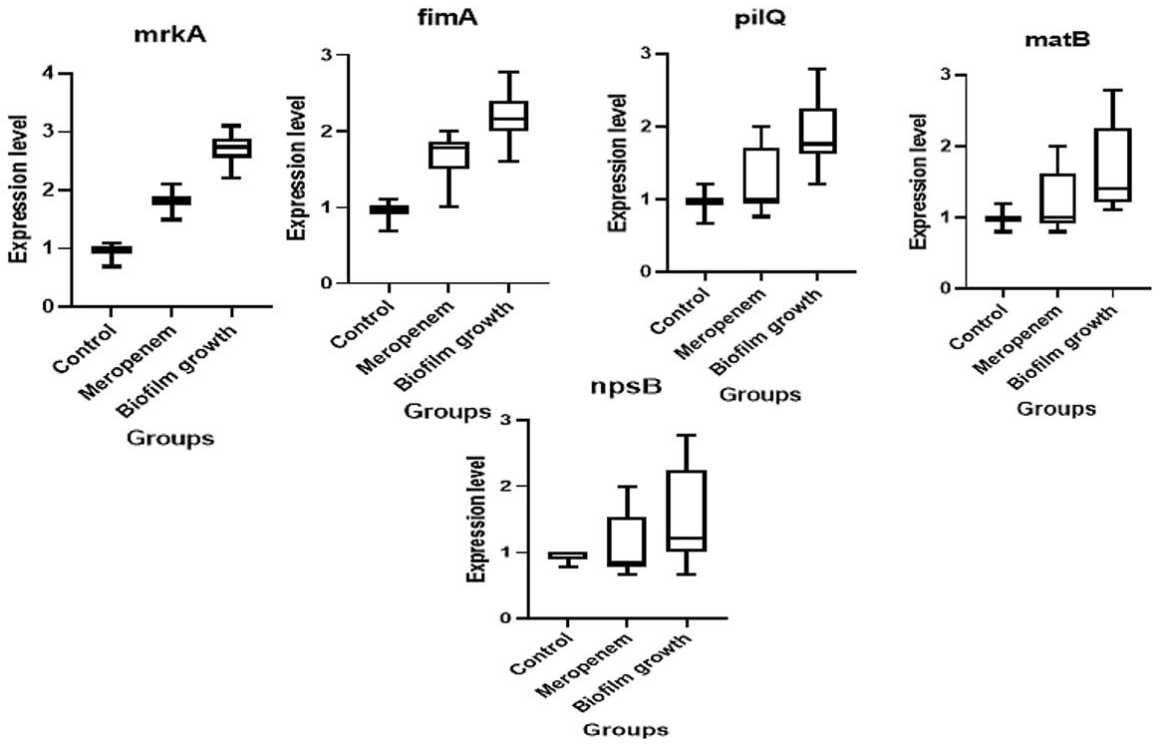

Figure 1. The regulation of adhesin gene expression across three different circumstances

The development of carbapenem-resistant *Klebsiella* species is considered one of the major problems of modern clinical microbiology. In the present study, all isolates were multidrug resistant, indicating that limited therapeutic options are available to treat infections caused by these pathogens. Hasan and Bakr (2022) observed that the highest antibiotic resistance pattern of *K. oxytoca* was 100% against Augmentin, Ampicillin, Cephalothin, Piperacillin, and Rifampin on one hand and 62.50%, 59.37%, 53.12%, and 50% against Ceftazidime, Cefixime, Cefotaxime, Trimethoprim, and Aztreonam on the other hand, respectively. Mustaf *et al.* (2025) showed that cefotaxime, tetracycline, and ciprofloxacin showed the lowest effect on *K. oxytoca* isolates, while imipenem and gentamicin showed the highest effect.

Moreover, a recent study in Iraq found that *K. oxytoca* isolates demonstrated a high level of resistance against amoxicillin (98.5%), cefotaxime (92.6%), cefepime (86.7%), and piperacillin (88.2%). Researchers reported a reduced level of resistance (65.4% and 59.5%) to tetracycline and ciprofloxacin, respectively. This study examined the effect of sub-MIC concentrations of meropenem on virulence

gene expression in CR-*K. oxytoca*. The data indicate that there is the potential for a significant alteration of virulence factors even at low levels of meropenem, which may influence pathogenicity. Importantly, exposure to sub-MIC levels of meropenem resulted in additional expression of some of the virulence genes. This observation suggests that low concentrations of antibiotics may act as environmental signals, which affect the expression of bacterial genes and increase pathogenic potential. More studies are necessary to provide researchers with insight into the mechanisms that regulate virulence gene expression in response to sub-MIC levels of antibiotics and to provide clinicians with recommendations on how to reduce or eliminate the risk of enhanced infections associated with sub-MIC exposure. Similar results have been obtained from other bacterial pathogens, in which sub-inhibitory concentrations of antibiotics were found to enhance virulence factor production and biofilm formation.

