Proteus mirabilis carrying \textit{NTE}_{KPC-IId}, \textit{bla}_{NDM-1}, \textit{bla}_{OXA-10}, \textit{aph}(3')-VI, \textit{qnrD1} and IncQ and \textit{Col3M} plasmids from a hospital in Recife-PE, Brazil

Proteus mirabilis portador de \textit{NTE}_{KPC-IId}, \textit{bla}_{NDM-1}, \textit{bla}_{OXA-10}, \textit{aph}(3')-VI, \textit{qnrD1} e plasmídeos \textit{IncQ} e \textit{Col3M} proveniente de paciente internado em hospital de Recife-PE, Brasil

Proteus mirabilis transportando \textit{NTE}_{KPC-IId}, \textit{bla}_{NDM-1}, \textit{bla}_{OXA-10}, \textit{aph}(3')-VI, \textit{qnrD1} e plasmídeos \textit{IncQ} y \textit{Col3M} de un hospital en Recife-PE, Brasil

Abstract

The present study objective to characterize the clinical aspects of a patient infected with two strains of \textit{P. mirabilis} and the presence of resistance determinants in the two isolates from a patient at a public hospital in Recife-PE, Brazil. The total DNA of the isolates was extracted and submitted to PCR and amplicon sequencing for the investigation of resistance genes, \textit{bla}_{KPC}, \textit{bla}_{OXA-10}, \textit{bla}_{OXA-23}, \textit{bla}_{OXA-58}, \textit{bla}_{VIM}, \textit{bla}_{IMP}, \textit{bla}_{GES}, \textit{bla}_{NDM}, \textit{qnrD} and \textit{aac}(6')-Ib. Isolate P21-A2 harbored the \textit{aac}(6')-Ib, \textit{bla}_{OXA-10} and \textit{qnrD} genes. One of the isolates, P20-A2, was selected for plasmid DNA sequencing. The results showed that the patient developed multiple infections with various pathogens including two strains of \textit{P. mirabilis}. The patient was hospitalized for 103 days, had septic shock of skin, abdominal, pulmonary and ulcer focus, and died. Isolate P20-A2 harbored the genes \textit{bla}_{NDM}, \textit{qnrD}, \textit{aph}(3')-VI, \textit{bla}_{KPC} and \textit{bla}_{OXA-10} and plasmids IncQ and Col3M, together with NTE\textit{KPC-IId}. To our knowledge, this is the first report of \textit{P. mirabilis} harboring NTE\textit{KPC-IId}. Although \textit{P. mirabilis} is standing out as a cause of nosocomial infections and a resistant multidrug pathogen, this species is still neglected, the emergence of these \textit{P. mirabilis} isolates harboring aforementioned resistance determinants and the plasmids IncQ and Col3M demonstrate the potential for dissemination of important resistance genes, mainly in the case of \textit{P. mirabilis}.

Keywords: \textit{Proteus mirabilis}; NTE\textit{KPC-IId}; IncQ; Col3M.

Resumo

O presente estudo teve por objetivo caracterizar aspectos clínicos de um paciente com infecção por duas cepas de \textit{P. mirabilis} e a presença de determinantes de resistência nos dois isolados provenientes de um paciente de um hospital público de Recife-PE, Brasil. O DNA total dos isolados foi extraído e submetido a PCR e sequenciamento dos amplicons para a investigação de genes de resistência, \textit{bla}_{KPC}, \textit{bla}_{OXA-10}, \textit{bla}_{OXA-23}, \textit{bla}_{OXA-58}, \textit{bla}_{VIM}, \textit{bla}_{IMP}, \textit{bla}_{GES}, \textit{bla}_{NDM}, \textit{qnrD} e \textit{aac}(6')-Ib. O isolado P21-A2 albergava os genes \textit{aac}(6')-Ib, \textit{bla}_{OXA-10} e \textit{qnrD}. Um dos isolados, P20-A2, foi selecionado para o sequenciamento do DNA plasmidial. Os resultados mostraram que o paciente desenvolveu diversas infecções por vários patógenos incluindo duas cepas de \textit{P. mirabilis}. O paciente ficou internado por 103 dias, teve choque séptico de foco cutâneo, abdominal, pulmonar e úlcera, e veio a óbito. O isolado P20-A2 albergava os genes \textit{bla}_{NDM}, \textit{qnrD}, \textit{aph}(3')-VI, \textit{bla}_{KPC} e \textit{bla}_{OXA-10}, e os plasmídeos IncQ e Col3M, juntamente com
Two clinical isolates of *P. mirabilis* recovered from a patient admitted to a tertiary hospital in Recife-PE, Brazil, were selected. Biochemical identification and antimicrobial susceptibility profile were performed by automated Phoenix-BD system.
and data were interpreted according to CLSI, 2018 specifications. The study was approved by the Research Ethics Committee involving human beings (CEP/Plataforma Brasil) and opinion number 3.007.636.

