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Coproduction of NDM-1 and KPC-2 in *Enterobacter hormaechei* from Brazil

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The most important resistance mechanism against β-lactam drugs is the production of carbapenemases. In this study, we report the first identification of *Klebsiella pneumoniae* carbapenemase (KPC)-2 and New Delhi metallo-β-lactamase (NDM)-1 in *Enterobacter hormaechei* subps. *oharae* from Brazil. The detection of carbapenemases was done by phenotypic assays, PCR, and DNA sequencing, whereas the identification was performed by conventional techniques, sequencing of the 16S rDNA gene, and *hsp60*-genotyping. Molecular typing was performed using pulsed-field gel electrophoresis, and antimicrobial susceptibility was surrogated by the Etest methodology. Using the whole genome sequencing approach, we searched for resistance genes, plasmid incompatibility group genes, and the genetic environment of bla_{NDM} and bla_{KPC}. The plasmid identification was done by restriction digests with the S1 nuclease followed by hybridization using digoxigenin labeled specific probes. The isolate was considered multiresistant, being susceptible to amikacin and polymyxin B. We observed the following resistance genes: $bla_{CTX\text{-}M\text{-}15}$, $bla_{ACT\text{-}7}$, $bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$, *aadA1*, *aadA2*, *strA*, *strB*, *aac(3)-II*, *qnrB1*, and *aac(6′)-Ib-cr* and incompatibility group plasmid genes IncA/C, IncHI2, and IncN. The bla_{KPC} gene was found associated to the transposon Tn*4401* isoform b in plasmid with 50 kb (IncN) and $bla_{NDM\text{-}1}$ was flanked by a truncated IS*Aba125* and ble_{MBL} in plasmid with 160 kb (IncA/C). This study showed the coproduction of two important carbapenemases (KPC-2 and NDM-1) associated with mobile genetic elements of worldwide epidemiological importance (Tn*4401* and IS*Aba125*, respectively), reinforcing the idea that urgent measures are necessary to reduce and prevent the spreading of these carbapenemases primarily in the hospital settings.

Introduction

Nowadays, the most important resistance mechanism against β-lactam drugs is the production of carbapenemases, such as *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM). The KPC enzyme has been described first in 2001,[13] in the United States, but since then it has been already described in almost all continents. The dissemination of this gene is usually associated to mobile genetic elements, which provide a high capacity to spread, such as *Tn*4401,[4] and the clonal spread of *Klebsiella pneumoniae* strains belonging to clonal complex 11 (ST11, ST258) in many different countries.[8] In Brazil, it is responsible for outbreaks and sporadic cases since its first description, in 2009.[7]

NDM-1 was first reported in 2009[14] and had already been detected in several countries worldwide, whose gene is also found correlated to insertion sequences and transposon, like IS*Aba125* and Tn*125*.[12] In Brazil, this carbapenemase was first described in a *Providencia rettgeri* isolate recovered from a surgical wound of a patient from Porto Alegre, Rio Grande do Sul state, in February 2013.[2] Then, in the same hospital were detected six, clonally related NDM-1-producing *Enterobacter hormaechei* isolates.[3] Coproduction of KPC-2 and NDM-1 was first observed in a *K. pneumoniae* isolate in India.[6] In this study, we report the first identification

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of these two enzymes in the *E. hormaechei* isolate from Brazil.

Materials and Methods

As part of the Bacterial Nosocomial Infection Resistance Surveillance network, the Laboratório de Pesquisa em Infecção Hospitalar (LAPIH–FIOCRUZ), which belongs to the Brazilian Ministry of Health, routinely receives clinical bacterial isolates from hospitals and Central Laboratories of Public Health (LACENs) from different Brazilian states to confirm the mechanisms of drug resistance. On October 10th, 2013, one strain coproducing NDM and KPC, identified by the conventional technique as belonging to *Enterobacter cloacae* complex (CCBH14397), was detected. This strain was recovered from a 39-year-old female inpatient, admitted in a public hospital in the city of Rio de Janeiro. This isolate was recovered from a rectal swab obtained during surveillance.

The detection of carbapenemases was done by phenotypic assays[9] and PCR followed by DNA sequencing. The identification confirmation was performed by sequencing of the 16S rDNA gene and *hsp60*-genotyping.[5] Molecular typing was performed using pulsed-field gel electrophoresis (*Xba*I) with the aim to compare this strain to NDM-1-producing isolates from the same species previously detected in Brazil.[3] Antimicrobial susceptibility was surrogated by the Etest methodology (AB Biodisk) according to the manufacturer's instructions.

To investigate the presence of other resistance genes, plasmid incompatibility group genes, and the genetic environment of bla_{NDM} and bla_{KPC}, we performed whole genome sequencing using the Illumina MiSeq approach. The libraries were prepared using the Nextera XT kit (Illumina) and the reads obtained were assembled using the Velvet algorism.[15] The contigs were submitted to the ResFinder, PlasmidFinder, and pMLST database at the Center for Genomic Epidemiology (www.genomicepidemiology.org/) to search for other acquired antibiotic resistance genes, plasmid incompatibility genes, and plasmid incompatibility group alleles.

