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Original Article

Distinct carbapenems susceptibility profiles in isogenic isolates of *Klebsiella pneumoniae* presenting Ompk36 disruption and expression of down-regulated bla_{KPC-2}

Perfis distintos de suscetibilidade a carbapenêmicos em isolados isogênicos de *Klebsiella pneumoniae* apresentando interrupção de Ompk36 e expressão de bla_{KPC-2} negativamente regulada

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Abstract

The isolation of multidrug-resistant *Klebsiella pneumoniae* in hospitals is a major public health threat, increasing patient hospitalization costs, morbidity and mortality. Therefore, this work investigated the resistance mechanisms that produced different carbapenems susceptibility profiles in two isogenic strains of *K. pneumoniae* isolated from the same patient in a public hospital in Recife, Pernambuco. The genes that encode the main porins in *K. pneumoniae*, *ompK*35 and *ompK*36, and several beta-lactamase genes were analyzed. The expression of these genes was evaluated by quantitative real time PCR (polymerase chain reaction) with reverse transcriptase (RT-qPCR). SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) was performed to analyze the outer membrane proteins. The analysis of the *ompK*36 genetic environment disclosed an *IS*903 insertion sequence disrupting this gene in the ertapenem resistant isolate (KPN133). The bla_{KPC-2} gene showed down-regulated expression in both isolates. Our findings show that **c**hanges in porins, especially OmpK36, are more determinant to carbapenems susceptibility profile of bacterial isolates than variations in bla_{KPC} gene expression.

Keywords: bacterial outer membrane proteins, antimicrobial drug resistance, beta-lactam resistance.

Resumo

O isolamento de *Klebsiella pneumoniae* multirresistente em hospitais é uma grande ameaça à saúde pública, aumentando os custos de internação, morbidade e mortalidade dos pacientes. Portanto, este trabalho investigou os mecanismos de resistência que produziram diferentes perfis de suscetibilidade aos carbapenêmicos em duas cepas isogênicas de *K. pneumoniae* isoladas do mesmo paciente em um hospital público em Recife, Pernambuco. Foram analisados os genes que codificam as principais porinas em *K. pneumoniae*, *ompK*35 e *ompK*36, e diversos genes de beta-lactamases. A expressão desses genes foi avaliada por PCR (reação em cadeia da polimerase quantitativa em tempo real) com transcriptase reversa (RT-qPCR). SDS-PAGE (dodecil sulfato de sódio-poliacrilamida gel eletroforese) foi realizada para analisar as proteínas da membrana externa. A análise do ambiente genético *ompK*36 revelou uma sequência de inserção *IS*903 interrompendo este gene no isolado resistente ao ertapenem (KPN133). O gene bla_{KPC-2} apresentou expressão negativamente regulada em ambos os isolados. Nossos achados mostram que alterações nas porinas, especialmente OmpK36, são mais determinantes no perfil de suscetibilidade aos carbapenêmicos de isolados bacterianos do que variações na expressão do gene bla_{KPC}.

Palavras-chave: proteínas da membrana externa bacteriana, resistência a drogas antimicrobianas, resistência a beta-lactâmicos.

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1. Introduction

Klebsiella pneumoniae is an important opportunistic pathogen in the *Enterobacterales* order which causes infections related to healthcare (Munoz-Price et al., 2013). The number of hospital outbreaks related to this microorganism is increasingly frequent, mainly due to the change in the pattern of susceptibility to antimicrobials (Castanheira et al., 2019).

Resistance to carbapenems in this species is mainly related to the acquisition of β-lactamases, the most common of which are carbapenemases, especially the *Klebsiella pneumoniae* carbapenemase (KPC). KPC-producing strains of *K. pneumoniae*, originally isolated in the USA, have spread and reached a wide geographical distribution (Villegas et al., 2006; Gupta et al., 2011; Nordmann et al., 2012; Melo et al., 2024; Zagui et al., 2022).

In addition to the enzymatic mechanisms, the reduced permeability of the outer membrane due to the loss or deficiency of transmembrane proteins (porins), may be necessary for the KPC-producing microorganism to reach high levels of carbapenem resistance, because It is through these channels that these antimicrobials penetrate the bacterial cell (Vera-Leiva et al., 2017; Wise et al., 2018).