For susceptibility tests, the following antimicrobials were tested: Amikacin, Amoxicillin-clavulanic acid, Ampicillin; Aztreonam, Cephalotin, Cefepime, Cefoxitin, Ceftazidime, Ceftriaxone, Ciprofloxacin; Gentamicin, Ertapenem, Imipenem, Meropenem, Levofloxacin, Pipercillin-tazobactam and Sulfametaxazol-trimetoprim.

Genomic DNA from the isolates was extracted using the Wizard Genomic DNA Purification Kit (Promega) and the resistance genes blaKPC, blaOXA-10, blaOXA-23, blaOXA-48, blaVIM, blaIMP, blaSPM, blaGES, blaNDM and qnrD, aac(6)-Ib were investigated by PCR. The amplification conditions and the used primers were shown in table 1. Positive and negative controls were included in each PCR.

The Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) technique was performed to determine the relationship between the two isolates, P20-A2 and P21-A2. Primers described in Table 1 were used. For the analysis of the ERIC results, the GelAnalyzer and DARwin 6.0 programs were used.

Table 1. Sequence of the PCR primers that were used in the study.

| Genes | Sequence of primers | Fragment Size | Annealing Temperature | Ref. |
|-------|---------------------|---------------|-----------------------|------|
| blaKPC | TGTCACTGTATCGCCGTCCTCAGTGCTCTACAGAAACC | 882bp | 63°C | (YIGIT et al., 2001a) |
| blaGES | ATCAAGCCACCTCTCAATGGTAGCATGGGACACATGAC | 860bp | 55°C | (BOYD et al., 2015) |
| blaNDM | TGCCCAATATATATGCACCCGGCGAAACCCTCTGCGTGTTGAG | 621bp | 60°C | (HUANG et al., 2017) |
| blaVIM | CAGATTGCGGTGGTGTGGAGGGGCGAGCTTGCCAGCGA | ND | 62°C | (CABRAL et al., 2012) |
| blaIMP | GGAATAGTGCTTTAATTCGTGTAGGCCTCAAYTTCACT | 232bp | 60°C | (CABRAL et al., 2012) |
| blaSPM | CCTAAATCTAAAGGCGACCCTGCGCTGTCCAGGATAACC | 271bp | 63°C | (GALES et al., 2003b) |
| blaOXA-10 | TCAACAAATCGCCAGAGATCCCCCACCGCAGAAACCAAG | 276bp | 62°C | (BERT et al., 2002) |
| blaOXA-23 | GATCGGATTGAGAACCAGATTTCGACCGCATTTCCA | 501bp | 57°C | (RANJBAR; ZAYERI; MIRZAIE, 2020) |
| blaOXA-48 | TTGGTGTCATCGATTATCGGACGACCTTCTGGATGAG | 743bp | 55°C | (POIREL et al., 2004) |
| blaOXA-58 | CGACTAGATGTTCAAGGCGACGATTCTCCCCCTGTGCC | 800bp | ND | (POIREL; NORDMANN, 2006b) |
| qnrD | CGAGATCAATTACCGGGGAAATAAACAGTGCTGACGCGCTGCTG | 500bp | 61°C | (CAVACO et al., 2009) |
| aac(6)-Ib | CCCGCTTTTCTGCTAGCAATGAGGCTAAATTGATGAT | 500bp | 52°C | (FIRMO et al., 2020) |
| ERIC | ATGTAAGCTCTGGGGATTTAACAAAGTAAAGTCAGCGCGC | ND | 36°C | (DUAN et al., 2009) |

ND – Not determined. Source: Authors.

To carry out the plasmid DNA sequencing, the isolate P20-A2 was selected because it harbors the blaKPC and blaNDM genes.

