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Proceeding Paper

In Silico Analysis and PCR Characterization of non-Tn*4401* Transposable Elements in *Pseudomonas aeruginosa* †

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Abstract: The multi-resistance presented by *P. aeruginosa* has greatly increased due to the presence of genes for carbapenemases such as bla_{KPC}. The dissemination of this gene has been associated with the Tn*4401*, the main mobile genetic element that carries bla_{KPC} in its structure. However, some non-Tn*4401* elements (NTE_{KPC}) associated with bla_{KPC} have been found in different bacteria. Here, we characterized in silico and in vitro bla_{KPC}-associated elements in *P. aeruginosa*. To identify these elements, a search algorithm was performed using NCBI databases; sequences were filtered and pair-aligned to describe the bla_{KPC} genetic environment. Additionally, a PCR-based strategy was designed to target Tn*4401* variants and NTE_{KPC} groups and assessed in 61 Colombian clinical isolates. Using an in silico approach, 51 bla_{KPC}-positive entries longer than 3kb (in the bla_{KPC} upstream region) were found; from these, 72.7% carried an NTE_{KPC}. On the PCR assay, Tn*4401* was the most frequent element among the *P. aeruginosa* in Colombia. However, NTE_{KPC}-IIf was presented on 29.5% of the isolates, in different genetic lineages and at least in four hospitals. These results show high NTE_{KPC} prevalence in *P. aeruginosa*.

Keywords: multi-resistance; *Pseudomonas aeruginosa*; bla_{KPC}; NTE_{KPC}; carbapenemases; Tn*4401*

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1. Introduction

The circulation of bacteria carrying beta lactamases genes is increasing, since these genes are continuously transmitted by horizontal transfer, which has caused the emergence of new classes of multi-resistant bacteria that have become a public health problem around the world [1]. The carbapenemase-encoding bla_{KPC} gene was initially reported in *K. pneumoniae* [2]. However, this gene has been transmitted to other enterobacteria, such as *Escherichia coli* or *Salmonella enterica* and, more recently (in 2007), to *Pseudomonas aeruginosa*, the first record in non-enterobacteria organisms [3–5].

The main mobile genetic element (MGE) associated with bla_{KPC} dissemination toward new genetic structures is Tn*4401*, a transposon commonly associated with high-risk plasmids and clones that facilitate the propagation of this gene, such as ST258 in *K. pneumoniae* [6–10]. Nonetheless, in recent years different elements unrelated to Tn*4401* surrounding bla_{KPC} have been identified. These elements are known as NTE_{KPC} (non-Tn*4401* elements) [7], and may play a relevant role in the spread of bla_{KPC} [7,11]. Based on the bla_{KPC} upstream structure, NTE_{KPC} can be classified in at least three subgroups (I, II and III) [7]. In *P. aeruginosa*, the information is mostly focused on anti-biotic resistance, so

the relevance of these new elements for bla_{KPC} dissemination has not been deeply studied; yet, this gene is showing a rapid expansion around the world in this species [12].

Therefore, the aim of this work was to characterize, in silico, the transposable elements associated with bla_{KPC} in *P. aeruginosa*, according to reports presented in the GenBank, to contribute information that allows us to elucidate the dissemination mechanisms of this resistance determinant. Subsequently, a method was designed for the identification by PCR of the Tn*4401* variants and NTE_{KPC} groups and used to determine the elements and frequency in a cohort of Colombian clinical isolates.

2. Methods

This research was divided into two phases: an in silico phase, which aims to analyze and characterize the genetic environment of bla_{KPC}-positive isolates in *P. aeruginosa* that have been reported in the GenBank; and an in vitro phase, which consisted of the experimental analysis of a cohort of bla_{KPC}-positive isolates of *P. aeruginosa*, to characterize the region that flanks upstream the bla_{KPC} gene and establish the frequency of circulation of NTE_{KPC} elements in this species in Bogotá, Colombia.

2.1. In Silico Phase

Exploration of the bla_{KPC} Genetic Environment for *P. aeruginosa* in the GenBank

Initially, a database was created for compiling information of the bla_{KPC} genetic environments in *P. aeruginosa* collected in the GenBank (reviewed until 13 October 2021). All partial or fully sequenced nucleotide entries with more than 3000 bp upstream bla_{KPC} were included. General information of the entries such as country, length, replicon type (linear or circular), bla_{KPC} variant, position in the genome, isolate name, and access information (GenBank and PMCID access numbers) were also registered. Nucleotide sequence for all entries was exported and compared against reference sequences of the Tn*4401* and its variants (a–i) and NTE_{KPC} and its different subgroups (I, II and III), whose classification criteria are based on the region upstream of bla_{KPC}.

