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Effects of sub-inhibitory concentrations of antibiotics and oxidative stress on the expression of type II toxin-antitoxin system genes in *Klebsiella pneumoniae*

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ABSTRACT

Objectives: Sub-inhibitory concentrations (sub-MICs) of antibiotics reflect the conditions that bacteria encounter in tissues and the natural environment. Sub-MICs of antibiotics can induce stress and alter the expression of different bacterial genes. Bacteria react to stress conditions using different mechanisms, one of which is the toxin-antitoxin (TA) system. This study investigated the expression of the TA system genes under oxidative and antibiotic stresses in *Klebsiella pneumoniae* (*K. pneumoniae*).
Methods: To determine the effects of sub-MICs of gentamicin, nalidixic acid, ceftazidime, and certain concentrations of H_2O_2 on bacterial survival and growth, colony forming units were quantitated and turbidity was assessed following the treatment of *K. pneumoniae* with $^1/_2$ MICs of antibiotics and 5 mM H_2O_2 at different time intervals. The expression of TA system genes in *K. pneumoniae* was evaluated 1 h after treatment using the quantitative real-time PCR (qRT-PCR) method.
Results: The results revealed reduced *K. pneumoniae* growth in the presence of sub-MICs of antibiotics and 5 mM H_2O_2 compared to the control. Furthermore, according to the results of the qRT-PCR assay, only the presence of gentamicin could increase the expression of TA system genes.
Conclusion: Although the exact role of the TA systems in response to stress is still unclear, this study provided information on the effect of the type II TA systems under oxidative and antibiotic stress conditions.

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is an important opportunistic pathogen frequently found in the human flora of the skin and digestive and respiratory tracts. It leads to a wide range of hospital-acquired and community-acquired infections, including: urinary tract infections, septicaemia, pneumonia, intra-abdominal infections, and respiratory tract infections [1]. Currently, multi-drug-resistant *K. pneumoniae* isolates have increased due to the widespread use of antibiotics and has become a major health concern [2,3].

In order for an antibiotic to act against bacterial pathogens, it should be used at a concentration above the minimum inhibitory concentration (MIC) in successive doses. However, after a specific period the concentration of antibiotics in different tissues generally becomes lower than the respective MIC. The sub-minimum inhibitory concentrations (sub-MICs) are defined as concentrations lower than the MIC values [4]. Many studies have shown that sub-MICs of antibiotics can act as signal molecules and may alter their physicochemical characteristics and expression of bacterial virulence [5,6]. The changes caused by sub-MICs of antibiotics are indicative of a condition that bacteria encounter in a wild environment and the way that they cope with it [7].

Klebsiella pneumoniae, similar to many other pathogens, faces many stress conditions during infection and colonisation of the human body. Oxidative stress is one of the most important stress conditions encountered by bacteria. During infections caused by aerobic bacteria, oxidative stress is induced by reduced oxygen levels or generated reactive oxygen species such as H_2O_2, leading to

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the disruption of the cellular membrane, DNA and proteins. According to recent studies, antibiotic treatment can also give rise to oxidative stress. Consequently, several bacteria have employed various mechanisms to reduce the effects of oxidative stress [8,9]. In fact, the increased antioxidant ability protects bacteria from the host immune response and antibiotic therapy. Therefore, in order to combat bacterial infections and failure in antibiotic therapy it seems critical to identify the factors involved in antioxidant protection [10].

Toxin-antitoxin (TA) systems contribute to bacterial survival during stress conditions [11]. Six types of TA systems have been identified, with the type II TA system being the most studied. In the type II TA system, two protein genes (toxin and antitoxin) are co-expressed [12]. In normal conditions the labile antitoxin inhibits the activity of the stable toxin by forming a tight protein-protein complex [13]. More than 3 decades ago, the first TA system was identified as an 'addiction module' and was shown to play an important role in plasmid maintenance. When a TA system-encoding plasmid leaves a cell, the labile antitoxin is degraded and the remained cognate toxin induces cell death [14]. After the initial discovery, a large number of TA systems were found on the chromosome of a wide variety of bacteria. The role of chromosomal type II TA systems is not yet clearly understood; however, it seems that they are involved in bacterial responses to stress conditions. In stress conditions, unstable antitoxin is degraded by proteases such as ClpXP and Lon [13,15]. Antitoxin degradation leads to the toxin activation, which subsequently interferes with various cellular processes (DNA or protein synthesis, cell wall synthesis and cell division), causes slow cellular growth and induces different responses to different stress conditions such as oxidative stress, nutrient deprivation and antibiotic stress [16,17].

