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Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results

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Objectives: The aim of this study was to evaluate the presence of carbapenemases in a *Klebsiella pneumoniae* collection and the performance of the modified Hodge test (MHT) to correctly identify this phenotype.

Methods: Twenty-eight *K. pneumoniae* clinical isolates with reduced susceptibility to carbapenems were evaluated. Antimicrobial susceptibility and molecular typing were performed by agar dilution and PFGE, respectively. The MHT was performed using both standard and high inoculum of test organisms. Imipenem hydrolysis was investigated by spectrophotometric assays and carbapenemase-encoding genes were identified by PCR and amplicon sequencing. Porin loss was investigated by both PCR and SDS–PAGE.

Results: Susceptibility rates for imipenem, meropenem and ertapenem were 93%, 57% and 11%, respectively. The PFGE analysis showed seven unrelated genotypes. By testing standard inoculum and ertapenem or meropenem discs, 25% ($n=7$) and 21% ($n=6$) of the isolates were classified as carbapenemase producers, respectively. When a higher inoculum was employed, these rates increased to 54% ($n=15$) and 43% ($n=12$), respectively. No imipenem hydrolysis was detected. PCRs identified $bla_{\text{CTX-M}}$ in 27 (96%) isolates, of which 2 isolates also carried $bla_{\text{GES-1}}$. SDS–PAGE and PCR assays revealed that all isolates had lost at least one outer membrane protein, except for a single isolate that was found to express both OmpK35 and OmpK36.

Conclusions: False detection of carbapenemase production was observed by the MHT possibly as a result of extended-spectrum β-lactamase (ESBL) production coupled with porin loss as reported before. Clinical laboratories must be aware of this fact, especially in geographical areas where ESBL-producing isolates are highly prevalent.

Keywords: carbapenem resistance, OmpK35, OmpK36, CTX-M, KPC

Introduction

Infections due to *Klebsiella pneumoniae* have emerged as an important challenge in healthcare settings. In Brazil, *K. pneumoniae* has been the fourth most frequent pathogen isolated from bloodstream infections according to the SENTRY Antimicrobial Surveillance Program.[1] High rates of extended-spectrum β-lactamase (ESBL) production have been observed in this species, making the carbapenems the main therapeutic option for treatment of serious infections caused by such pathogens. Although the rates of resistance to carbapenems are low, they have been increasing. The most common mechanism for carbapenem resistance in *Klebsiella* spp. is the production of carbapenemases belonging to Ambler class A, especially *K. pneumoniae* carbapenemase (KPC), or B, metallo-β-lactamases (MBLs) such as IMP and VIM types. Production of ESBL and/or AmpC β-lactamases coupled with porin loss has also been shown to be responsible for carbapenem resistance.[2,3]

The detection of KPC has become a real challenge for clinical laboratories since some carbapenemase-producing Enterobacteriaceae may have carbapenem MICs that are elevated but still within the susceptible category according to currently defined breakpoints.[4] To address this challenge, in January 2009, the CLSI published a recommendation in which carbapenem-susceptible Enterobacteriaceae with elevated MICs or reduced disc diffusion inhibition zones should be tested for the production of carbapenemases.[4] For this purpose, the Cloverleaf test[5] [modified Hodge test (MHT)] was suggested due to its acceptable sensitivity and specificity for carbapenemase detection.[4,5] To date, the BSAC and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) do not routinely recommend carbapenemase detection in Enterobacteriaceae.

The aim of this study was to evaluate the presence of carbapenemase production in a *K. pneumoniae* collection with reduced susceptibility to carbapenem, and the performance of

the MHT in correctly identifying the presence of such resistance determinants.

Methods

Collection

In 2009, the Laboratório ALERTA, a research laboratory from the Universidade Federal de São Paulo, received 28 *K. pneumoniae* clinical isolates with reduced susceptibility to carbapenems. These isolates were recovered from eight hospitals located in three distinct Brazilian regions. A PFGE assay was performed to study the genetic relatedness among these isolates.

Antimicrobial susceptibility testing and MHT

Antimicrobial susceptibility testing was performed using the CLSI agar dilution technique. Cefepime, ceftazidime, ertapenem, imipenem and meropenem MICs were interpreted following the CLSI recommendations.[4] All isolates fulfilled the CLSI criterion for performing carbapenemase detection by the MHT.

MHT was performed with both a CLSI standard inoculum (three to five colonies) and a high inoculum (a full 1 μL loop) of test organisms. Results were interpreted by two blinded observers.

β-Lactamase detection

Investigation of carbapenemase activity in crude extracts was performed by UV spectrophotometric assays. Briefly, a full 10 μL loop of the test organism was inoculated into 500 μL of 100 mM phosphate buffer (pH 7.0) and disrupted by sonication. The cells were removed by centrifugation and the supernatants were used for further experiments. Protein quantification in crude extracts was performed using the Bradford stain, and the results were used to normalize the volume to be tested for each isolate. The hydrolytic activity of crude extracts was determined against 100 μM imipenem in 100 mM phosphate buffer (pH 7.0), and measurements were carried out at a wavelength of 297 nm. Positive controls included SPM-1-producing *Pseudomonas aeruginosa* and GES-5-producing *P. aeruginosa* for high- and low-level hydrolysis, respectively. CTX-M-2-producing *P. aeruginosa* was included as a negative control.[6]