In the case of no association with previously reported genetic environments, the entry was characterized by manual curation using the Artemis Comparison Tool (ACT), BLASTn and BLASTp [13,14] and specialized databases for mobile genetic elements (TnRegistry and ISFinder) and resistance genes (CARD) [15,16]. Paired alignments were developed and plotted using Easyfig [17], showing identity between pairs in a window of 300 bp.

2.2. PCR Essay for Tn4401 or NTE_{KPC} Identification

Primer Design

Using default parameters in the NCBI Primer BLAST platform [14], several primer sets were designed, which aimed to amplify bla_{KPC} upstream regions and differentiate, by amplicon size, the Tn*4401* subtypes or NTE_{KPC} subgroups. Briefly, to amplify the different upstream regions, the reverse primer must align with the bla_{KPC} gene, and forward primers were designed to align with specific regions (for each group) that were absent on the other possible MGEs. For the recognition of the Tn*4401*, the forward primer was designed to align with the *istB* gene, which has not been reported on the NTEs. However, for the NTE_{KPC} subgroups, the primers were designed to align with the IS*Kpn27* (initially misreported, like IS*Kpn8*) (NTE_{KPC}-I), the resistance gene bla_{TEM} (NTE_{KPC}-II) and the Tn*5563* resolvase—genes that are unique for each subgroup.

For the PCR assays, bacterial isolates were cultured in 3 mL of LB broth;later, total DNA was extracted by phenol-chloroform method [18] and purified with 70% ethanol. Finally, the DNA was resuspended in 50 µL of molecular-biology-grade water. Using the designed oligonucleotides, PCRs were performed to identify and classify bla_{KPC} upstream surroundings as NTE_{KPC} (either I, II, or III) or as a Tn*4401* variant. PCR products were evaluated by agarose electrophoresis (1% agarose in 1 × TBE buffer) and stained with ethidium bromide (0.01 µg/mL). With the results, the frequency of circulation of these genetic structures in the Colombian *P. aeruginosa* was reported.

3. Results and Discussion

3.1. In Silico Analysis of the Collected Reports

Using an in silico approach, 60 bla_{KPC}-positive *P. aeruginosa* sequences longer than 3 Kb in the upstream flanking region were retrieved, of which, 73.3% (n = 44) carried bla_{KPC} in an NTE_{KPC} environment, and the remaining 26.6% (n = 16) in a Tn*4401* transposon. This is a remarkable result, since, around the world, Tn*4401* has been reported as the main element associated with the dissemination of bla_{KPC} [19]. These results suggest the dynamics of dissemination of this resistance gene in *P. aeruginosa* present different behavior to that observed in *Klebsiella pneumoniae*. Additionally, all the sequences were associated with the bla_{KPC-2} isoform, which historically is the predominant variant in the world [8]. In *P. aeruginosa*, NTE_{KPC} elements were identified in three countries, in South America and Asia, whilst Tn*4401* was identified in North America, South America and Asia (Table 1).

Table 1. KPC distribution based on the collected reports to date (21 May 2022).

MGE	Genetic Landmark	Location	ST
NTE_{KPC}-I n = 32	IS*Kpn27*	China (n = 30)	Chromosome (n = 5)
			Plasmid (n = 25)
		Brazil (n = 2)	Plasmid (n = 2)
NTE_{KPC}-II n = 9	bla_{TEM}	China (n = 5)	Plasmid (n = 5)
		Brazil (n = 1)	Plasmid (n = 1)
		Colombia (n = 3)	Plasmid (n = 3)
NTE_{KPC}-III n = 0	Tn*5563* resolvase	Not reported	Not reported
NTE_{KPC} n = 3	ND	China (n = 1)	Plasmid (n = 1)
		Brazil (n = 1)	Plasmid (n = 1)
		France (n = 1)	Plasmid (n = 1)
Tn*4401* n = 16	IS*Kpn7*	France (n = 2)	Plasmid (n = 2)
		Argentina (n = 2)	Plasmid (n = 2)
		Colombia (n = 2)	Chromosome (n = 1)
			Plasmid (n = 1)
		USA (n = 2)	Plasmid (n = 2)
		Brazil (n = 1)	Plasmid (n = 1)
		Chile (n = 2)	Plasmid (n = 2)
		China (n = 4)	Plasmid (n = 4)
		Nepal (n = 1)	Not reported

Group I was the most frequent subgroup (77.7%, n = 32) among the NTE_{KPC}, which is characterized by having an IS*Kpn27* upstream bla_{KPC}. However, majority of these reports (93.75%) come from China, suggesting NTE_{KPC}-I is locally disseminated in this country. Unlike the rest of the world, China has reported that its main KPC disseminator, not only for *P. aeruginosa* but for many different bacteria, is a chimera of Tn*3*-Tn*4401* that presents an IS*Kpn27* (misreported as *ISKpn8*) upstream of the bla_{KPC} gene; therefore, this genetic environment is also considered to be an NTE_{KPC}, most probably of group I [20]. Interestingly, the only subgroup of NTE_{KPC} that was presented on the chromosome of a *P. aeruginosa* (n = 5) was the NTE_{KPC}-I, which may indicate the vertical transmission of bla_{KPC} through this type of element.