These results are concerning in that they show how inappropriate antibiotic usage can have ramifications and the importance of antibiotic stewardship. This research evaluated the effect of sub-MIC levels of meropenem on the expression of virulence genes of CR-*K. oxytoca*. The study focused on analyzing the expression levels of the *matB*, *mrkA*, *npsB*, *fimA*, and *pilQ* genes under both planktonic and biofilm growth conditions, which demonstrated significantly higher expression rates in the latter conditions. Additionally, meropenem treatment increased the expression of these virulence genes significantly compared to the planktonic mode of growth. Biofilm formation is crucial in bacterial persistence and antimicrobial agents' resistance. The results of this study showed that all isolates were capable of forming moderate biofilms, and this is consistent with previous studies reporting a strong relationship between biofilm formation and increased antibiotic resistance among *Klebsiella* species. Gene expression analysis showed that there is a significant gene upregulation of adhesion-related genes during biofilm growth in comparison with planktonic conditions. These results are consistent with previous reports showing that genes like *mrkA* and *fimA* are involved in the basic development of biofilms and bacterial adhesion [14]–[18]. Additionally, *K. oxytoca* with the ability to produce toxins are responsible for AAHC, and we observed the *npsB*-positive for all of the isolates, having caused AAHC in patients [3], [20]. Antibiotic exposure may increase bacterial pathogenicity, as seen in this research where the expression of *mrkA* and *matB* genes enhanced significantly following meropenem treatment. It has been unveiled that the virulence of bacteria like Pseudomonas aeruginosa and Acinetobacter baumannii can be affected by antibiotic exposure [13], [23]. Consequently, the unregulated consumption of antibiotics should be controlled to intercept the development of resistance and effects of bacterial virulence. In this in vitro study, antibiotic exposure could elevate the virulence of CR-*K. oxytoca* isolates by upregulating adhesin and capsular polysaccharide biosynthesis genes. This raises concerns regarding the use of low-dose antibiotic therapy for infections.

Moreover, previous research indicates that sublethal antibiotic levels can promote bacterial resistance and induce changes in molecular signaling pathways [25]. The precise mechanisms behind these effects remain to be entirely appreciated. For example, a study demonstrated that sublethal ciprofloxacin doses altered the Staphylococcus saprophyticus and Escherichia coli isolates' pathogenicity in experimental conditions, increasing adhesin expression crucial for urothelial colonization and biofilm formation in a mouse urinary tract model [5]. However, in this study there are a number of limitations. The relatively low number of isolates may not allow generalizability of the results. In addition, gene expression was only assessed at the transcriptional level, and no evaluation of protein expression was made.

CONCLUSION

The present study shows that growth in the biofilm dramatically increases expression of virulence-associated genes in the carbapenem-resistant *Klebsiella oxytoca*. Moreover, exposure to sub-MIC levels of meropenem may also lead to further stimulation of the expression of certain adhesion genes. These results indicate that sub-therapeutic exposure to antibiotics may potentially cause an increase in bacterial virulence, which may contribute to infection persistence. Further studies with higher numbers of animals and analyses of protein expression are needed to gain a better understanding of the mechanisms that underlie antibiotic-induced virulence regulation.

SUPPLEMENTARY MATERIAL

None.

AUTHOR CONTRIBUTIONS

Istabreq Muhammed Ali Salman: Conceptualization and Validation. Shaymaa Khudhr Al-Alak: Investigation. Saba Saadoon Khazaal: Methodology and Formal Analysis. Hussein T. Abdulabbs: Writing – original draft. Ali A. Hanoone: Writing – review and editing.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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CONFLICTS OF INTEREST

The author declares no conflicts of interest.

DECLARATION OF GENERATIVE AI USE

During the preparation of this work, the authors used Grammarly for grammar checking and language polishing. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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