In *K. pneumoniae*, the main porins of the outer membrane protein (OMP), OmpK35 and OmpK36, homologous to the well-studied OmpF and OmpC, respectively, in *Escherichia coli*, have been implicated in reduced susceptibility to carbapenem (Doménech-Sánchez et al., 2003).

Although the occurrence of changes in porins are frequently reported in *K. pneumoniae*, studies that relate these findings to the expression of OMPKs and beta-lactamases genes have been reported uncommonly.

2. Materials and Methods

2.1. Bacterial strains and susceptibility profile

The bacterial isolates of *K. pneumoniae* used, KPN132 and KPN133, were obtained from blood samples from a single patient admitted to the intensive care unit in a public hospital from Recife, Pernambuco, Brazil. Species identification and antimicrobial susceptibility tests were performed using the automated Vitek2® system (BioMerieux, Marcy l'Etoile, France). After isolation and identification, the strains were transferred to preservation medium, 15% glycerol and Müeller Hinton broth (HIMEDIA®) containing the Meropenem (8 µg/mL), and kept in frozen stock, at -80 ºC, until the day of use**.**

The interpretation of the results was performed according to Clinical and Laboratory Standards Institute (CLSI, 2021).

2.2. Clonal relatedness analysis

The PFGE (pulsed field gel electrophoresis) of the *K. pneumoniae* isolates was performed using CHEF 415 DR II (Bio-Rad, Richmond, CA, USA). The restriction patterns were determined by the *Xba*1 enzyme, analyzed, and interpreted according to the criteria of Tenover and colleagues (Tenover et al., 1995). The determination of the sequence type (ST) was performed by PCR for the constitutive expression genes (*rpoB*, *gapA, mdh, pgi, phoE, infB, and tonB*) according to Diancourt and colleagues (Diancourt et al., 2005) and compared using the Institut Pasteur platform.

2.3. Plasmid analysis and transformation

Plasmid DNA was isolated using the Plasmid Mini Kit (QIAGEN). *E. coli* R861 and *E. coli* Top10 isolates were used as size determinants and chromosomal DNA patterns, respectively. The plasmid transfer was carried out using *E. coli* DH5alfa as a receptor strain. The transformant cells were selected in Müeller Hinton medium with 100 µg/mL ampicillin.

2.4. PCR amplification and DNA sequence analysis

The genomic DNA of the bacterial isolates was extracted using the Wizard® Genomic DNA Purification Kit (Promega) and quantified by spectrophotometry on NanoDrop 2000® equipment (Thermo Scientific). The analysis of the genes of porins (*ompK*35 and *ompK*36) and beta-lactamases (bla_{KPC}, bla_{TEM}, bla_{CTX-M}, bla_{SHV}) was performed by PCR (Hangzhou Bioer Techonology). The primers used in the reactions are described in Table 1**.** The amplification products were purified using the Clean-Up Purification Kit (Promega) and sequenced using a 3500 Genetic Analyzer and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences from the GenBank database were used as the reference for the analysis of mutations in gene sequences (ATCC 13883 KN046818.1 and KCTC 2242 CP002910.1). The *IS* Finder (https://isfinder.biotoul.fr/) was used to identify and analyze insertion sequences.

2.5. Analysis of outer membrane proteins

For analysis of porins OmpK35 and OmpK36 by SDS-PAGE, bacterial cells were cultured in Müeller Hinton broth for a period of 5 h at 37 °C and later lysed by sonication. The OMPs were isolated with Sarcosil 20% and recovered by centrifugation at 14000 rpm for 60 minutes at 4 °C. Protein quantification was performed using Coomassie Blue G-250 and 20 µg of protein was applied on a 12.5% SDS-PAGE gel. The images of the one-dimensional gels were analyzed in Gel Analyzer 2010 (http://www.gelanalyzer.com/index.html).