PCRs targeting genes encoding CTX-M, GES, KPC, plasmid-mediated AmpCs, MBL and OXA-type carbapenemases were performed using primers described previously.[6,7] For direct DNA sequencing, PCR products were purified using PCR purification columns (Qiagen, Hilden, Germany). Sequencing reactions were performed using the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an automated ABI 3130 sequencer (Applied Biosystems). The nucleotide and deduced protein sequences were analysed with software available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Outer membrane proteins

The presence of outer membrane proteins (OMPs) was analysed by SDS–PAGE as previously described.[8] Altered OmpK35- and OmpK36-encoding genes were investigated by PCR.[7]

Results

Twenty-eight *K. pneumoniae* clinical isolates were investigated. None of these isolates was susceptible to cefepime but 7% were susceptible to ceftazidime. Susceptibility rates for imipenem, meropenem and ertapenem were 93%, 57% and 11%, respectively. MIC_{50}/MIC_{90} values (mg/L) were as follows: cefepime, 128/>256; ceftazidime, 32/128; ertapenem, 16/64; imipenem, 2/4; and meropenem, 4/16. PFGE, interpreted according to Tenover *et al.*,[9] showed seven unrelated genotypes. By testing standard inoculum and ertapenem or meropenem discs, 25% ($n=7$) and 21% ($n=6$) of the isolates were classified as carbapenemase producers, respectively. When a higher inoculum was employed, these rates increased to 54% ($n=15$) and 43% ($n=12$), respectively. Carbapenemase production by MHT was detected among *K. pneumoniae* isolates belonging to distinct genotypes. No imipenem hydrolysis was detected among the isolates classified as carbapenemase producers by the MHT. PCRs targeting genes encoding KPC, plasmid-mediated AmpCs, MBL and OXA-type carbapenemases did not show any amplicons. $bla_{CTX\text{-}M}$ was detected in 27 (96%) isolates, in which 24 (89%) were $bla_{CTX\text{-}M\text{-}2}$, 2 (7%) were $bla_{CTX\text{-}M\text{-}15}$ and 1 (4%) was $bla_{CTX\text{-}M\text{-}59}$. Two $bla_{CTX\text{-}M\text{-}2}$-positive isolates also carried $bla_{GES\text{-}1}$. Both OmpK35 and OmpK36 were expressed in a single isolate (Kpn 27) according to the SDS–PAGE results. Interestingly the MICs of imipenem, meropenem and ertapenem for this isolate were ≤0.12 mg/L, ≤0.12 mg/L and 2 mg/L, respectively. Five isolates (18%) presented two bands, probably corresponding to OmpA and either OmpK35 or OmpK36. Finally, 22 isolates (79%) presented one single band, probably corresponding to OmpA, suggesting that they have lost both porins. These findings were corroborated by PCR analysis of OMP-encoding genes, in which 24 isolates (86%) presented altered amplicons of at least one OMP-encoding gene, being either the lack of amplification or enhanced amplicon size.

Discussion

In this study, we have observed the occurrence of false detection of carbapenemase production by the MHT among isolates in which carbapenem reduced susceptibility or resistance was detected. Therefore, a false positive MHT probably results from

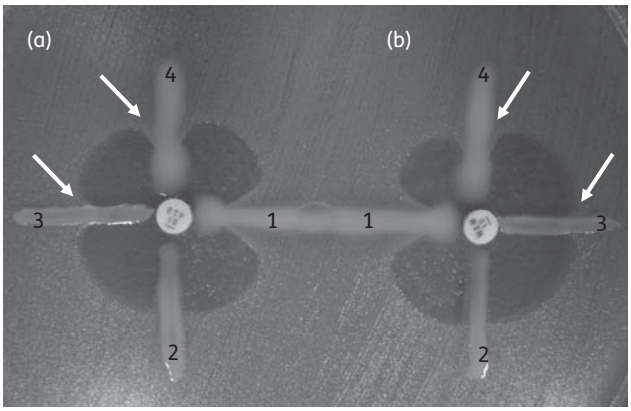

Figure 1. Phenotypic carbapenemase detection by MHT applying the inoculum recommended by CLSI for ertapenem (a) and meropenem discs (b). 1, *K. pneumoniae* ATCC BAA-1705, positive result; 2, *K. pneumoniae* ATCC BAA-1706, negative result; 3, CTX-M-producing *K. pneumoniae* clinical isolate; 4, KPC-producing *K. pneumoniae* clinical isolate. Arrows indicate the similar size of *E. coli* ATCC 25922 grown within the carbapenem disc inhibition zones when testing both carbapenemase producer and carbapenemase non-producer isolates.

low-level carbapenem hydrolysis by ESBLs, particularly those of the CTX-M type.[10] This hypothesis is strengthened by the fact that the frequency of false positive results was directly related to the inoculum tested.[11] Our results corroborated with those observed by Doumith *et al.*[3] in which carbapenem resistance due to ESBL production coupled with porin loss was reported.

When testing true carbapenemase producers, *Escherichia coli* ATCC 25922 usually grows better within the carbapenem disc inhibition zones. For this reason, we thought that the true production of carbapenemase could be discriminated by looking at the growth size produced in the MHT. However, we have observed that KPC-producing *K. pneumoniae* isolates might also show a weak positive result on MHT, as shown in Figure 1.

Clinical laboratories must be aware that false positive results may occur, especially in geographical areas where ESBL-producing isolates are prevalent. Currently, a new study is being carried out to determine the frequency of false detection of carbapenemase production in Enterobacteriaceae by the MHT in the clinical microbiology routine setting.

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Transparency declarations

None to declare.

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