**Klebsiella pneumoniae blaNDM-1 carrying a class 1 integron causing a hospital outbreak in a Mexican attention center**

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**Abstract**

Introduction: Infections acquired in hospitals are the cause of high morbidity and mortality and with the emergence of resistant bacteria, the problem is greater. The aim of this work was to determine the genetic characteristics and timeline of *Klebsiella pneumoniae* blaNDM-1 carrying a class 1 integron involved in an intrahospital outbreak.

Methodology: Investigation was made from the first detection of *K. pneumoniae* blaNDM-1, strain “466”, and the last clone “423”. 16S rRNA gene analysis showed that 466 strain and clones were related to *K. pneumoniae*. Extended-spectrum β-lactamases (ESBL) was detected according to the Clinical and Laboratory Standards Institute (CLSI) and real time-PCR. Typing of *K. pneumoniae* blaNDM-1 strains was carried by ERIC-PCR and sequencing the variable region of the integrons were performed.

Results: A cluster of six resistant isolates of *K. pneumoniae* blaNDM-1 was detected in intensive care unit (ICU), internal medicine (IM) and orthopedics (OT). Timeline revealed that the first bacterial identification was in ICU and the last clone in OT service. The array genetic of variable region was “IntI/aadA5-dfrA17/qacEΔ1-Sul1”.

Conclusions: The evidences highlight the importance of the epidemiological surveillance of Extended-spectrum β-lactamases (ESBL) strains, as well as the need for molecular epidemiological studies to identify the routes of transmission and the contamination sources within health personnel.

**Key words:** Hospital outbreak; *Klebsiella pneumoniae*; blaNDM-1; class 1 integron.

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**Introduction**

Bacterial infections acquired in hospitals are the cause of high morbidity and mortality rates worldwide and with the emergence of antibiotic-resistant bacteria, the problem is greater, and it represents a challenge for health personnel and immunosuppressed patients [1-3]. It has been reported that inanimate surfaces are the main sources of contamination [4,5]. Moreover, cross contamination between patients and medical personnel, as well as patients releasing bacteria to the environment are determinant in the transmission of pathogens [6,7]. Reports indicate that 20 to 40% of pathogens causing hospital infections come from cross contamination by health personnel [8,9]. Gram-negative bacilli, especially species of the genus *Klebsiella*, are among the main bacteria that cause outbreaks in the intensive care units (ICU) [10-13]. With the recent emergence of multi-resistant strains due to extended-spectrum β-lactamases (ESBL), the persistence of pathogens in the hospital environment plays an important role in morbidity and mortality [14].

Carbapenemases are increasingly detected in *Klebsiella pneumoniae* and other members of *Enterobacteriaceae* family, such as class A carbapenemase (KPC)-type, class B metallo-β-lactamases (MBLs mainly *blaIMP*, *blaVIM* and *blaNDM*-
type enzymes) and class D blaOXA-48-like enzymes [15-17]. Another type of important genetic elements that are associated with antibiotic resistance are class 1 integrons. These structures are versatile for the capture of resistance cassettes, therefore, their identification and characterization in outbreaks associated with antibiotic resistant bacteria is of great relevance and could be a potential supplementary tool in the characterization of nosocomial outbreaks [18-20]. Additionally, the molecular typing has been widely used in the study of clonal dissemination of pathogens in the hospital environment to investigate nosocomial outbreaks. In the present study, we present the characterization of an outbreak in the “Hospital Juárez de México”, caused by K. pneumoniae blaNDM-1 strain 466 that colonized six patients. This strain was able to produce a carbapenemase type blaNDM-1 and carry a class 1 integron with the genetic arrangement (IntI1/aadA5-dfrA17/qacEA1-Sul1) located in genome and plasmid respectively. Implications of the propagation of multiresistant strains in hospitalized patients and their attempt to eradicate them are discussed.