2.6. Analysis of gene expression

The bacterial total RNA was extracted using the TRIzol® Reagent Kit (Thermo Fisher Scientific). The synthesis of complementary DNA (cDNA) was carried out using the EasyScript ™ Reverse Transcriptase/EasyScript ™ cDNA Synthesis Kit (Applied Biological Materials Inc. ABM®). The relative expression of the *ompK*35, *ompK*36, and bla_{KPC} genes was evaluated by RT-qPCR, using 25 ng from each sample tested. The constitutive expression gene *rpoB* was used as an endogenous control, and a sensitive strain of *K. pneumoniae* was used as a reference sample (KPN077). The reactions were performed in the StepOne TM Real-time PCR System (Applied Biosystems), using SYBR Green I as the detector. All tests were done in technical triplicates. The expression levels of the genes were analyzed by the method $2^{-\Delta\Delta Cq}$ (Livak and Schmittgen, 2001).

Ethics. This work was approved by the Research Ethics Committee of the University of Pernambuco, Brazil (reference nº 265.604).

3. Results

3.1. Clonal relationship and susceptibility to antimicrobials

The PFGE showed that the isolates have an indistinguishable restriction pattern (Figure S1, Supplementary Material) and the analysis of the allelic profile revealed that they belong to ST11. According to the susceptibility profile, the isolates showed an MDR (multidrug resistance) phenotype with resistance to beta-lactams and quinolones. KPN133 isolate showed a higher resistance profile, especially to carbapenems (Table S1, Supplementary Material).

3.2. Transfer of resistance determinants

The plasmid profile of the clinical isolates KPN132 and KPN133 showed that they harbored four plasmids (39 kb, 11 kb, and two plasmids smaller than 6.9 kb). An *Inc*K 39kb plasmid was transferred by transformation to recipient *E. coli* cells using plasmid DNA from both clinical samples. The bla_{KPC-2}, bla_{TEM}, and $bla_{CTX-M-2-like}$ genes were detected in transformant cells, TF132 and TF 133, suggesting their localization in the *Inc*K plasmid.

3.3. Disruption of porin genes

The analysis of the *ompK*36 genetic environment (Figure S2, Supplementary Material) showed the presence of the *IS*903-like insertion sequence interrupting this gene in the KPN133 isolate. Our results indicated that the *ompK35* gene sequence was preserved.

3.4. Gene expression

The expression of the bla_{KPC-2}, *ompK*35, and *ompK*36 genes was negatively regulated in both clinical isolates when compared to passive reference levels (Table 2).

Table 1. Oligonucleotides used for beta-lactamases and porin analysis.

Primer		Sequence 5' 3'	Target gene	Using	Length	Reference
KPC	F	TGTCACTGTATCGCCGTC	bla_{KPC-2}	coding region	1011	Yigit et al., 2001
	R	CTCAGTGCTCTACAGAAAACC				
	F	TCGCTAAACTCGAACAGG		expression	59	This study
	R	TTACTGCCCGTTGACGCCCAATCC				
CTX-M	F	SCSATGTGCAGYACCAGTAA	bla_{CTXM-2}	coding region	500	Cao et al., 2002
	R	CCGCRATATGRTTGGTGGTG				
TEM	F	TCGGGGAAATGTGCGCG	bla_{TEM}		700	
	R	TGCTTAATCAGTGAGGCACC				
SHV	F	TTATCTCCCTGTTAGCCACC	bla_{SHV-11}		900	
	R	GATTTGCTGATTTCGCTCGG				
*ompK*35	F	GCGCAATATTCTGGCAGTGG	*ompK*35	genetic environment	1996	Doumith et al., 2009
	R	TAAACGATACCAACCGCAGCCT				
	F	TCCCTGCCCTGCTGGTAG		expression	93	This study
	R	CTGGTGTCGCCATTGGTGG				
*ompK*36	F	GACCCGCCAGAAGGTGCCCA	*ompK*36	genetic environment	2159	Doumith et al., 2009
	R	TGATGTTGCCGGGGATCAGGGA				
	F	AACCCAGATCAACGACCAGC		expression	98	This study
	R	ATCGCTGGAGCTTTCAGTGT				
rpoB	F	CTGATGCCTCAGGATATGATCAAC	*rpoB*	expression	72	This study
	R	CTGGCTGGAACCAAAGAACTCT				

Table 2. Characteristics of KPC-producing *Klebsiella pneumoniae*.

