Klebsiella pneumoniae

Subjects: Microbiology
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Klebsiella pneumoniae is an opportunistic pathogen that causes nosocomial and community-acquired infections. The spread of resistant strains of *K. pneumoniae* represents a growing threat to human health, due to the exhaustion of effective treatments. *K. pneumoniae* releases outer membrane vesicles (OMVs). OMVs are a vehicle for the transport of virulence factors to host cells, causing cell injury. Previous studies have shown changes of gene expression in human bronchial epithelial cells after treatment with *K. pneumoniae* OMVs. These variations in gene expression could be regulated through microRNAs (miRNAs), which participate in several biological mechanisms. Thereafter, miRNA expression profiles in human bronchial epithelial cells were evaluated during infection with standard and clinical *K. pneumoniae* strains. Microarray analysis and RT-qPCR identified the dysregulation of miR-223, hsa-miR-21, hsa-miR-25 and hsa-let-7g miRNA sequences. Target gene prediction revealed the essential role of these miRNAs in the regulation of host immune responses involving NF-κB (miR-223), TLR4 (hsa-miR-21), cytokine (hsa-miR-25) and IL-6 (hsa-let-7g miRNA) signalling pathways. The current study provides the first large scale expression profile of miRNAs from lung cells and predicted gene targets, following exposure to *K. pneumoniae* OMVs. Our results suggest the importance of OMVs in the inflammatory response.

Keywords: Klebsiella pneumoniae ; outer membrane vesicles ; inflammatory ; miRNAs ; antibiotic resistance

1. Introduction

K. pneumoniae is a significant opportunistic pathogen, mainly associated with hospital-acquired infections [1]. Studies have estimated that it causes 8% of all nosocomial bacterial infections in Europe and in the United States [2][3]. This bacterium is responsible for a broad spectrum of extraintestinal diseases such as sepsis, pneumonia, urinary tract, lungs, abdominal cavity and soft tissue infections [4]. *K. pneumoniae* has important virulence factors, such as lipopolysaccharides, a capsule, adhesins and siderophores, required for its mechanism of colonization, adherence, invasion and to enable the progression of infection [2][5]. In addition, hemolysins, tyrosine kinase, heat-stable enterotoxins and heat-labile exotoxins participate in the pathogenicity [6][7]. *K. pneumoniae* rapidly acquires antibiotic resistance mechanisms making the selection of the appropriate antibiotic treatment more challenging [1][8]. Carbapenem resistance appears to have the greatest impact on the effectiveness of the treatment. The European Centre for Disease Control and Prevention (ECDCP) assumed that 15.2% of *K. pneumoniae* strains are carbapenem resistant in Italy [9][10]. In this scenario, nosocomial *K. pneumoniae* infections reflect a 50% mortality rate if untreated [11][12]. Given the clinical significance of this pathogen, a better understanding of other mechanisms of virulence is fundamental for designing new strategies to treat Klebsiella infections. It is well established that one of the characteristics of Gram-negative bacteria is their ability to form vesicles from the outer membrane, called outer-membrane vesicles (OMVs) [13][14][15]. OMVs are lipid bilayer spherical nanostructures with a diameter of 20–250 nm that are released into the host environment [16][17][18]. The surface of these vesicles is composed of lipopolysaccharide (LPS), phospholipids and outer membrane proteins [19][20]. The vesicular lumen, however, contains periplasmic and cytoplasmic components, including genetic material and virulence factors, such as invasion associated factors, toxins, and immune response modulators [21][22][23]. Thermolabile toxins and cytolysin have been identified in OMVs produced by *Escherichia coli* [24][25][26]. Haemolytic phospholipase C and alkaline phosphates have been detected in the OMVs of *Pseudomonas aeruginosa* [27][28][29]. Keenan et al. have found vacuolating cytotoxin A in the OMVs produced by *Helicobacter pylori* [18][30][31]. Since OMVs consist of toxins and several virulence determinants, it was postulated that the vesicles play a crucial role in bacteria–host interactions [32]. Previously, we demonstrated that OMVs of *K. pneumoniae* induce a strong inflammatory response in human bronchial epithelial cells (BEAS-2B) [13][14][15]. In these cells, OMVs strongly upregulate the expression of genes, encoding cytokines and chemokines [32]. In addition, the effect of the inflammatory cascade leads to pathogen clearance and host homeostasis [33][34][35]. Therefore, understanding cellular and molecular factors in response to the exposure of OMVs could be highly relevant for susceptibility to infection.