Methodology

Outbreak investigation

This work describes the methodology in the characterization of an outbreak due to K. pneumoniae blaNDM-1 and carrier of a class 1 integron in the “Hospital Juárez de México”. The investigation of the outbreak included the first detection of K. pneumoniae blaNDM-1, strain “466” (June 21st, 2016; beginning of the outbreak) and the identification of the last clone “423” (September 14th in the same year). On December 31st, the outbreak was declared as finished. Phenotypic and molecular assays were performed on all positive isolates of K. pneumoniae together with prospective epidemiological surveillance and data collection. Additionally, information (file number, diagnosis, antimicrobial treatment, gender/age of the patient, hospital service and source of isolation) was analyzed. Bacterial cultures were obtained and characterized from routine diagnosis and demographic analyses were performed retrospectively, so neither Institutional Review Board (IRB) approval nor informed consent from patients was required.

Bacterial identification

Thirty-six substrates were used for the identification of strains at the genus and species level through a phenotypic test by using the system Phoenix-100 (Becton Dickinson, USA), according to the manufacturer protocol. In order to confirm, sequence analysis of the 16S rRNA gene was performed as follow. 16S rRNA gene was amplified by using universal primers (27F and R1492) according to DeSantis et al. [21] (Table 1). Reactions were performed in a SEEAMP™ PCR system (Seegene, Seoul, Korea). The nearly complete 16S rRNA gene sequence was determined as described by Kwon et al. [22]. Sequencing was carried out by the Biology Institute, at the “Universidad Nacional Autónoma de México” by using an ABI PRISM® 310 Genetic Analyzer sequencer (Applied Biosystems, CA, USA). Sequences were compared with the nucleotide sequence database (GenBank) by means of the Blast algorithm [23]. Phylogenetic analysis was carried out by maximum-likelihood after multiple alignments of data by Thompson et al. [24].

Antimicrobial resistance assay

Antimicrobial resistance was determined by Clinical and Laboratory Standards Institut (CLSI) [25]. Antimicrobial susceptibility tests was performed by the use to Cefotaxime (CTX, 30 μg), Cefotaxime-Clavulonate (CTX-CLA, 30/10 μg), Ceftazidime (CAZ, 30 μg), Ceftazidime-Clavulonate (CAZ-CLA, 30/10 μg), Cefepime (FEP, 30 μg), Cefoxitin (FOX, 30 μg), Cefotetan (CTT, 30 μg), Ceftriaxone (CRO, 30 μg), Ertapenem (ETP, 10 μg), Meropenem (MEM, 10 μg), Imipenem (IPM, 10 μg), Aztreonam (ATM, 30 μg).

Table 1. Primers used in this study.

| Primer   | Target              | Amplicon size (bp) | 5' / 3' | Reference |
|----------|---------------------|--------------------|---------|-----------|
| 27F      | 16S rRNA            | 1465               |         | [21]      |
| 1492R    | Variable region     | Variable           |         | [39]      |
| in-F     | qacEA1/Sul1 3’CS    | 800                |         | [40]      |
| in-B     | IntI 5’CS           | 923                |         | [41]      |
| qacEA1-F | Intergenic consensus| Variable           |         |           |
| sul1-B   |                     |                    |         |           |
| IntI1-F  |                     |                    |         |           |
| IntI1-R  |                     |                    |         |           |
| ERIC1R   |                     |                    |         |           |
| ERIC2    |                     |                    |         |           |
coli ATCC 25922 was used as control. Results were inferred as susceptible or resistant by measuring the diameter of inhibition.

**Carbapenemase-coding genes detection by real time-PCR**

Detection of *blaKPC*, *blaOXA-48* and *blaNDM-1* was performed by real time-PCR, using the BD MAX™ CRE ASSAY set in the system BD™ MAX (Becton Dickinson, NJ, USA). Additionally, a macroarray assay based in reverse hybridization (Sepsis Flow Chip™ Kit) was performed in order to confirm the presence of *blaNDM-1* marker (Master Diagnóstica, Granada, Spain). Location of carbapenemase-coding gene was done by plasmid DNA isolation and used as template in PCR assays.