Therefore, to provide more information about the role of TA systems and Lon protease in oxidative and antibiotic stresses, the current study evaluated the expression levels of the type II TA system and *lon protease* genes of *K. pneumoniae* treated with sub-MICs of gentamicin, nalidixic acid, ceftazidime, and H_2O_2.

2. Material and methods

2.1. Bacterial strain and growth conditions

K. pneumoniae ATCC 13883 was used in this study. The bacterial strain was stored in brain heart infusion broth containing 20% glycerol at −80 °C.

2.2. Identification of type II TA systems

The nucleotide sequence of *K. pneumoniae* ATCC 13883 was downloaded from the National Center for Biotechnology Information. Type II TA systems were determined by the TADB database [18] and specific primers were designed using primer3 software (Table 1). Lastly, PCR assay was used to determine the presence of the genes coding for Lon protease and type II TA system (*relE1/relB1, relE2/relB2, hipA/hipB, vapC/vapB, doc/phd, mazF/mazE*) in the *K. pneumonia* strain. The PCR assay was performed in a DNA thermal cycler (PeqLab, Germany) in a final volume of 25 μL. Thermal cycling included an initial denaturation step at 94 °C for 4 min; 30 × 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 30 s, with a final extension step at 72 °C for 5 min.

2.3. Determination of minimum inhibitory concentration

The microdilution method was used to determine the MIC of *K. pneumoniae*, according to the Clinical and Laboratory Standards Institute (CLSI). To determine the MICs of gentamicin, nalidixic acid and ceftazidime (Sigma Aldrich), 100 μL of 0.5 MacFarland overnight bacterial culture (dilution of 1:100 in Mueller Hinton broth) and different concentrations of antibiotic solution were added to the 96-well microtiter plates and incubated at 37 °C for 18 h. The MIC was considered as the lowest concentration of antibiotic that prevented visible bacterial growth after 18 h of incubation. MIC values for the studied antibiotics were independently determined three times.

2.4. Growth curves and viability assessment

Growth curves and viability of *K. pneumoniae* strain were determined in the presence of sub-MIC (1/2 MIC) of each antibiotic and 5 mM of H_2O_2. The *K. pneumoniae* strain was cultivated on Luria-Bertani (LB) agar and incubated at 37 °C for 24 h. One colony from an overnight culture was inoculated in 5 mL of LB broth and incubated for 24 h at 37 °C. A second subculture was diluted 100-fold in 50 mL of LB broth and grown at 37 °C on a shaker at 200 rpm until reaching an optical density of 0.4 at 600 nm (OD600). Then, bacterial culture was diluted three-fold to reach an OD of 0.08-0.1 at 600 nm. These cultures were divided and antibiotic solutions as well as H_2O_2 were added to yield the sub-MIC of antibiotics and the concentration of 5 mM of H_2O_2. After 1, 2, 3, 4, 5, and 24 h, growth curves were turbidimetrically estimated. Then, cultures were washed twice with 0.85% sterile saline solution and serial dilution was prepared and plated onto LB agar. Colonies were counted after 24 h of incubation at 37 °C. Untreated bacterial cells were used as controls at different time intervals. All experiments were performed independently at least three times.