MicroRNAs (miRNAs) are small non-coding RNA molecules that are involved in the post-transcriptional regulation of gene expression [36][37]. These molecules are essential in different biological processes, such as development, proliferation, differentiation, cell death and disease [38]. In infected epithelial cells, downregulation of miRNAs increases the expression of cytokines, chemokines, adhesion factors and costimulatory molecules [39][40][41][42]. Little is known about the function of miRNAs in the human bronchial epithelial cells after OMV interaction.

2. Characterization of K. pneumoniae-Derived OMVs

In order to define the structural and functional characteristics of OMVs produced by *K. pneumoniae*, vesicles were purified from three different strains: *K. pneumoniae* ATCC 10031, MS *K. pneumoniae* (clinical isolate) and KPC-producing *K. pneumoniae* (clinical isolate). The strains were cultured to stationary phase and their OMVs were collected. To guarantee precise accuracy of the analyses, three independent purifications of the OMVs were performed for each strain. All vesicles were analysed in terms of diameter and size distribution, through DLS. DLS analysis showed that most OMVs of *K. pneumoniae* ATCC 10031 presented a diameter of 273.3 ± 1.3 nm and were characterized by a slightly heterogeneous size distribution, confirmed by the polydispersity index of 0.329 ± 0.021. OMV vesicles from isolated clinical strains showed an increase in size and a greater heterogeneity of vesicular populations. The OMVs of MS *K. pneumoniae* predominately exhibited a diameter of 427.1 ± 0.9 nm and the vesicle population showed a high heterogeneity, demonstrated by a polydispersity index of 0.417 ± 0.017. Similar results were obtained for OMVs produced by KPC-producing *K. pneumoniae*. The majority of these vesicles presented with a diameter of 483.3 ± 1.7 nm and a polydispersity index of 0.333 ± 0.132, suggesting a heterogeneous population in size distribution (Table 1). All purified OMVs were quantified based on protein yield. Protein concentrations of 0.08 ± 0.06 mg/mL, 0.14 ± 0.03 mg/mL and 0.21 ± 0.01 mg/mL had been generated by *K. pneumoniae* ATCC 10031, MS *K. pneumoniae* and KPC-producing *K. pneumoniae*, respectively, for 600 mL of LB culture (Table 2).

Table 1. Dynamic light scattering (DLS) analysis measurements of the Z-average size (Z-ave) and polydispersity index (PDI) of the outer membrane vesicles (OMVs).

Bacterial Strain	Z-Ave (d.nm)	PDI
K. pneumoniae ATCC 10031	273.3 ± 1.3	0.329 ± 0.021
MS *K. pneumoniae*	427.1 ± 0.9	0.417 ± 0.017
KPC-producing *K. pneumoniae*	483.3 ± 1.7	0.333 ± 0.132

Table 2. Protein concentration of OMVs purified from different strains *of K. pneumoniae.*

Bacterial Strain	Protein Concentration[mg/mL]
K. pneumoniae ATCC 10031	0.08 ± 0.06
MS *K. pneumoniae*	0.14 ± 0.03
KPC-producing *K. pneumoniae*	0.21 ± 0.01

3. SDS-PAGE and LC-MS/MS Analysis of OMVs

To evaluate the protein profile of the OMVs purified from *K. pneumoniae* ATCC 10031, MS *K. pneumoniae* and KPC-producing *K. pneumoniae*, 3.3 µg of protein was subjected to 10% SDS-PAGE (Figure 1). Two major bands, in the range of 30–40 KDa, were detected in the OMVs from *K. pneumoniae* ATCC 10031, MS *K. pneumoniae* and KPC-producing *K. pneumoniae*, with a clear difference from the bacterial lysate protein profile, confirming the absence of bacterial contaminants. The main protein bands were digested with trypsin and mass spectrometry-based proteomic analysis was performed. Mass spectra analysis identified eight proteins common to all purified OMVs. The list of OMV proteins is reported in Table 3 in which identification name, function, molecular weight, and the total score values are indicated.