One entry presented upstream bla_{KPC}, both a Tn*3* resolvase and an unknown resolvase that did not match the current nomenclature [7], preventing it to be classified in any of the established groups. In another case, the upstream region showed an IS26 insertion

sequence which is not related to any established group. Lastly, the presence of the IS*Kpn27* (of NTE_{KPC}-I) and IS*6100* (of NTE_{KPC}-III) prevented group discrimination in another entry. The remaining seven entries (11.36%) belonged to NTE_{KPC}-II and were reported in China, Brazil and Colombia [7,21,22].

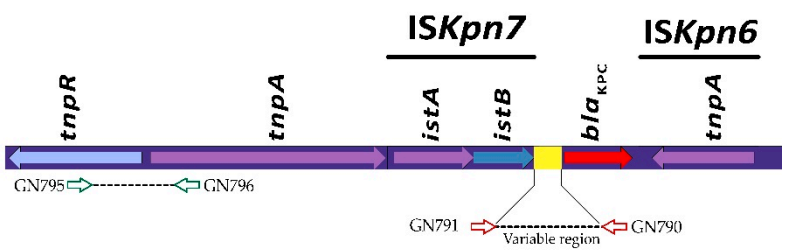
Sixteen entries reported a sequence type for the *P. aeruginosa*, and from these, six (37.5%) were ST463—all presented in China. These isolates carried NTE_{KPC}-I; ST1006 was reported by two isolates (12.5%) and three isolates (18.75%) reported ST235. The rest of the STs (ST381, ST697, ST316, ST277 and ST308) presented in just one isolate and in different countries; this also suggests the dissemination of local clones. Sequence types 235, ST308 and ST1006 were found in Colombia; ST277 and ST381 in Brazil; and ST463 and ST697 in China. The appearance of the diverse genetic backgrounds associated with NTE_{KPC} in *P. aeruginosa* suggests these types of elements may play a preponderant role in bla_{KPC} dissemination in this species. However, the characteristics of these genetic environments must be studied to elucidate the role they play in the genetic mobility of this resistance gene.

3.2. In Vitro Results

3.2.1. Tn*4401* and NTE_{KPC} Primers Design

Two pairs of primers were designed to identify the Tn*4401* and its variants. The first pair of oligonucleotides was designed to detect the bla_{KPC} and *istB* genes (Figure 1), as they are part of a conserved region in the different Tn*4401* isoforms and is absent in all NTE_{KPC} reported structures. The size of the amplicons generated in this PCR depends on the Tn*4401* variant, since some of the isoforms of this transposon have deletions in this region and are mostly distinguishable by the size of the deleted bases (Figure 1).

A.



B.

Variant	*istB-bla*KPC product* (GN790-GN791)	*tnpR-tnpA* product* (GN795-GN796)	*istB-bla*KPC deletion size±
Tn*4401a*	424	909	99 pb
Tn*4401b*	523	909	0 bp
Tn*4401c*	307	909	216 bp
Tn*4401d*	NA	909	Δ*bla*KPC e IS*Kpn7* absent
Tn*4401e*	268	NA	255 bp
Tn*4401f*	523	NA	0 bp
Tn*4401h*	335	909	188 bp
Tn*4401i*	268	909	255 bp

Figure 1. PCR for Tn*4401* detection and variants discrimination. (**A**) Tn*4401* and location of PCR oligonucleotides for Tn*4401* variants detection. Genes and their coding orientations are indicated by horizontal arrows; these are enclosed in a purple box indicating the boundaries of Tn*4401*. The white arrows represent the primers and the product generated by them is denoted by a dotted line. (**B**). The size of the product generated by primers GN790/GN791 is specific for all variants except "e" and "i", which generate products of the same size, and "d" which do not predict to amplify. Primers GN795/GN796 do not predict to amplify for the variants "e" and "f". * NA = Not amplify. ± The number indicates the size of the deletion between *istB* and bla_{KPC}. Variants b and f have no deletion.

