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Molecular characterization of *Enterobacteriaceae* resistant to carbapenem antimicrobials

Caracterização molecular de enterobactérias resistentes aos carbapenêmicos

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ABSTRACT

The present study aimed to genotypically and phenotypically characterize clinical isolates of carbapenem-resistant *Enterobacteriaceae* collected from inpatients at the University Hospital of Santa Maria, during seven months. Among the clinical isolates subjected to the modified Hodge test (MHT), 62.5% were positive, indicating possible production of carbapenemase. Polymerase chain reaction (PCR) demonstrated that bla_{KPC} was the most frequently found gene (31%), followed by bla_{IMP} (12.5%). Combined use of the methods is needed to identify carbapenem resistance in enterobacteria to prevent their spread and control the infections caused by these organisms.

Key words: *Enterobacteriaceae*; carbapenemase; phenotypic methods; molecular methods.

INTRODUCTION

Antimicrobial resistance in *Enterobacteriaceae* is a serious threat to public health due to the association of different resistance mechanisms and the insufficient development of new drugs. Carbapenemase production, added to other resistance mechanisms already described in enterobacteria – such as the extended-spectrum betalactamases (ESBL) –, makes these microorganisms resistant to almost all available antimicrobials[1]. Thus, they are frequently associated with pandemic outbreaks, being responsible for thousands of deaths from infections that persist in spite of treatment[2, 3].

Three major classes of carbapenemases are known: 1) metallo-beta-lactamases (imipenemase [IMP], Verona imipenemase [VIM], New Delhi metallo-beta-lactamase [NDM]); 2) oxa-carbapenemases (OXA-48); and 3) *Klebsiella pneumoniae* carbapenemase (KPC)-type serine carbapenemases[2]. From an epidemiologic point of view, these enzymes are extremely relevant because of their rapid and wide dissemination, once they are encoded by genes located in mobile genetic elements, such as plasmids and transposons[4].

In Brazil, since the first detection of KPC in 2006, dissemination throughout the country is described, in outbreaks caused by *K. pneumoniae* and other enterobacteria. Other enzymes have already been found, such as IMP, VIM and, more recently, NDM[5, 6].

Although phenotypic tests are widely used in many clinical laboratories, it is necessary to confirm the production of enzymes that confer resistance to carbapenems by means of molecular tests, such as the polymerase chain reaction (PCR), which presents high sensitivity and specificity. The detection of carbapenemase-producing enterobacteria is extremely important to control infection and prevent dissemination of these resistance mechanisms[2, 4].

MATERIALS AND METHODS

Clinical isolates

Clinical isolates of carbapenem-resistant *Enterobacteriaceae* (CRE) were collected at the clinical laboratory of Hospital Universitário de Santa Maria (HUSM), from June to December 2013. Thirty-two positive culture samples obtained from any infection

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site were analyzed, identified by the automated method Vitek® 2 (BioMérieux) and/or by disk diffusion assay. For the conduction of experiments, from patients who had more than one sample testing positive for CRE, only the first positive culture was included.

This study was approved by the research ethics committee of Universidade Federal de Santa Maria (UFSM) under report nº 27094514.7000-5346.

Modified Hodge test

The modified Hodge test (MHT) was carried out as described by the Clinical and Laboratory Standards Institute (CLSI) (2013)[7]. The test was conducted using ertapenem (10 µg) or meropenem (10 µg) disks, the standard strain *Escherichia coli* (ATCC 25922) and the KPC-type carbapenemase-producing *K. pneumoniae* strain. The latter was kindly provided by Prof. Dr. Anna Sara Levin, of the medical investigation laboratory (LIM54), bacteriology laboratory of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP).

PCR for detection of carbapenemase-encoding genes

The extraction of deoxyribonucleic acid (DNA) was done by the boiling technique described by Woodford and Johnson (1998)[8]. All enterobacteria isolates with carbapenem resistance profile underwent PCR for the investigation of the following carbapenemase-encoding genes: bla_{KPC}, bla_{OXA-48}, bla_{NDM}, bla_{SPM}, bla_{IMP}, bla_{VIM} and bla_{GIM}. The identification of bla_{KPC}, bla_{OXA-48} and bla_{NDM} genes was made by PCR; the detection of bla_{SPM}, bla_{IMP}, bla_{VIM} and bla_{GIM} genes, by multiplex PCR. The primers used in the detection of serine carbapenemases were those described by Lomaestro *et al*. (2006)[9] and Poirel *et al*. (2011)[10]; for the detection of metallo-beta-lactamases, those indicated by Nordmann *et al*. (2010)[11] and Mendes *et al*. (2007)[12]. In both reactions, the 2x PCR kit LGC Biotecnologia was used. The strains used as positive control for reactions were provided by Prof. Dr. Anna Sara Levin, and had been obtained from the LIM54/FMUSP culture collection.

The PCR conditions for the detection of bla_{KPC}, bla_{OXA-48}, bla_{NDM} genes and other metallo-beta-lactamases were: denaturation for five minutes at 94ºC, 35 cycles of five minutes at 94ºC, annealing for 30 seconds at 58ºC, and extension for one minute at 72ºC, followed by a final extension step of 10 minutes at 72ºC.

The PCR products were added to GelRed™ (Biotium) and *gel-loading buffer (6x)* and underwent electrophoresis on 1.5% agarose gel run at 110 V/cm. The gels were visualized under an ultraviolet (UV) transilluminator (Hoefer MacroVue UV20) and photographed using the Loccus Biotecnologia LPix® and software L-Pix Image ST® (data not exhibited).