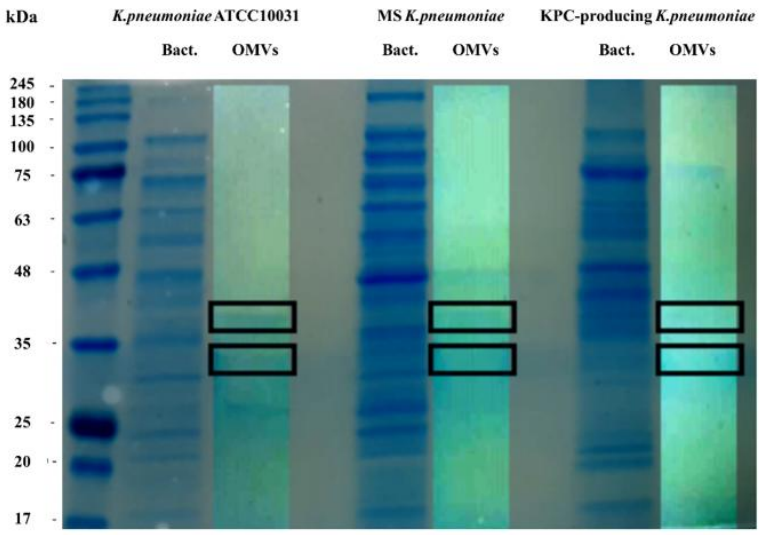


Figure 1. Coomassie-stained SDS-PAGE (10%) protein profiles of *K. pneumoniae* ATCC 10031, MS *K. pneumoniae* and KPC-producing *K. pneumoniae* and relative OMVs. Molecular mass marker (MW) is expressed in kilodaltons (kDa). The rectangles indicate the bands subjected to trypsin digestion and MS and MS/MS analysis.

Table 3. Protein concentration of OMVs purified from different strains *of K. pneumoniae.*

Protein	Function	Theoretical MW (Da)	Score
Outer membrane protein A	Action of colicins K and L	37,152	1501
Outer membrane porin C	Passive diffusion across the outer membrane	39,639	1298
Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	Enzyme in glycolysis	32,457	680
Nucleoside-specific channel-forming protein	Receptor for colicin K	33,486	348
Malate dehydrogenase	Enzyme in Krebs cycle	32,549	180
Glucokinase	Enzyme in glycolysis	34,756	106
2-dehydro-3-deoxyphosphooctonate aldolase	Enzyme in aminoacids synthesis	31,033	97
Aminomethyltransferase	enzyme in the glycine cleavage complex	39,904	88
L-threonine 3-dehydrogenase	Enzyme in L-threonine catabolism	37,559	33

Elongation factor Ts	Protein biosynthesis	30,545	18

4. K. pneumoniae-Derived OMVs Affect miRNA Expression Profile in BEAS-2B Cells

The evaluation of the expression profiles of miRNAs was carried out after treating BEAS 2B cells with OMVs purified from *K. pneumoniae* ATCC 10031, MS *K. pneumoniae* and KPC-producing *K. pneumoniae*. Using the TaqMan miRNA Array CARD, we screened the expression level of 384 miRNA sequences [43]. Raw microarray data were filtered and analysed. To give an illustrative and informative depiction, the results are shown via heatmap, using MeV software (MultiExperiment Viewer) (Figure 2). Transcripts with upregulated expression are indicated in red, while downregulated transcripts are indicated in green. In particular, the analysis revealed 115 miRNA sequences that were differentially regulated in treated samples compared to untreated controls ($p < 0.05$; cut-off > 1.5 or < −0.5). *K. pneumoniae* ATCC 10031 derived OMVs induced the upregulation of 81 and downregulation of 13 miRNAs. In cells treated with MS *K. pneumoniae* derived OMVs, 57 miRNAs were upregulated and 16 were downregulated. Incubation with KPC-producing *K. pneumoniae* derived OMVs altered the expression of 71 miRNAs (58 upregulated and 13 downregulated). The differential analysis of miRNome profiling in response to each of the three treatments was compared and shown in the Venn diagram in Figure 3. The dysregulated miRNAs were common to all the three samples and the individually dysregulated miRNAs in each sample were used for gene ontology, biological function, and pathway analysis.