2.5. Quantitative real-time PCR

The expression levels of genes coding for Lon protease and type II TA systems (*relE1/relB1, relE2/relB2, hipA/hipB, vapC/vapB, doc/phd, mazF/mazE*) upon antibiotic and H_2O_2 exposure were analysed using the quantitative real-time PCR (qRT-PCR) method. Total RNA from the bacteria was extracted using high pure RNA isolation kit (Roche kit, Germany) 1 h after adding antibiotics and H_2O_2. The quantity and quality of RNA were assessed using the NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and gel electrophoresis analysis. In order to remove DNA contamination, extracted RNA was treated with DNase1 (Roche, Germany) according to the manufacturer's protocol. Total RNA was reverse transcribed to cDNA using the cDNA Synthesis Kit (Takara, Japan). The qRT-PCR (three replicates) assay for each gene was performed on Rotor-Gene thermal cycler according to the following program: one cycle of 95 °C for 12 min, 40 cycles of 95 °C for 15 s and one cycle of 60 °C for 45 s. The *kphs* gene was used as an internal control for normalisation of mRNA levels and fold changes. mRNA expression was calculated by the $2-\Delta\Delta Ct$ method [19].

2.6. Statistical analysis

Data of growth curves and viability assessment were expressed as the mean of the three independent experiments. Data obtained from the mRNA expression analysis were presented as means ± standard error of three independent assessments. Values obtained for the expression of each individual gene exposed to antibiotics and H_2O_2 were compared using one-way analysis of variance (ANOVA) test followed by Tukey's post-hoc test for multiple comparisons by Prism 8 (GraphPad Software, Inc.).

Table 1
Primers used for both PCR and qRT-PCR studies.

Primer name	Primer sequence	Product size (bp)	Reference
Kphs	F: GTCAGAGGAGTGCTTTGTCC R:GACCTTTATCGCCCTCAAGC	120	In this study
lon Protease	F:GACCGGTGAAATCACTCTGC R:GGTCGCGTTTGTTCTCATCA	122	In this study
relE1	F:CAAATGGTTCGCCAGAGAGG R: GCGATTCTTGTTGAGTCGCT	136	In this study
relB1	F:ATCCCACAGGAAACAATGCG R:ACACGGGTTGGCTAACATTG	115	In this study
relE2	F:TGTCCTCTCCCGAAAACCAC R:CCACGACGTAGCAGGTATCG	96	In this study
relB2	F:CTCTGCTGGAGTACGACGAC R: ACTTCGCCTGGTAGCATGAC	94	In this study
vapB	F:CCTGGATGGAGGTGATGGTT R:GTTCCAGACGATGGAGTTGC	192	In this study
vapc	F:ACTGTTACGTGAAGCGGTTG R: CCGTCTTCTTCGCAGTCTTG	102	In this study
MazF	F:CACGGTTTCACTGGAAGAGG R: GTATGCGTTCCAGACGCTTG	110	In this study
mazE	F:GACAGCGAAGTGGGTATGAC R: TCCATTCCCGATCCTCTTCG	136	In this study
doc	F:CGTGCCGAAGCGATAATGTA R:GATAAACAGTGCCGTACGCT	186	In this study
phd	F:CGTGCCGAAGCGATAATGTA R:GATAAACAGTGCCGTACGCT	147	In this study
hipA	F:GGAGGATCTGTGTCAGGCTT R:AGCCACTGGAAGACCATGAA	156	In this study
hipB	F:ATGGTGAGGAACAAGGCGAT R: CAGCGTGGTTAAGGTGGTTT	186	In this study

3. Results

3.1. Identification of type II TA systems

This study investigated the existence of type II TA systems in *K. pneumoniae* ATCC 13883. The results revealed the presence of all of the studied genes coding for the type II TA system and as Lon protease in this strain.

3.2. Growth curves and viability assessments

This study first determined the MIC ranges of gentamicin, nalidixic acid and ceftazidime against *K. pneumoniae* ATCC 13883 using broth microdilution assay. The MIC ranges for the selected antibiotics were 0.5 μg/mL, 0.5 μg/mL and 0.25 μg/mL, respectively. To evaluate the effects of antibiotics and oxidative stress on cell survival, the exponential phase of *K. pneumoniae* was exposed to sub-MICs of antibiotics and 5 mM H_2O_2. Both the viable counts and OD600 values were measured in five consecutive hours and 24 h post exposure.

