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Detection and analysis of different interactions between resistance mechanisms and carbapenems in clinical isolates of *Klebsiella pneumoniae*

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ABSTRACT

Carbapenems are considered last-line agents for the treatment of serious infections caused by *Klebsiella pneumoniae*, and this microorganism may exhibit resistance to β-lactam antibiotics due to different mechanisms of resistance. We evaluated 27 isolates of *K. pneumoniae* resistant to carbapenems recovered from inpatients at the University Hospital of Santa Maria-RS from July 2013 to August 2014. We carried out antimicrobial susceptibility, carbapenemase detection, testing for the presence of efflux pump by broth microdilution and loss of porin by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Genetic similarity was evaluated by ERIC-PCR. High levels of resistance were verified by the minimum inhibitory concentration for the antimicrobials tested. The bla_{KPC} gene was present in 89% of the clinical isolates. Blue-Carba and combined disk with AFB tests showed 100% concordance, while the combined disk test with EDTA showed a high number of false-positives (48%) compared with the gold-standard genotypic test. Four isolates showed a phenotypic resistance profile consistent with the overexpression of the efflux pump, and all clinical isolates had lost one or both porins. The ERIC-PCR dendrogram demonstrated the presence of nine clusters. The main mechanism of resistance to carbapenems found in the assessed isolates was the presence of the bla_{KPC} gene.

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Introduction

Cephalosporins used to be the antibiotics commonly prescribed for treatment of severe *Klebsiella pneumoniae* infections. However, due to the high frequency of extended spectrum β-bactamases (ESBL) producing *K. pneumoniae*, carbapenems have become the most common antibiotics prescribed for the treatment of these infections. Unfortunately, the high consumption of carbapenems has been accompanied by the emergence and spread of carbapenem-resistant *K. pneumoniae*.[1]

Resistance to carbapenems can be associated with the production of carbapenemases, loss of porins and overexpression of the efflux pump. *K. pneumoniae* have acquired genes encoding for carbapenemases, which are enzymes capable of breaking down most β-lactams antibiotics, including carbapenems, and thus conferring resistance to these drugs, which can result in treatment failure.[2] The carbapenemases are classified in A, B and D Ambler classes. The most common carbapenemase class A is *K. pneumoniae* carbapenemase (KPC), the most common carbapenemase class B is New Delhi metalo β-lactamase (NDM) and the most common carbapenemase class D is Oxacillinase-48 (OXA-48), occurring in *K. pneumoniae*.[3]

K. pneumoniae presents two main porins, OmpK35 and OmpK36. The loss of these porins (OmpK35 and OmpK36) may play an important role in the development of resistance to carbapenems in *K. pneumoniae*.[4] Furthermore, overexpression of the AcrAB efflux pump has also been proposed as being responsible for reduced susceptibility to ertapenem and meropenem antibiotics in some strains.[5]

The aim of this study was to evaluate mechanisms involved in carbapenem resistance among *K. pneumoniae* isolates. As carbapenemases are most often acquired by horizontal transfer between strains, whereas change in carbapenem permeability or efflux is a result of point mutations, it is important to study these different resistance mechanisms in locally collected strains, as these resistance mechanism profiles might vary temporally and geographically.

Materials and methods

Bacterial strains

Twenty-seven non-duplicated *K. pneumoniae* clinical isolates resistant to carbapenems collected from July 2013 to August 2014 at the Santa Maria University Hospital, Brazil were included in this study. Species identification was carried out on the Vitek® 2 automated identification system (Biomérieux, France) and confirmed with MALDI-TOF MS in a Microflex LT apparatus (Bruker Daltonics, Germany) considering a score value between 2.0 and 2.299.[6]

Susceptibility testing

The antimicrobials used for susceptibility testing were imipenem, meropenem, ertapenem, cefepime, ceftazidime, and cefoxitin. The tests were performed by broth microdilution according to CLSI guidelines.[7] *Escherichia coli* ATCC 25922 was used as a quality control.

Carbapenemases

The detection of genes bla_{KPC},[8] bla_{OXA-48}[9] and bla_{NDM}[10] was carried out using the multiplex PCR technique, as well as the detection of genes bla_{SIM}, bla_{SPM}, bla_{IMP}, bla_{VIM} and bla_{GIM}.[11] The detection of bla_{GES} was carried out with simplex PCR.[12]

Disk diffusion assay using phenyl boronic acid, EDTA and cloxacillin

For phenotypic detection of the KPC- and metallo-β-lactamases (MBLs), we used disk diffusion assays with phenylboronic acid (AFB)[13] and ethylenediamine tetra-acetic acid (EDTA), respectively.[14] In both tests, an increase of 5 mm in zone diameter in the presence of AFB or EDTA compared with either meropenem or imipenem tested alone was considered to represent a positive result for the presence of KPC β-lactamase or MBL enzyme, respectively.

For the detection of plasmid-mediated AmpC, we used test results from AFB and cloxacillin (CLOXA)[13] in combination with meropenem and imipenem, compared with carbapenem disks alone. An increase of 5 mm in zone diameter for both AFB and CLOXA was considered to be a positive test result for the presence of AmpC. Strains KPC2-*K. pneumoniae*, IMP1-*K. pneumoniae* and *E. coli* ATCC 25922 were used as quality controls.

Blue-Carba test

The Blue-Carba test consists of the detection of hydrolysis of the carbapenem β-lactam ring in a bacterial extract through the acidification of bromothymol blue indicator. A loop of a pure bacterial culture was directly suspended in 100 μL of the test solution (aqueous solution of bromothymol blue, $ZnSO_4$ and imipenem) and in the negative-control solutions (without imipenem), followed by incubation at 37 °C for 2 h, with the first reading at 15 min. Carbapenemase activity was revealed when the test and negative-control solutions were (i) yellow versus blue, (ii) yellow versus green, or (iii) green versus blue. Noncarbapenemase producers remained blue or green on both solutions. Strains harboring the genes bla_{KPC}, bla_{IMP} and bla_{OXA-48} were used as positive controls, and *E. coli* ATCC 25922 was used as a negative control.[15]

Efflux pump

The technique was carried out by broth microdilution using antimicrobial alone and associated with the efflux pump inhibitor carbonyl cyanide m-cholorophenyl hydrazone (CCCP), using half of the minimal inhibition concentration (MIC) for CCCP. The reduction in MIC of the antimicrobial sample, associated with the efflux pump inhibitor, was indicative of efflux pump overproduction.[16]

OMP analysis

Outer membrane protein (OMP) profiles were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis

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