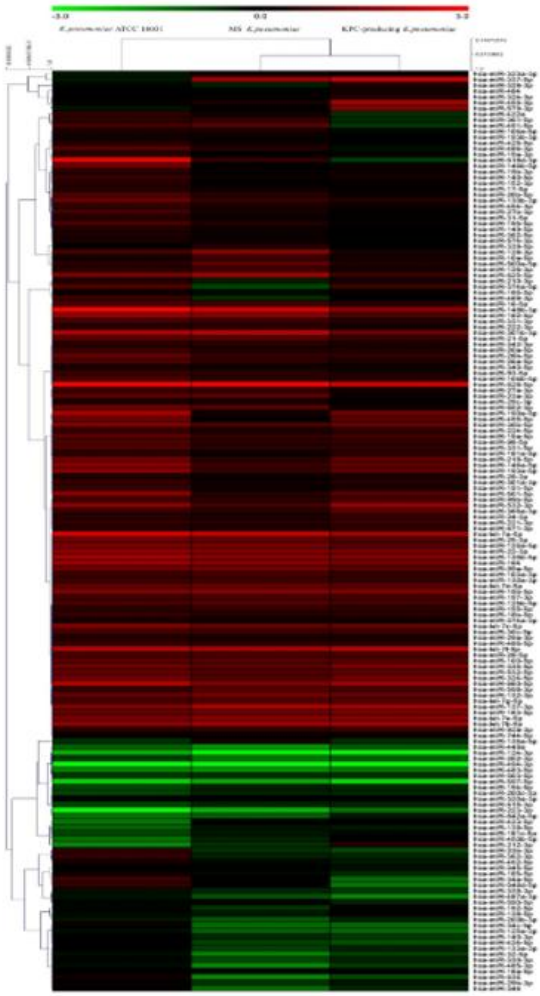


Figure 2. Heat map and hierarchical analysis of clusters. Heat map based on microarray results filtered and processed by bioinformatics analysis. Red indicates a greater expression than the control and green indicates a lower expression.

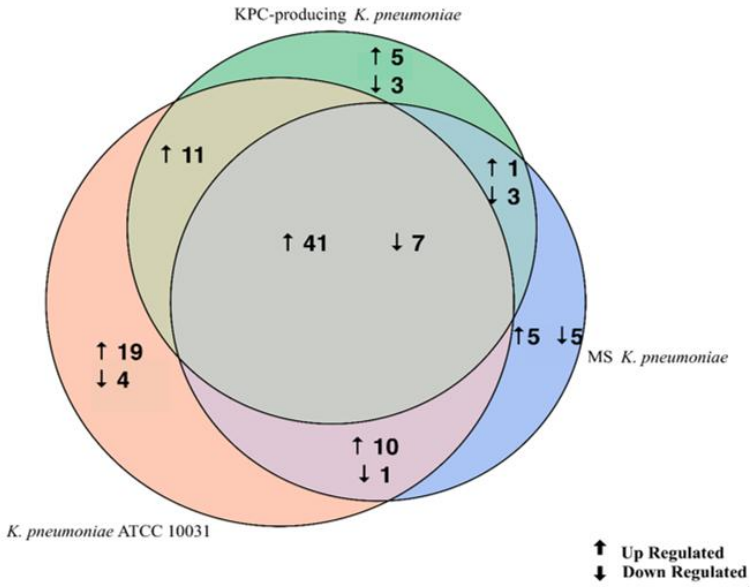


Figure 3. Venn diagram of differentially expressed miRNAs in three samples. The Venn diagram shows the different expressions of miRNAs after exposure of BEAS-2B cells to OMVs purified from three different strains of *K. pneumoniae* (ATCC 10031, MS and KPC-producing) compared to the control sample. The numbers in the intersecting circles represent the miRNA sequences commonly dysregulated in the different samples.