As shown in Fig. 1, the growth of *K. pneumoniae* ATCC 13883 was severely inhibited in the presence of 1/2 MIC of gentamicin. After 24 h exposure to gentamicin, $\log_{10}$ cfu/mL reduced from 8.5 to 4.3 and the value of OD600 reached 0.085, showing the lowest value among the studied antibiotics. In the case of ceftazidime, a slight increase in the number of bacterial cells was observed in the early hours of exposure with 1/2 MIC of ceftazidime. However, after 3 h a notable decrease in the number of bacterial cells was observed. As such, after 24 h 1/2 MIC of ceftazidime induced a 4.3 log reduction in bacterial count compared with 3 h, and the OD600 value was reduced from 0.51 to 0.096. The 1/2 MIC of nalidixic acid showed the least effect on growth inhibition. In addition, within 24 h the number of living cells remained almost constant in the presence of this antibiotic. These results indicate the variations in bacterial survival following antibiotic treatment depending on the type of antibiotic. In the presence of 5 mM of H_2O_2, over a period of 5 h, a 3.3 log reduction of bacterial growth was observed and the OD600 value was reduced from 0.092 to 0.072. However, after 24 h the bacterial growth showed 2 log reduction compared with 5 h, suggesting the short-term effect of oxidative stress and bacterial adaptation to this stress condition.

3.3. Quantitative real-time PCR

To evaluate the expression of *lon protease* and type II TA system genes under antibiotic and oxidative stresses, *K. pneumoniae* ATCC 13883 was exposed to sub-MICs of selected antibiotics and 5 mM of H_2O_2 for 1 h. The results showed reduced expression levels of genes coding for the Lon protease and type II TA systems in the presence of ceftazidime. Bacterial exposure to nalidixic acid and H_2O_2 slightly increased the expression level of *lon protease* gene, while reducing the expression levels of type II TA system genes. Analysis of relative gene expression in the presence of sub-MICs of gentamicin demonstrated upregulation of *lon protease* and *relE1/relB1, hipA/hipB, doc/phd,* and *mazF/mazE* loci and decreased expression levels of the *relE2/relB2* and *vapC/vapB* loci (Fig. 2).

4. Discussion

K. pneumoniae is an important pathogen leading to a high incidence of opportunistic infections. In the last decade, the emergence of multidrug-resistant K. pneumoniae isolates has become a worldwide public health concern [1,20]. Although several mechanisms have been attributed to stress responses in K. pneumoniae, few studies have investigated the contribution of the TA systems. Therefore, this study aimed to determine the effects of H_2O_2 and sub-MICs of different antibiotics on growth and expression of the type II TA system genes in K. pneumoniae. The results showed bacterial cell reduction following exposure to the sub-MICs of gentamicin and ceftazidime compared to nalidixic acid, suggesting greater impact of gentamicin and ceftazidime sub-MICs on the growth of K. pneumoniae. Since antibiotic concentrations in different tissues are often below the MIC values, exposure to lethal doses of antibiotics seems essential for the effective treatment of K. pneumoniae infections [21]. The current study investigated the effect of H_2O_2 and sub-MICs of antibiotics on the expression of genes coding for Lon protease and type II TA systems. According to the results only gentamicin was able to upregulate the expression of the type II TA system genes, while other antibiotics and H_2O_2 downregulated the expression of type II TA system genes. Moreover, the concentration of 5 mM of H_2O_2 and 1/2 MIC of nalidixic acid slightly affected the *lon protease* expression.