RESULTS

Based on the analysis of patients' records, it is possible to associate *Enterobacteriaceae* species with their respective isolation sites. The most frequent species, *K. pneumoniae*, was mainly isolated from stool and urine; *K. oxytoca*, from blood, tracheal secretion, and sputum. Data on resistance to ertapenem, imipenem and meropenem by each clinical isolate were associated with the MHT results and the presence of bla_{KPC} gene. These pieces of information are displayed in the **Table**, in which 12 different resistance profiles are observed.

Among the isolates subjected to MHT, 62.5% ($n = 20$) were positive, indicating a possible carbapenemase production. About the number of studied microorganisms, the genotypic tests evidenced that bla_{KPC} was the most encountered gene, in 31% ($n = 10$) of the samples, followed by bla_{IMP}, in 12.5% ($n = 4$). MHT was associated with the detection of resistance genes, and four main groups were observed, what can be seen in the Table.

TABLE – Profiles of isolates according to the modified Hodge test and bla_{KPC} gene detection

Species	MHT+ bla_{KPC}+	MHT+ bla_{KPC}-	MHT- bla_{KPC}+	MHT- bla_{KPC}-	Total
Enterobacter aerogenes		1		1	2
Enterobacter cloacae		3		1	4
Escherichia coli	1				1
Klebsiella oxytoca	4	1	1	1	7
Klebsiella pneumoniae	4	6		8	18
Total	9	11	1	11	32

KPC: K. pneumoniae *carbapenemase; MHT: modified Hodge test.*

DISCUSSION

Enterobacteria present multiple mechanisms of antimicrobial resistance, of which the production of carbapenemases stands out, as its dissemination is made easy by resistance gene transfer through plasmids and transposons[3]. Rapid identification of these enzymes is, thus, essential, and molecular tests able to identify specific carbapenemase-encoding genes must be included in laboratory routines[13].

Analysis of the different groups after association of MTH results and detection of resistance genes allows the discussion of some aspects. The first group encompasses the samples in which there was agreement between phenotypic (MHT) and genotypic methods. The second comprises samples that were positive in MHT, but negative in PCR. The third group includes a small number of samples, for which MHT resulted in low sensitivity, not detecting genes found when molecular tests were performed. The fourth group includes samples with negative results, both in MHT and PCR, evidencing that these isolates may show another mechanism of resistance that does not involve carbapenemases, as they show resistance to at least one carbapenem in the sensitivity test. In the cases encompassed by the second and fourth groups, it is possible that the isolates present a combination of resistance mechanisms, such as AmpC beta-lactamase hyperproduction, presence of efflux pumps or porin loss. However, in the first and third groups, it is not possible to exclude the existence of mechanisms other than carbapenemase production[14, 15].

Even if new CLSI guidelines have minimized the importance of phenotypic tests for clinical purposes, their use for identification of carbapenem resistance mechanisms may help in infection control, as they are sensitive and able to detect the activity of carbapenemases in *K. pneumoniae*, *K. oxytoca* and *E. coli* species[16]. Confirming other studies, the disk diffusion method, using ertapenem disks, proved more sensitive than that using meropenem in screening for resistant samples, demonstrating that screening may be optimized by the use of two carbapenems in combination[15, 17-19].

In Brazil, most reported CRE isolates contain the bla_{KPC}[5, 6, 12] gene. However, this study revealed low prevalence of this gene in the tested isolates. In addition, the concomitant presence of bla_{IMP} and bla_{KPC} genes was found in four isolates of *K. oxytoca*, *K. pneumoniae* and *E. cloacae*. The coexistence of these genes was reported by Wei *et al*. (2011)[20]. Pournaras *et al*. (2010)[21] verified a clinical isolate with simultaneous presence of bla_{VIM} and bla_{KPC} genes, which evidenced a higher minimal inhibitory concentration (MIC) for meropenem and imipenem. The authors point that the spread of these associated genes may have even more serious consequences for the treatment of such infections.

The variability demonstrated by phenotypic tests, both with ertapenem and MHT, means that it is not advisable to employ a single screening criterion for carbapenemase, but to associate methods to the confirmatory PCR test[15]. Similarly, it is necessary to research other genes and mechanisms of resistance, as the concomitant presence of several types of beta-lactamases in enterobacteria makes it difficult to identify individual resistance mechanisms[18].

In spite of its limitations, this study issues an alert to health professionals and clinical laboratories, so that they establish new criteria for the detection of carbapenemases. With the application of molecular techniques associated with traditional phenotypic tests, the detection of carbapenemases will become faster and reliable, aiding diagnosis and treatment, as well as providing information that directly influences the control measures for infections caused by these microorganisms.

RESUMO

Objetivou-se caracterizar fenotípica e genotipicamente isolados clínicos de enterobactérias resistentes aos carbapenêmicos (CRE) provenientes do Hospital Universitário de Santa Maria (RS). Entre os isolados clínicos submetidos ao teste modificado de Hodge (MHT), 62,5% apresentaram positividade, indicando possível produção de carbapenemase. A reação em cadeia da polimerase (PCR) demonstrou que o bla_{KPC} *foi o gene mais encontrado (31%), seguido de* bla_{IMP} *(12,5%). O uso conjunto de distintas metodologias faz-se necessário para identificar a resistência aos carbapenêmicos produzida pelas enterobactérias, de modo a auxiliar o controle de infecção prevenindo a disseminação desses microrganismos.*

Unitermos: *enterobactérias; carbapenemases; métodos fenotípicos; métodos moleculares.*

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