Plasmid DNA from isolate P20-A2 was extracted using the PureYieldTM Plasmid Miniprep System kit (Promega) according to the manufacturer's specifications. Isolates were characterized by Illumina MiSeq sequencing (Nextera XT libraries).
Data were processed to remove low quality readings using the Trimmomatic tool. Subsequently, the filtered readings were used for reassembly by applying the Velvet tool, whose parameters were optimized using the Velvet Optimiser program. Velvet results were also used as input to another assembly program, CAP3, in order to improve the assemblies. Gene prediction and annotation were performed using the Prokka program. Plasmid DNA sequences were analyzed using Artemis Sanger software. In addition, the Resfinder and PlasmidFinder platforms were used.

3. Results

Clinical patient information

In December 2017, a 43-year-old man was admitted for preoperative bariatric surgery. The patient had grade III obesity (216kg at admission) with a BMI of 71.4, hypertensive crisis, edema (with a restrictive disorder), Systemic Arterial Hypertension (SAH) and depression. After 16 days of hospitalization, the patient underwent open Roux-en-Y gastroplasty under balanced general anesthesia. The patient used a Blake drain in the abdominal cavity and elastic stockings in the lower limbs. The length of stay was 103 days. After surgery, the patient had several bacterial infections and died. The reason for death was pulmonary insufficiency, septic shock of skin, abdominal, pulmonary and ulcer focus. During the 103-day hospital stay, the patient used meropenem, vancomycin, polymyxin B, ampicillin, amikacin, daptomycin and amphotericin.

Proteus mirabilis isolates profile

A tissue sample and a sample of tracheal secretions from the patient were sent to the hospital's microbiology laboratory within 3 days of collection of both samples. Two clinical isolates of *P. mirabilis* (P20-A2 and P21-A2) multidrug resistant and possibly ESBL producers were recovered (Table 2). Isolate P20-A2 showed resistance to most antimicrobials, including first, second and third generation cephalosporins, sulfonamides, monobactams, carbapenems and quinolones. Isolate P21-A2 differed by not having resistance to carbapenems (Table 2). Isolates P20-A2 and P21-A2 did not show clonal relationship by Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR), by previous study (Beltrão et al., 2021).
Table 2. Phenotypic resistance profile and presence of resistance genes investigated by PCR for the two Proteus mirabilis isolates (P20-A2 e P21-A2). Id.: Identification; ICU – Intensive care unit, UCO –Coronary Unit; Int – intermediary.

| Id.   | Harvest date (dd/mm/yyyy) | Insulation sample | Sector           | Resistance profile | Resistance genes |
|-------|---------------------------|-------------------|------------------|--------------------|------------------|
|       |                           |                   |                  | Antimicrobial groups                               |                  |
|       |                           |                   |                  | Gentamicine (=8)(Int)                               |                  |
|       |                           |                   |                  | Cefepime (>16); Cefotaxime (>16); Ceftazidime (>32); Cefuroxime (>16) |                  |
|       |                           |                   |                  | Imipenem (>8); Meropenem (=6); Ertapenem           |                  |
|       |                           |                   |                  | Ampicillin (>16); Amoxicillin-acid clavulanic (>16/8); Piperacillin-tazobactam (=64/4)(Int) |                  |
|       |                           |                   |                  | Levofloxacin (>4); Ciprofloxacin (>2)                |                  |
|       |                           |                   |                  | Sulfamethoxazole-trimethoprim (>4/76) Aztreonam     |                  |
| P20-A2 | 23/03/2018                | Tissue            | ICU              | Aminoglycosides                                          | aph(3')-VI |
|       |                           |                   |                  | Cephalosporins                                          | blaoxa-10 |
|       |                           |                   |                  | Carabapenems Penicillins                                  | blaoxa-2 |
|       |                           |                   |                  | Quinolones and fluoroquinolones                          | blasme-1 |
|       |                           |                   |                  | Sulfonamide                                              | qnrD1        |
|       |                           |                   |                  | Monobactam                                               |               |
| P21-A2 | 25/03/2018                | Tracheal secretion | UCO             | Aminoglycosides                                          | aac(6')-Ib |
|       |                           |                   |                  | Cephalosporins                                          | blaoxa-10   |
|       |                           |                   |                  | Carabapenems Penicillins                                  | qnrD1        |
|       |                           |                   |                  | Quinolones and fluoroquinolones                          |               |
|       |                           |                   |                  | Sulfonamide                                              |               |
|       |                           |                   |                  | Monobactam                                               |               |

Source: Beltrão et al., (2021).