5. Functional Characterization of Target Genes

Gene ontology enrichment analysis was performed using Metascape software. The DIANA gene, Target Scan gene and MirTarBase gene software were exploited to filter predicted data. There were 41 miRNAs that were upregulated and seven that were downregulated in all samples following exposure to OMVs from three different *K. pneumoniae* strains. Predicted target genes of upregulated miRNA sequences in BEAS-2B cells were significantly associated with “miRNA metabolic processes” (GO: 0010586), “cell division” (GO: 0051301), “developmental processes involved in reproduction” (GO: 0003006), “chromatin remodelling” (GO: 0006338) and “response to growth factor” (GO: 0070848) (Figure 4A). Target genes of seven downregulated miRNA sequences were involved in “regulation of acute inflammatory response” (GO: 0002673) (Figure 4B). All identified biological processes are closely related (Figure 4C). Our analysis also assessed the differences in expression of miRNA induced by *K. pneumoniae* ATCC 10031, MS *K. pneumoniae* and KPC-producing *K. pneumoniae* OMVs. After exposure with *K. pneumoniae* ATCC 10031, 19 and four miRNA sequences are upregulated and downregulated, respectively. Upregulated miRNA sequences were involved in “glandular epithelial cell development” (GO: 0002068), “DNA damage response, detection of DNA damage” (GO:0042769) and “positive regulation of apoptotic process” (GO: 0043065) (Figure 5A). The downregulated miRNAs were strongly associated with “cellular response to hormone stimulus” (GO: 0032870) (Figure 5B). The treatment with the MS strain upregulated and downregulated five different miRNA sequences in BEAS-2B cells. Five upregulated miRNA sequences were involved with the “adaptive immune system” (R-HSA-1280218) (Figure 5C) while the five downregulated miRNA sequences were related to the “cellular response to growth factor stimulus” (GO: 0071363), “regulation of neuron differentiation” (GO: 0045664) and “disease of signal transduction by growth factor receptors and second messengers “(R-HSA-5663202) (Figure 5D). The exposure to KPC-producing *K. pneumoniae* OMVs resulted in the upregulation and downregulation of five and three miRNA sequences, respectively. Upregulated sequences were involved in “FOXO-mediated transcription of cell death genes” (R-HSA-9614657), “positive regulation of organelle organization” (GO: 0010638) and “regulation of protein complex assembly” (GO: 0043254) (Figure 5E). Downregulated miRNA sequences were mainly associated with “columnar/cuboidal epithelial cell differentiation” (GO: 0002065) (Figure 5F).

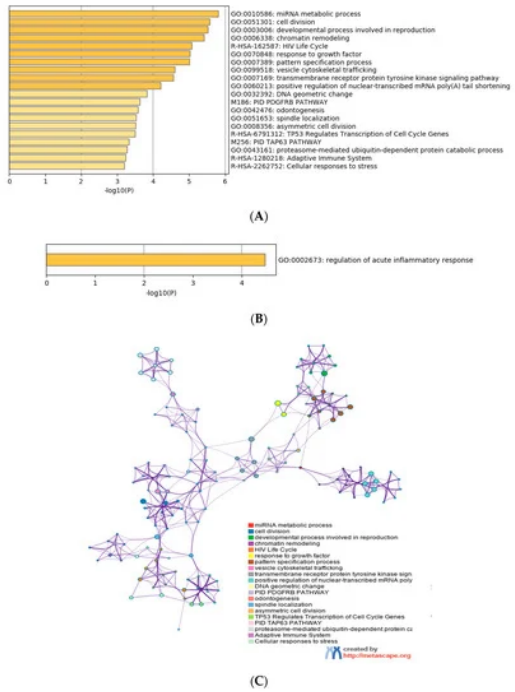
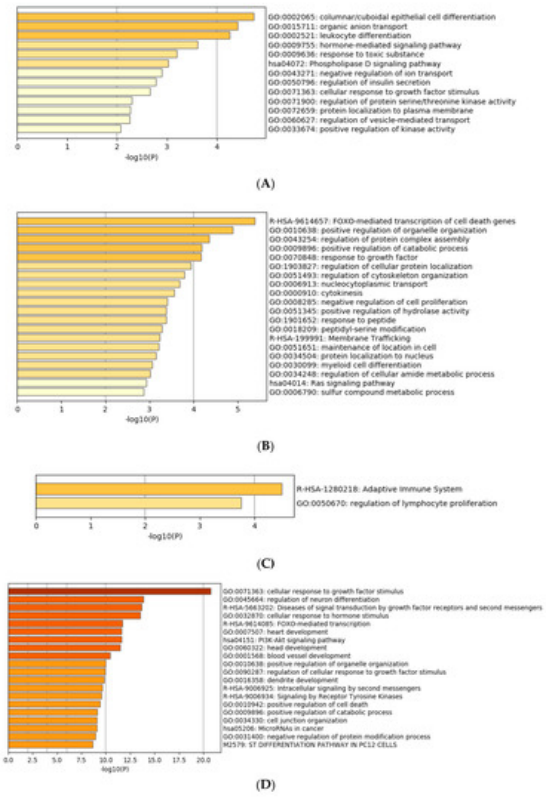


Figure 4. Analysis of the functional enrichment of target genes. (**A**, **B**) The main enrichment analysis clusters detected by Metascape of genes associated with upregulated miRNA after treatment with OMVs; (**C**) interaction network of the clusters detected by Metascape. The nodes of the same colour belong to the same cluster. Terms with a similarity score > 0.3 are linked by an edge. The network is visualized with Cytoscape (v3.1.2) with a “force-directed” layout and edge bundled for clarity.