The identification of the size and the plasmid incompatibility groups of the plasmids carrying bla_{KPC} and bla_{NDM} was done by restriction digests with S1 nuclease, followed by hybridization using digoxigenin-labeled specific probes.[1]

Results and Discussion

The CCBH14397 isolate was characterized as *E. hormaechei* subps. *oharae*. On performing DNA sequencing, it was found that the isolate carried the allelic variants NDM-1 and KPC-2. Until now, in Brazil, these two variants have been the ones described.[10]

On performing molecular typing, it was observed that the isolate CCBH14397 had a different genomic digest DNA profile when compared to six, clonally related NDM-1-producing isolates of this same species in the Rio Grande do Sul state, sharing just 67.5% similarity.[3]

According to CLSI breakpoints (2013), the isolate was considered resistant to all the β-lactams tested (ertapenem, meropenem, imipenem, cefotaxime, and ceftazidime) with MIC ≥ 32 mg/L, ciprofloxacin (32 mg/L), gentamicin (48 mg/L), and being susceptible to amikacin (4 mg/L). According to EUCAST breakpoints, the isolate was resistant to tigecycline (4 mg/L) and susceptible by the broth microdilution method to polymyxin B (0.5 mg/L).

Whole genome sequencing performed originated 1,925,906 paired-end reads from a 500 cycle run, generating 73 contigs after the reads assemble. By ResFinder analyses, we observed other β-lactam resistance genes: $bla_{CTX\text{-}M\text{-}15}$, $bla_{ACT\text{-}7}$, $bla_{TEM\text{-}1}$, and $bla_{OXA\text{-}1}$ a part of the $bla_{KPC\text{-}2}$, $bla_{NDM\text{-}1}$; different aminoglycoside resistance genes (*aadA1, aadA2,strA, strB,* and *aac(3)-II*); and two plasmid-mediated quinolone resistance genes, *qnrB1* and *aac(6′)-Ib-cr* gene (that provide resistance to aminoglycosides and quinolones), contributing to the multidrug-resistant profile.

By PlasmidFinder analyses, it was possible to observe replicons belonging to groups IncA/C, IncHI2, and IncN. Performing pMLST analysis, the replicon of the incompatibility group IncN belonged to ST15.

Plasmid analysis showed three plasmids of approximately 50 kb, 160 kb, and 290 kb. Southern blotting hybridization, using probes for $bla_{KPC\text{-}2}$, $bla_{NDM\text{-}1}$, IncN, and IncA/C showed that the $bla_{KPC\text{-}2}$ was found in the 50 kb plasmid belonging to the IncN group, whereas the $bla_{NDM\text{-}1}$ was observed in the IncA/C plasmid of 160 kb.

Analyzing the genetic environment surrounding these genes, the $bla_{NDM\text{-}1}$ was found in a 5,932 bp contig and $bla_{KPC\text{-}2}$ in a 47,755 bp contig. Performing blast searches, we observed that the NDM-1 contig shared 100% identity and 6% coverage with the 94,794 bp region present in a plasmid carried by the previously mentioned *E. hormaechei* subps. *oharae* CCBH10892 isolate from Brazil (GenBank accession number KF727591). In this region, the $bla_{NDM\text{-}1}$ gene was found flanked by a truncated IS*Aba125* and a bleomycin resistance gene (ble_{MBL}). Despite having the same flanking genetic structure, using the sequence of CCBH10892 as a reference to map the reads of the KPC-2- and NDM-1-producing isolate (CCBH14397) with Geneious software R6.1 (Biomatters) was not possible to recover the entire plasmid region. Furthermore, the $bla_{NDM\text{-}1}$ gene was found in the CCBH14397 isolate on a different plasmid from those previously encountered in Brazilian strains of the same species (plasmids ranging from 420 to 490 kb).[3]

The 47,755 bp contig carrying $bla_{KPC\text{-}2}$ shared 100% identity with the 54.6 kb plasmid pKPC_FCF3/SP belonging to IncN recovered in Brazil from *K. pneumoniae* isolated in September, 2009, from a blood culture (GenBank accession number CP004367.2).[11] Using the sequence of pKPC_FCF3/SP as a reference to map the reads of our isolate with Geneious software R6.1 (Biomatters), we succeeded to recover the whole plasmid sequence. In this plasmid, $bla_{KPC\text{-}2}$ was found associated to the transposon Tn*4401* isoform b. In Brazil, there are reports of IncN plasmids carrying $bla_{KPC\text{-}2}$ associated to the same flanking structure in strains of *K. pneumoniae*.[10]

This study showed the coproduction of two important carbapenemases (KPC-2 and NDM-1) associated to other resistance genes in mobile genetic elements of worldwide epidemiological importance (Tn*4401* and IS*Aba125*, respectively) resulting in very broad-spectrum antibiotic resistance profiles. These findings reinforce the idea that urgent measures are necessary to reduce and prevent the spreading of these carbapenemases primarily in the hospital settings.

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Ethical Approval

The study was approved by the Fiocruz Ethics Committee and Brazilian National Committee for Research Ethics–CONEP under the reference number CAAE-000012/011-02.

Disclosure Statement

All authors report no conflicts of interest relevant to this article.

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