In addition to P. mirabilis, the patient also acquired infections at different sites. Morganella morganii and Klebsiella pneumoniae were isolated from abdominal cavity fluid, P. mirabilis (P21-A2) and Acinetobacter baumannii were isolated from tracheal secretions, from blood culture Enterococcus faecalis, from Morganella morgannii wound secretion and from a tissue sample from P. mirabilis (P20-A2).

Analysis of the genetic environment of the blaKPC gene and Plasmidial Incompatibility group (Inc)

The plasmid sequencing results showed that the P20-A2 isolate had a GC content of 47.8%, with a total of 31,899 bases. In summary, all sequenced content was assembled into 27 contigs, produced with 333.0x coverage. And 33 CDS were obtained (Table 3).

Table 3. Plasmid DNA characterization of Proteus mirabilis isolate P20-A2.

| Isolated | P20-A2 |
|----------|--------|
| GC content          | 47.8%  |
| Plasmid DNA sequence size | 31.899bp |
| Contigs          | 27     |
| CDS             | 33     |
| Found plasmids  | IncQ1  |
|                 | Col3M  |
| Resistance Genes | aph(3')-VI |
|                 | qnrD1  |
| Other genes found in plasmids | tnpA; tnpR; mobA; mobC; oriV; repA; repC; maze; mazF; traU; higA; dinG |

Source: Authors.
Analysis using the Resfinder and GenBank databases showed 100% identity for the \textit{bla}_{KPC-2}, \textit{aph(3')-VI} and \textit{qnrD1} resistance genes in the plasmid DNA of the P20-A2 isolate. Incompatibility replicons were found for plasmid IncQ and a small plasmid from the Col3M family (Figure 1).

**Figure 1.** Complete sequence of plasmid IncQ1 and non-Tn4401 mobile genetic element (NTE\textsubscript{KPC-IIId}) that harbors the \textit{bla}_{KPC-2} gene in the study isolate P20-A2 and comparison with reference sequences from GenBank (NTE\textsubscript{KPC-IIId}:MG786907 (Beltrão et al., 2020)). Protein coding sequences were represented as arrows and marked with the gene name. Gray dashed represents shared homologous regions (>95%) The NTE\textsubscript{KPC-IIId} IR sequence is represented by a circle.

The \textit{bla}_{KPC-2} gene was found inserted between the partial IS\textsubscript{Kpn6} insertion sequence (ΔIS\textsubscript{Kpn6}) with an associated left IR (IRL) and \textit{tnpR} resolvase (Figure 1). A 21 bp fragment corresponding to the Δ\textit{bla}_{TEM} gene was found upstream of the \textit{bla}_{KPC-2} gene. When comparing the genetic environment of the \textit{bla}_{KPC-2} gene with the sequences deposited in GenBank, we observed the NTE\textsubscript{KPC-IIId} variant (GenBank accession number: MG786907) with approximately 100% identity for the isolates.

The other genes \textit{mobA}, \textit{mobC}, \textit{oriV}, \textit{repA}, \textit{repC}, \textit{mazE}, \textit{mazF}, \textit{traU}, \textit{higA} and \textit{dinG} were identified using Blast/NCBI.

**4. Discussion**

In addition to other HAIs, \textit{P. mirabilis} can cause infections in the colonized skin and oral mucosa of hospitalized patients (Wasfi et al., 2020). It can cause serious infections, in addition to contributing to the increase in hospitalization time and the use of various antimicrobials. The patient in the present study acquired infections by \textit{Morganella morganii}, \textit{K. pneumoniae}, \textit{P. mirabilis} (P21-A2), \textit{Acinetobacter baumannii} and \textit{Enterococcus faecalis} in different infection sites, for a period of 103 days. Patients who acquire infections in the hospital have a history of recurrent infections, especially if the hospital stay is prolonged (Wasfi et al., 2020). The most common clinical manifestations caused by \textit{P. mirabilis} are urinary tract infections (UTIs), but little is known about other infections caused by this pathogen, as in the case of the patient in the present study with respiratory tract and tissue infections.

In addition to the establishment of serious infections in the host by pathogenic strains, bacterial resistance to antimicrobials has been a matter of great concern to world health agencies, especially in strains that harbor resistance determinants to carbapenems. The isolates analyzed in the present study harbored resistance determinants, including the \textit{aph(3')-VI}, \textit{aac(6')-Ib} and \textit{bla}_{OXA-10} genes, widely reported in other species such as \textit{K. pneumoniae} or \textit{Pseudomonas aeruginosa} (Firmo et al., 2020).