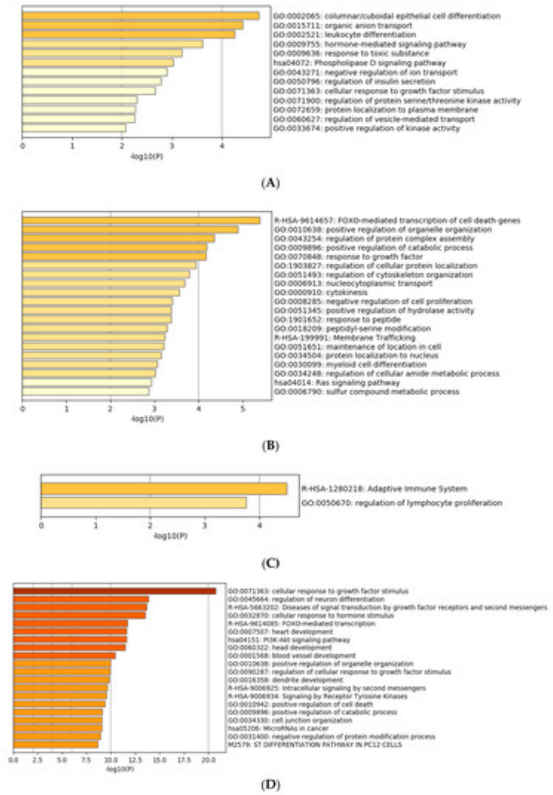


Figure 5. Gene prediction analysis. MiRNA sequences up and downregulated in the BEAS-2B sample exposed to OMVs of *K. pneumoniae* ATCC 10031 (**A**,**B**), MS *K. pneumoniae* (**C**,**D**) and KPC-producing *K. pneumoniae* (**E**,**F**).

6. miRNAs Validation

RT-qPCR was used to confirm the gene expression results obtained from microarray analysis. Four miRNAs (hsa-miR-223, hsa-miR-21, hsa-miR-25, hsa-let-7g) were selected for validation. The expression of the analysed miRNAs showed a good compliance with microarray data (Figure 6). These findings suggest that the microarray data were reliable, supported by similar fold changes. The expression levels in the miRNAs hsa-miR-223, hsa-miR-21 and hsa-let-7g were significantly higher in the treatment with KPC-producing *K. pneumoniae* OMVs. In contrast, the OMVs from MS strain induced higher expression levels than OMVs from ATCC 10031 strain. For hsa-miR-25, there were no significant variations in expression between treatment with OMVs derived from KPC-producing *K. pneumoniae* and MS strain.

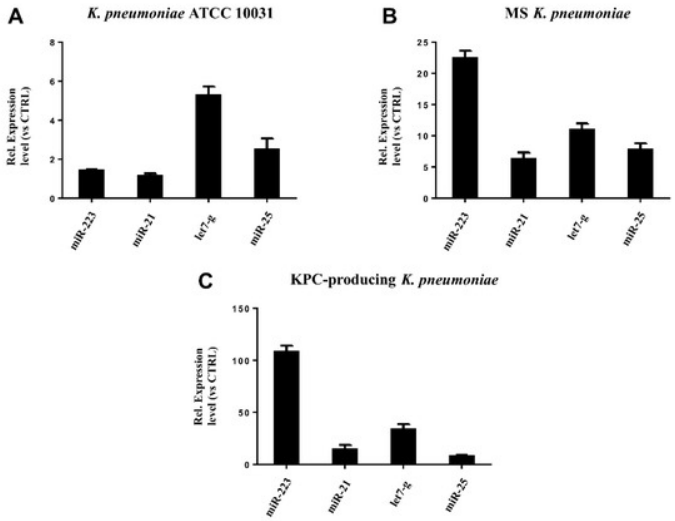


Figure 6. Validation of miRNA microarray data by real-time PCR. The expression levels of miR-21, miR-25, miR223, and let-7g were consistent with the miRNA microarray results after treatment with *K. pneumoniae* ATCC 10031 (**A**), MS *K. pneumoniae* (**B**) and KPC-producing *K. pneumoniae* (**C**).

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