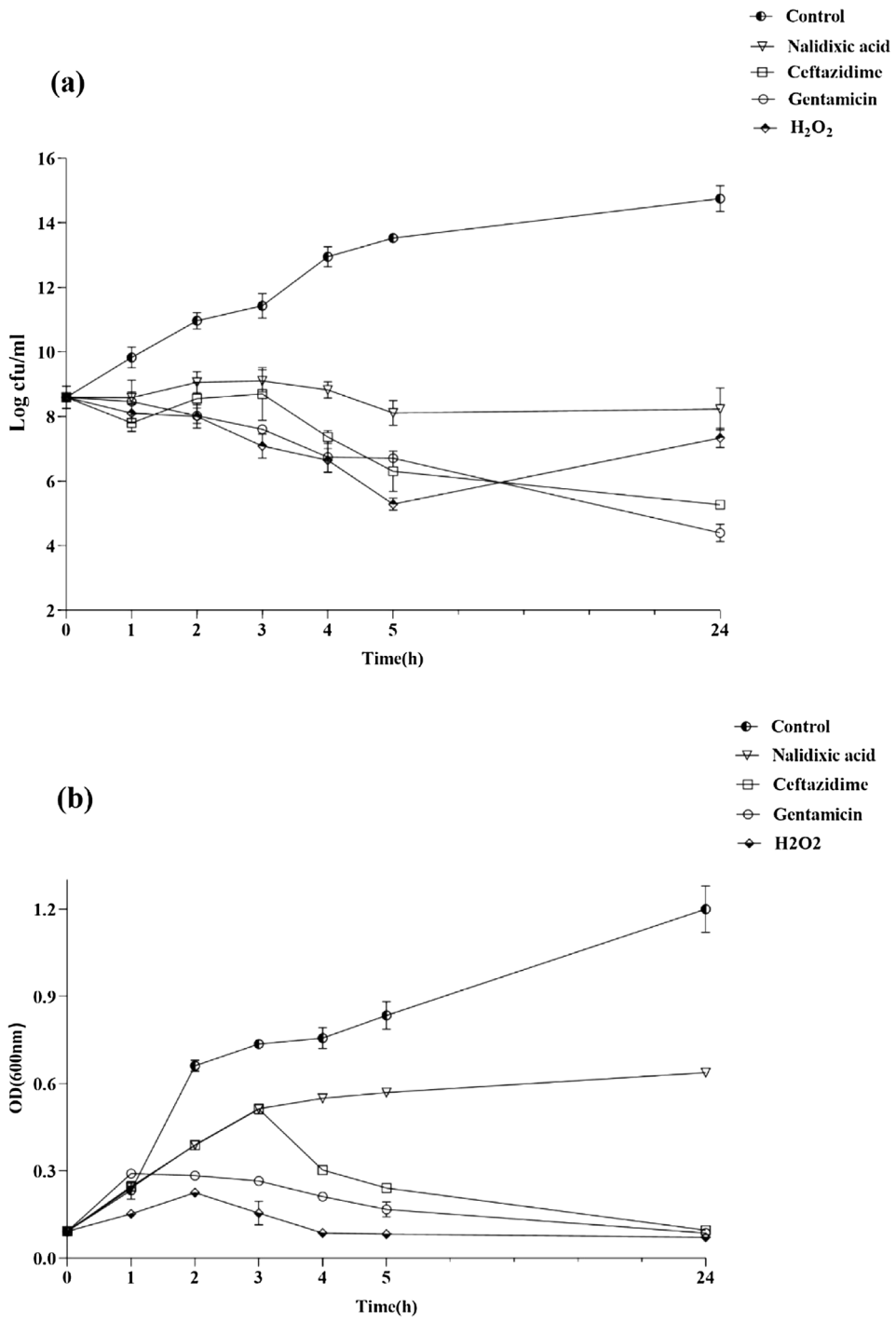

Fig. 1. Effects of sub-MICs of gentamicin, nalidixic acid and ceftazidime, and 5 mM of H_2O_2 on the survival of *Klebsiella pneumoniae* ATCC 13883. (a) Growth curves (b) viability curves. Data are indicated as the means ± SD of three independent replicates.