**Detection of class 1 integrons and their gene cassettes**

Strains were screened in both templates (genomic DNA and plasmid) for class 1 integron elements: 5´ integrase (*Int1I*)/variable region/*qacEΔ1–sulI*) 3´ using primers previously described (Table 1). Products of variable region were purified, sequenced and compared with the nucleotide sequence database (GenBank) by using the BlastX algorithm by using strict filter parameters with more than a 99% of nucleotide identity, and at least 80% query coverage. *Escherichia coli* W3102 carrying pAr–32 plasmid (*IncU*, Class 1 integron (*IntI1–aadA2–qacEA1/sulI*, *In6 (catA2)*)), *Aeromonas salmonicida* (Amp*) carrying pRAS1 plasmid (*IncU*, Class 1 integron (*IntI1–dfrA16–qacEA1/sulI*), *Tn1721 (TetA)*) were used as positive control and *E. coli* J53–1 (NalR F *pro met*) as negative control in the detection of class 1 integrons. *A. salmonicida* subsp. *salmonicida* 718 and *E. coli* J53–1 were provided by Dr. Glenn Rhodes from the Centre for Ecology and Hydrology in Lancaster, UK. Strains were screened in both templates (genomic DNA and plasmid) for class 1 integron elements: 5´ integrase (*IntI1*)/variable region/*qacEΔ1–sulI*) 3´ using primers previously described (Table 1).

**Molecular typing of Klebsiella pneumoniae blaNDM-1 466 strain and clones**

*Klebsiella pneumoniae* blaNDM-1 466 and the five derived clones were analyzed by ERIC–PCR.

**Table 2.** Characteristics of patients involved in the *Klebsiella pneumoniae* blaNDM-1 outbreak in a mexican hospital.

| Isolate       | Patient gender | Age | Hospital service | Isolation source | Patient survival | Diagnostic                                                                 | Treatment before detection of Kpn blaNDM-1                                                                 | Treatment after detection of Kpn blaNDM-1 |
|---------------|----------------|-----|------------------|------------------|-----------------|----------------------------------------------------------------------------|--------------------------------------------------------------------------------|----------------------------------------|
| *K. pneumoniae* 466 | Female         | 81  | ICU*             | Expectoration    | No              | Liver abcess and pneumonia                                                 | Levofloxacin, metronidazole, cefepime and ciprofloxacin Ceftriaxone, piperacilllin, tazobactam and vancomycin | Colistin                               |
| *K. pneumoniae* 391 | Female         | 45  | ICU              | Catheter         | Yes             | Subarachnoid hemorrhage                                                     | Ciprofloxacin, ceftazidime and amikacin Piperacillin, tazobactam, vancomycin and imipenem | Colistin                               |
| *K. pneumoniae* 332 | Male           | 28  | ICU              | Urine            | No              | Testicular cancer and pulmonary metastasis Gastric G1 neuroendocrine tumor and septic shock | Imipenem, levofloxacin, metronidazole, meropenem, piperacillin, azobactam, ceftazidime and cefepime Imipenem, levofoxacin, ceftriaxone and metronidazole | Colistin                               |
| *K. pneumoniae* 381 | Male           | 34  | ICU              | Purulent wound   | No              | Soft tissue sepsis, pneumonia and urinary tract infection                  |                                                                                          | Colistin                               |
| *K. pneumoniae* 208 | Female         | 32  | IM**             | Blood            | No              | Soft tissue infection and uncontrolled diabetes mellitus                    |                                                                                          | Colistin                               |
| *K. pneumoniae* 423 | Male           | 53  | OT***            | Expectation      | No              |                                                                             |                                                                                          | Colistin                               |

* Intensive care unit; ** Internal medicine; *** Orthopedic.
Amplification conditions were: pre-denaturation at 95 °C for 5 minutes, denaturation at 90 °C for 30 seconds, annealing at 50 °C for 1 minute, and extension at 72 °C for 1 minute for 30 cycles with a final extension at 72 °C for 8 minutes. Products were analyzed by genetic pattern profiles and dendrogram analysis with the BioNumerics V4.5 program was done.

Conjugal transfer experiments
Donors *K. pneumoniae blaNDM-1* and recipient *E. coli* J53-1 strains were adjusted to 10⁸ CFU/mL, mixed (1:1) and spotted on the surface of 0.22 μm nitrocellulose filters, positioned on LB agar and incubated overnight at 37 °C. Bacteria were suspended in 100 μL and were diluted serially and spread on MacConkey agar plus 10 μg/mL nalidixic acid and 25 μg/mL streptomycin or 1 μg/mL meropenem for transconjugants and nalidixic acid for recipients. Conjugal transfer frequencies were calculated by dividing the CFU transconjugants by the CFU recipients.