Isolates	Carbapenem MIC (μg/mL)			β-Lactamases	Codify region		Relative Expression		
	IPM	MER	ETP		*ompK35*	*ompK36*	*ompK35*	*ompK36*	bla_{kpc}
KPN 132	64 R	32 R	0.12 S	bla_{KPC-2}, bla_{TEM}, $bla_{CTX-M-2}$, bla_{SHV-11}	Preserved	Preserved	-46.945 SD: 0.002	-17.963 SD: 0.0019	-416.575 SD: 0.00045
TF 132	32 R	32 R	ND	bla_{KPC-2}, bla_{TEM}, $bla_{CTX-M-2}$	-	-	-	-	-
KPN 133	256 R	64 R	> 32 R	bla_{KPC-2}, bla_{TEM}, $bla_{CTX-M-2}$, bla_{SHV-11}	Preserved	Disruption by IS 903	-106.352 SD: 0.034	-58,193.964 SD: 9.317	-3,924.585 SD: 5.194
TF 133	32 R	8 R	ND	bla_{KPC-2}, bla_{TEM}, $bla_{CTX-M-2}$	-	-	-	-	-

TF—transformant; IMI—imipenem; MER—meropenem; ETP—ertapenem; R—resistant; S—susceptible.

3.5. Outer proteins

The analysis of OMPs by SDS-PAGE revealed that both isolates have a decrease in the production of OmpK36 compared to the control isolate. Moreover, the KPN133 isolate showed a more evident decrease. For the OmpK35 porin, there were no visible differences between the isolates (Figure 1).

4. Discussion

This work reported a high carbapenem resistance level in ST11 *K. pneumoniae* isogenic isolates belonging to clonal complex 258 (CC258), one of the most widespread clonal complexes in the world. This high-risk international CC is of concern for its contribution to the spread of multiple β-lactamases, such as KPC (Yu et al., 2019).

The susceptibility profiles of the isolates indicated that alterations in the *ompK*36 gene may be the main cause of the different MIC values for carbapenems presented by the isolates, especially for ertapenem. We propose that the increase in MIC for ertapenem in isolate KPN133 was due to the inactivation of the *ompK*36 gene by an *IS*903, resulting in a significant decrease in the availability of unaltered DNA to be transcribed and translated.

The results of the relative expression of bla_{KPC} indicate that there may be a strict control in the mechanisms of regulation of the expression of this gene, mainly in strains that present alterations in the porins, because the decrease of these proteins disturbs the entry of the antimicrobial in the cell. A similar hypothesis was inferred by Doumith and colleagues previously (Doumith et al., 2009).

Other studies that evaluated the expression of OMPs and KPC genes mentioned a codependency relationship between them. The increase in the expression of porin genes results a decrease in carbapenem MICs, especially to ertapenem, despite the bla_{KPC} gene expression (Jacoby et al., 2004; Landman et al., 2009; Wong et al., 2022).

These data are reinforced by the decrease in the expression of the bla_{KPC} gene in the KPN133 isolate that also presented *ompK*36 expression down-regulated, that resulted in resistance to carbapenems, mainly to meropenem and ertapenem as also demonstrated by Fernandéz and Hancock (Fernandéz and Hancock, 2012).

According to our findings, it is estimated that the membrane permeability in the KPN132 isolate was less affected than in KPN133 and that, for this reason, a greater amount of the antimicrobial will enter the cell. However, given the less down-regulated expression of bla_{KPC-2} in KPN132, even reaching the bacterial intracellular medium, part of the drug can be hydrolyzed by KPC but not reaching resistance levels.

The results of this study reinforce the role of outer membrane proteins in the resistance to beta-lactams, such as ertapenem. Lumbreras-Iglesias and colleagues already detected that ertapenem can be particularly affected by the concomitant loss of these two main porins and this fact may reflect the slower penetration of this drug through the smaller porins, if present in the outer membrane (Lumbreras-Iglesias et al., 2021).