Considering the function of the TA systems, it was assumed that this system could be involved in responding to antibiotic and oxidative stress in *K. pneumoniae*. TA system genes are abundantly present in different bacterial genomes, and according to previous studies, under stress conditions, toxins can target diverse cellular processes such as translation, DNA replication and cell wall synthesis, leading to inhibition of cell growth, a switch to a dormant state and response to various stress conditions [22,23]. Several studies have indicated the impact of MqsA, the antitoxin of the MqsAR TA system, on rpoS transcription and its association with oxidative stress. In addition, previous studies have shown the involvement of the TA systems, especially the type II TA system, in persister cell formation and antibiotic tolerance in many bacteria [24,25].

Moyed et al. discovered that *hipA7* mutation in the toxin gene of the HipAB TA system led to a 1000-fold increase in *Escherichia coli* (*E. coli*) persistence [26]. In another study, it was shown that overexpression of RelE, the toxin of the RelBE TA system, led to the increased level of persister cell formation, while deletion of RelBE, MazEF andDinJ/YafQ did not affect persister cell formation in E. *coli* after antibiotic treatment [27]. Curtis et al. indicated that the mazEF TA system had no effect on persister cell formation during

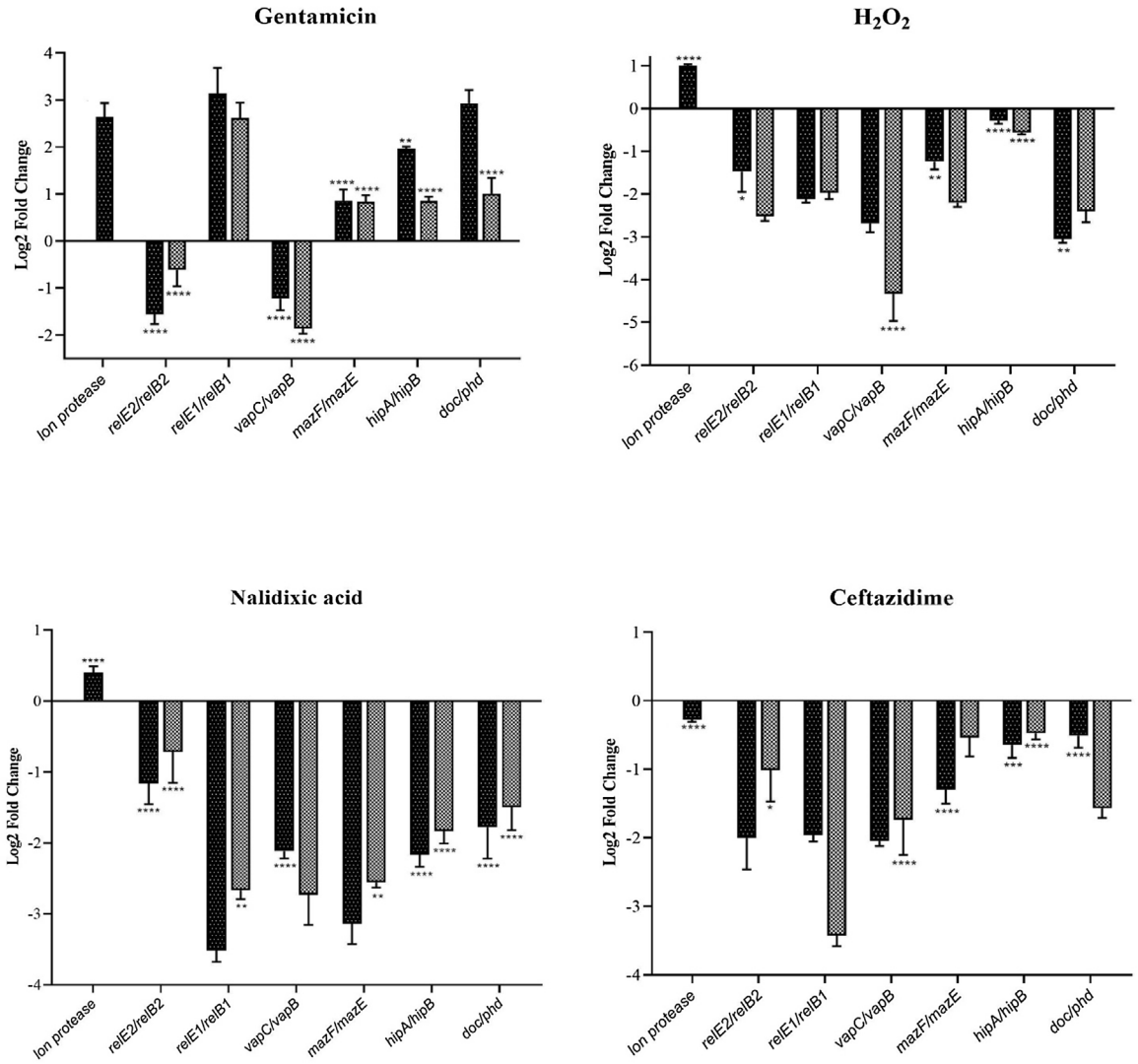

Fig. 2. Analysis of relative expression level of the *lon protease* and type II TA system genes in the presence of gentamicin, nalidixic acid and ceftazidime at sub-MICs and 5 mM of H_2O_2 in *Klebsiella pneumoniae* ATCC 13883. The expression of *lon protease* and type II toxin genes (*relE2, relE1, vapC, mazF, hipA, and doc)* and of type II antitoxin genes (*relB1, relB2, vapB, mazE, hipB and phd,)* are represented by black and grey histograms, respectively. Graph data are indicated as the means ± SD of three independent replicates. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$ by One-way ANOVA and Tukey's post-hoc test for multiple comparisons.

exposure to lethal doses of antibiotics. They also showed that following ΔmazEF mutation in *Listeria monocytogenes,* sub-MICs of norfloxacin did not affect the bacterial survival and growth rate relative to the wild type, whereas the growth of ΔmazEF *Listeria monocytogenes* decreased after exposure to the sub-MICs of ampicillin and gentamicin [28]. In the present study, the expression of all the studied type II TA loci decreased in the presence of ceftazidime, nalidixic acid and H_2O_2. On the other hand, the presence of gentamicin increased the expression of *relEB1, mazEF, hipAB,* and *doc/phd,* while decreasing the expression of *relEB2* and *vapCB.*