Results
Outbreak investigation
Six patients infected with *K. pneumoniae blaNDM-1* were identified in a period of 4 months. This is equivalent to 0.137% of a total of 4,355 cultures of all Gram-negative bacteria during 2016. According to the timeline in the occurrence of cases associated with *K. pneumoniae blaNDM-1*, the origin of the outbreak was associated with strain 466 (June, 2016) in the intensive care unit (ICU). *K. pneumoniae blaNDM-1*(466 strain and 391, 332, 381, 208 and 423 clones) were detected in three hospital services at HJM: ICU (n=4), internal medicine (IM) (n=1) and orthopedics (OT) (n=1). From this first finding, epidemiological and microbiological surveillance was performed in hospital services of the HJM. Only one patient of the female gender in the ICU survived. A summary of microbiological detection, patient characteristics and isolation origin of 466 strain and its clones are shown in Table 2. Finally, an outbreak investigation timeline was carried out from the first identification in ICU (Figure 1).

Phenotypic and genetic bacterial identification
Biochemical reactions revealed that 23 substrates out of 43 were assimilated by isolates with a 99% confidence assignment. The analysis by system assigned the genus and species to strain 466 and clones 391, 332, 381, 208 and 423 as *K. pneumoniae*. Phylogenetic analysis and maximum-likelihood indicated that 466 strain and clones 391, 332, 381, 208 and 423 were closely related to *K. pneumoniae* subsp. *pneumoniae* strain Z-24 (KP135420.1) with 99.99% of similarity, correlating with the phenotypic assay.

Antimicrobial susceptibility assay
The results of antimicrobial susceptibility assay, revealed that 466 strain and clones 391, 332, 381, 208 and 423 showed resistance profile in both methods probed. All isolates were resistant to CTX, CAZ, FEP, FOX, CTT, CRO, ETP, MEM, IPM, and ATM. No inhibition halo was detected for CTX-CLA and CAZ-CLA.
Plasmid DNA isolation

Plasmid extraction assays in the isolates revealed in a plasmid of ≈ 15 Kb in 466 strain and clones 391, 332, 381, 208 and 423.

Detection of class 1 integron and cassettes

The PCR products of intI1 (923 bp), variable region and qacEΔ1–sulI (800 bp) were identified in 466 strain and 391, 332, 381, 208 and 423 clones. Variable region identified was 1.8 kb, indicating possible identical genetic arrangements (Figure 2). The sequences revealed inserted cassettes corresponding to genes coding for adenyllyl transferases (aadA5), dihydrofolate reductases (dfrA17). The final array in all isolates was as follow: “intI1-aadA5-dfrA17-qacEΔ1–sulI” (Figure 2). Elements of class 1 integron were detected in plasmid DNA.

Typing of K. pneumoniae blaNDM-1 466 strain and clones

Profiles of the intergenic products revealed sizes of amplicons ranged from slightly more than ≈300 bp to about ≈1700 bp (Figure 3). Intergenic region diversity not allowed the differentiation of 6 isolates, therefore were clustered in one genetic group. According to above, the six strains were indistinguishable from each other (100% similarity).

Conjugal transfer experiments

Conjugal transfer frequencies were 2.27×10⁻⁶ and identical plasmid profile was detected in all tranconjugants compared with strains donor. Also, PCR assays were performed in order to detect all elements of class 1 integron. According to above, class 1 integron was located in plasmid and it is transferable to recipient bacteria.

Discussion

The ESBL-producing Enterobacteriaceae causing hospital outbreaks continues to be a major public health problem mainly in developing countries, such as Mexico. Here, we describe the K. pneumoniae blaNDM-1 isolation in six patients from June to the end of September 2016. The presence of the molecular markers of carbapenemases has been previously identified in several pathogens belonging to the family Enterobacteriaceae, being K. pneumoniae one of the most important in the hospital environment [12,13,26,27].

According to the Mexican national system of epidemiological surveillance, the notification of the detection of carbapenemase-producing pathogens is mandatory along with the implementation of prospective epidemiological surveillance. In particular, the identification of the molecular marker “blaNDM-1” is relevant because the β-lactamase encoded by this gene is capable of inactivating all the antibiotics of the carbapenemic family, except aztreonam [28]. In this sense, the hospital outbreaks related to K. pneumoniae blanNDM-1 are alarming, due to the fact that all broad-spectrum antibiotics are not effective in antimicrobial therapy.