The importance of porins in antimicrobial susceptibility over other β-lactam resistance mechanisms is supported by the results of a recent multicenter study by Wise et al., which analyzed 487 isolates of *K. pneumoniae* that did not have carbapenemase genes but were resistant to ertapenem. In 83% of the strains investigated, the presence of interruptions in one or both *ompK*35 and *ompK*36 genes was detected (Wise et al., 2018).

The coding region of the *ompK*35 gene was conserved in both isolates and it was observed that expression of this gene in KPN133 isolate was only – 2.26-fold level in relation to KPN132 isolate. On the other hand, the *ompK*36 gene was disrupted by an *IS*903 in KPN133. Furthermore, its expression was – 3423.17-fold level in this isolate, compared to KPN132 isolate. Codify regions analyzes and relative expression porin genes data suggesting that OmpK36 has an important role in a susceptibility profile to carbapenems, in isolates reported here, as well as those, reported by some authors (Doumith et al., 2009; Landman et al., 2009).

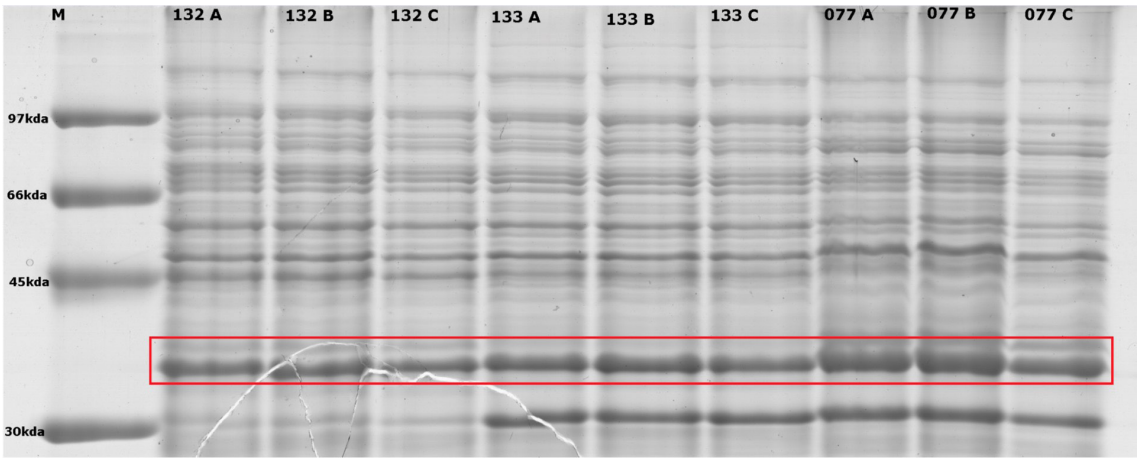

Figure 1. One-dimensional gel SDS-PAGE. The bands highlighted by the red frame correspond to 35 KDa (OmpK35) and 36 KDa (OmpK36), respectively. There is a lower abundance of the 36 KDa band in isolate KPN133 compared to strain KPN132 and the control (KPN077). **Legend:** M— Marker; 132A, 132B, 132C— KPN132 Triplicates; 133A, 133B, 133C — KPN133 Triplicates; 077A, 077B, 077C — Control triplicates.

Finally, as demonstrated in this work, high levels of resistance to carbapenems, as well as to other β-lactams, may require the association of different resistance mechanisms, such as production of β-lactamases, inactivation or decrease in the expression of porin genes and overexpression of efflux systems, as suggested by some studies (Doménech-Sánchez et al., 2003; Kitchel et al., 2010; Fernández and Hancock, 2012).

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. Antimicrobial susceptibility profile shown in isolates KPN132, KPN133 and their transformants.

Figure S1. PFGE. The indicative columns of KPN132 and KPN133 (only column 12 and 13, respectively) show an identical pattern of bands in both samples, which classifies them as indistinguishable (isogenic), according to the criteria of Tenover et al. (1995). The other columns refer to samples unrelated to this study.

Figure S2. Schematic representation of the genetic environment of ompK36 in isolate KPN 133, showing the integration site of the IS903-like insertion sequence. The arrow represents the direction of the transcript.

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