However, in \textit{P. mirabilis}, these resistance genes are little investigated when compared to other bacterial species. In the present study, clinical characteristics of the patient and genetic and resistance aspects of the two isolated strains were analyzed. Isolate P20-A2 harbored the \textit{bla}_{KPC-2} and \textit{bla}_{NDM-1} genes. The presence of these genes represents a real challenge, since the identification of this resistance phenotype by routine laboratory tests in the hospital does not have the sensitivity of molecular
The genetic environment of the bla\textsubscript{KPC}-2 gene has been widely investigated around the world. The Tn4401 transposon is commonly reported to harbor this gene and has been widely studied. To date, this transposon has nine variants named Tn4401a to Tn4401i, differentiated by deletions in its structure. The structure of Tn4401 comprises two tnpA, one tnpR, the insertion sequences ISKp6 and ISKp7, the bla\textsubscript{KPC} gene and the istA and istB genes, all these structures help in the transferability of this transposon. In addition to the Tn4401 transposon, other transposons may harbor the bla\textsubscript{KPC}-2 gene, such as the Tn3000 transposon and the non-Tn4401 mobile element (NTE). NTE\textsubscript{KPC-IId} has been reported in Brazil in K. pneumoniae and Klebsiella aerogenes, including in hospitals in Recife-PE, Brazil (Cerdeira et al., 2017; Beltrão et al., 2020; Lima et al., 2020; Peña et al., 2020; Lima et al., 2020). There is evidence that NTE\textsubscript{KPC-IId} is the variant circulating in Recife harboring the bla\textsubscript{KPC} gene in K. pneumoniae and K. aerogenes, together with the plasmid IncQ (Beltrão et al., 2020b; Lima et al., 2020; Oliveira et al., 2020). Since bla\textsubscript{KPC} is widely disseminated in our country (Almeida et al., 2012; Pereira et al., 2013, 2015; Dalmolin et al., 2018; Oliveira et al., 2020). In addition, NTE\textsubscript{KPC-IId} may decrease or enhance the spread of bla\textsubscript{KPC} (Beltrão et al., 2020). In addition, to our knowledge NTE\textsubscript{KPC-IId} has not yet been reported in P. mirabilis.

Plasmid IncQ1 found in isolate P20-A2 is a small, promiscuous, non-conjugative plasmid. However, this plasmid has the ability to bind to conjugative plasmids at the time of conjugation, which can facilitate its dissemination in pathogenic bacteria of the same species and different species (Beltrão et al., 2020; Lima et al., 2020; Oliveira et al., 2020). In addition to plasmid IncQ1, a small plasmid belonging to the Col3M family was found harboring the qnrD1 gene, which confers resistance to quinolones, in isolate P20-A2. The presence of qnrD transported by plasmid Col3M has been little reported, what is known is that the qnrD gene is widely disseminated in P. mirabilis isolates causing infections in humans and animals (Sanches et al., 2019). Plasmid-mediated resistance mechanisms are of concern as they have a greater capacity to spread by horizontal gene transfer (Rozwandowicz et al., 2018; Lerminiaux e Cameron, 2019).

5. Conclusion

In conclusion, although P. mirabilis is gaining prominence as a cause of nosocomial infections and resistant multidrug pathogen, this species is still neglected. The emergence of these P. mirabilis isolates harboring resistance determinants such as qnrD1, bla\textsubscript{KPC}-2, bla\textsubscript{NDM-1}, aph(3’)-VI, aac(6’)-Ib and bla\textsubscript{OXA-10} and the plasmids IncQ and Col3M demonstrates the potential for dissemination of important resistance genes, especially in the case of P. mirabilis. Additionally, the mobile genetic element NTE\textsubscript{KPC-IId}, together with IncQ may be related to the high spread of the bla\textsubscript{KPC} gene in Recife. Co-infections can contribute to a poor prognosis, especially in immunocompromised patients with prolonged hospitalization, as in the present study. Additionally, further studies are needed on the transferability of the non-Tn4401 mobile element (NTE\textsubscript{KPC-IId}) and investigations of the genetic environment of the bla\textsubscript{KPC} gene in other circulating isolates from Pernambuco.

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Conflict of interest

The authors declare that there are conflicts of interest.
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