Molecular tools play an important role in the identification and dissemination of pathogens in the hospital environment. The analysis of the sequences of the 16S rRNA gene allowed classifying the pathogen detected in K. pneumoniae, with an identity of 99.99% with sequences described previously. It is interesting to know if the isolates reported in this work are associated with similar clones detected in other hospital outbreaks. In Mexico, K. pneumoniae harboring blaNDM-1 was firstly identified in an hospitalized pediatric patient [29] and more recently, it has been reported as a causative agent of intrahospital outbreaks [30-33], suggesting an emerging role of this multiresistant strain in nosocomial environments. For this reason, futures studies are needed to determine the possible genetic association of strain 466 with other K. pneumoniae blanNDM-1 identified in Mexico and other parts of the world by sequencing additional genes such as recA and gyrB and even the complete genome. Clonal relation assays reported in the present work are important to identify the relationship between isolates with similar phenotypic characteristics. The results of the clonal relationship provide evidence to strengthen the hospital epidemiological surveillance system in making decisions about infection control measures associated with health care. The evidence generated by ERIC-PCR
showed a clonal dissemination of K. pneumoniae bla<sub>NDM-1</sub> in six patients from three direct hospital departments. Even though the patients hospitalization periods did not overlap each other (according to the timeline), the analysis revealed that all the isolates were genetically related. Cross contamination by medical personnel or by environmental contamination is suggested. It has been shown that the ICU is one of the hospital areas with the highest incidence in the detection of hospital outbreaks [10,11]. Sources of contamination derived from cross-contamination by staff, and the use of equipment that is not subject to a high-level cleaning process, are the main sources of pathogens [4,5]. Plasmids encoding antimicrobial resistance are diverse in K. pneumoniae and can also carry other resistance genes, including carbapenemase genes [34]. Conjugational transfer experiments were performed to demonstrate that the bla<sub>NDM-1</sub> marker was located in plasmid. Our results showed that the resistance marker bla<sub>NDM-1</sub> was not able to transfer because this marker was located in the bacterial chromosome, correlating with previous studies where chromosomal bla<sub>NDM-1</sub> has been observed, such as existence of integration events of carbapenemic markers, mediated by presence of transposons [35]. Other elements associated with antibiotic resistance in Gram-negative bacteria are the class 1 integrons [36]. These structures, having the capacity to capture resistance genes, assumed that the bla<sub>NDM-1</sub> gene could possibly be found in an integron. Under this hypothesis, search of class 1 integrons in K. pneumoniae bla<sub>NDM-1</sub> was done.

PCR assays and sequence analysis revealed an identical genetic arrangement in the six isolates (intI1/aadA5-dfrA17/qacE1-Sul1), located in plasmid. The genetic arrangements constituted by aadA and dfrA cassette variants have been previously reported in other members of the Enterobacteriaceae family that cause hospital outbreaks and that carry class 1 integrons [37]. One of the main causes of the spread of pathogens with resistance markers is the border transfer of infected patients [38]. Under this argument, the clinical record of the patient from where strain 466 was isolated was analyzed and did not contain information on the history of travel abroad. The control measures implemented after the detection of K. pneumoniae bla<sub>NDM-1</sub>, included the exhaustive disinfection of the areas of patients infected with the subsequent search for the carbapenemase producing pathogen, and extreme isolation precautions of the patients to avoid possible cross-contamination.

In conclusion, the temporality of the six cases, the genetic relationship determined by molecular typing, as well as the phenotypic resistance profile and the genetic elements associated with resistance shared between the isolates allowed to define K. pneumoniae as the causing of a nosocomial outbreak that included fatal cases. This strain was able to produce a carbapenemase type bla<sub>NDM-1</sub> and carry a class 1 integron with the arrangement (intI1/aadA5-dfrA17/qacE1-Sul1) located in genome and plasmid respectively.

Conclusions
The evidences presented in this work, highlight the importance of the constant epidemiological surveillance of strains producing EBLs at local level, as well as the need for epidemiological studies of national coverage to identify the possible routes of transmission and the possible sources of contamination within health personnel.

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