Lon protease is an ATP-dependent protease with a crucial role in protein quality control and regulation of various cellular processes through the degradation of misfolded, unstable and abnormal proteins [29]. Lon protease is crucial for bacterial survival and adaptation under different stress conditions [30]. Studies have shown the increased expression of this protease under antibiotic stress conditions, such as exposure to the sub-MICs of gentamicin and ciprofloxacin leading to increased antibacterial resistance [31,32]. Also, following the exposure of bacterial cells to antibiotics or other stress conditions, Lon protease contributes to the activation and regulation of TA systems by cleaving antitoxins and releasing the cognate toxins [13]. In the current study, the *lon protease* gene expression showed 6.06-fold, 1.98-fold and 1.31-fold increases in the presence of gentamicin, H_2O_2 and nalidixic acid, respectively, compared with the control samples. Expression of this protease showed 1.2-fold reduction after exposure to ceftazidime, indicating the significant effect of gentamicin on *lon protease* gene expression.

5. Conclusions

Generally, bacteria employ diverse strategies for survival following stress conditions. Bacteria can adapt to harsh conditions by alterations in different gene expression. Considering the limited information regarding the role of type II TA systems in response to various stresses in *K. pneumoniae*, the expression levels of the TA system genes in *K. pneumoniae* were studied under oxidative and antibiotic stress. The results showed that the expression levels of some of these systems increased following gentamicin treatment, while the expression levels of all the studied TA system genes decreased in the presence of ceftazidime, nalidixic acid and H_2O_2.

Therefore, these results indicate the diverse impacts of these systems depending on the type of stress, which necessitate further studies for a better understanding of the importance of these systems following stress conditions.

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

Not required.

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