Additionally, a PCR was designed to differentially amplify NTE_{KPC} groups (I, II and III). For this, the reverse primer is located on the bla_{KPC} gene as in the specific PCR for Tn*4401*. However, forward oligonucleotides target group-specific hallmarks; then, in combination with the conserved reverse primer targeting bla_{KPC}, specific products for the

NTE_{KPC}-I, NTE_{KPC}-II, and NTE_{KPC}-III elements (Figure 2) were generated. For NTE_{KPC}-III amplification, the primer was designed to target the *tnpR* present on Tn*5563*, unique to this element. For the differential amplification of NTE_{KPC}-II, the primer targets the bla_{TEM} resistance gene and for NTE_{KPC}-I targets the *tnpA* of IS*Kpn27* (Figure 2). The IS*Kpn27 tnpA* gene is found in both NTE_{KPC}-I and II (Figure 2), so amplification with this oligonucleotide generates a product in both groups. However, the size of the amplicon and the presence of bla_{TEM} allow for differentiation between them.

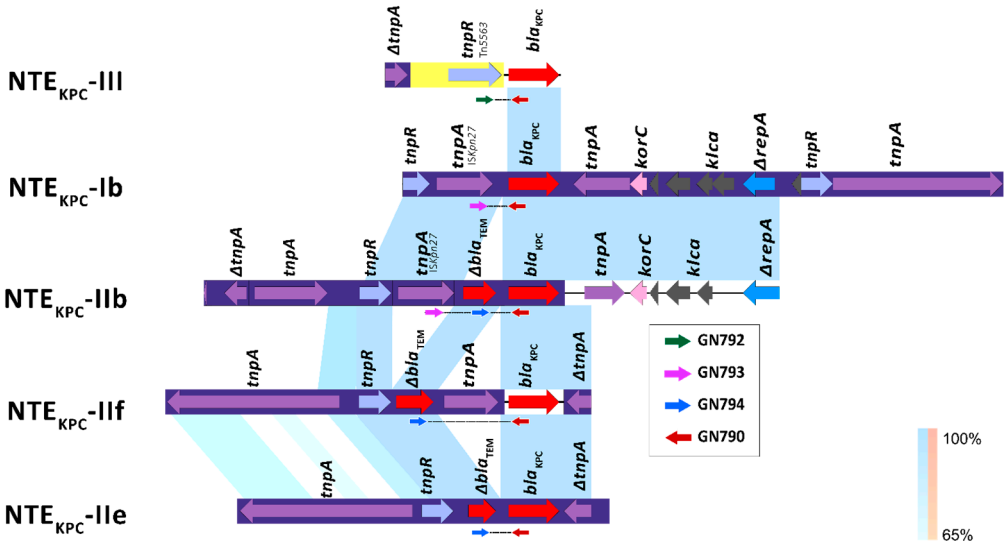


Figure 2. Schematic representation of the location of primer GN790 in conjunction with GN792, GN793 and GN794 for the differential amplification of NTE_{KPC}-III, NTE_{KPC}-II and NTE_{KPC}-I, respectively. Light-blue bars between sequences indicate areas of identity. White arrows with colored outlines represent primers; these are below the target sequence.

This strategy is the first reported that allows us to identify and differentiate groups of NTE_{KPC} and eight of the nine Tn*4401* variants (Tn*4401g* was not included). This method can be used for the rapid genetic screening of bla_{KPC} harboring isolates, not only in *P. aeruginosa*, and in clinical settings or research, to contribute to the surveillance of this resistance gene.

3.2.2. Characterization of the Genetic Environment Associated with bla_{KPC} in Colombian Clinical Isolates of *P. aeruginosa*

The different PCRs were standardized and implemented for the characterization of the region upstream of the bla_{KPC} gene in 61clinical isolates, from five hospitals, in Bogota, Colombia. None of the isolates amplified for more than one PCR, suggesting that they did not have multiple copies of the bla_{KPC} gene. In the analyzed population, two mobilization platforms associated with bla_{KPC} were identified—Tn*4401* (n = 37, 60.7%) and NTE_{KPC}-II (n = 19, 31.1%). In addition, five (8.2%) isolates did not amplify for any of the PCRs (Figure 3). Although the primers were designed to determine the NTE_{KPC} group (either I, II or III), but not the specific variants among them (the product generated for the NTE_{KPC}-II variants a, b, c and e is the same—371 bp), it is possible to differentiate the NTE_{KPC}-IIf, since this variant has a *TnpA* gene inserted between bla_{TEM} and bla_{KPC} (Figure 2). Here, of 19 NTE_{KPC}-II-positive isolates identified, 18 (94.7%) harbored NTE_{KPC}-IIf elements, whilst the remaining isolate amplified the 371 bp product.

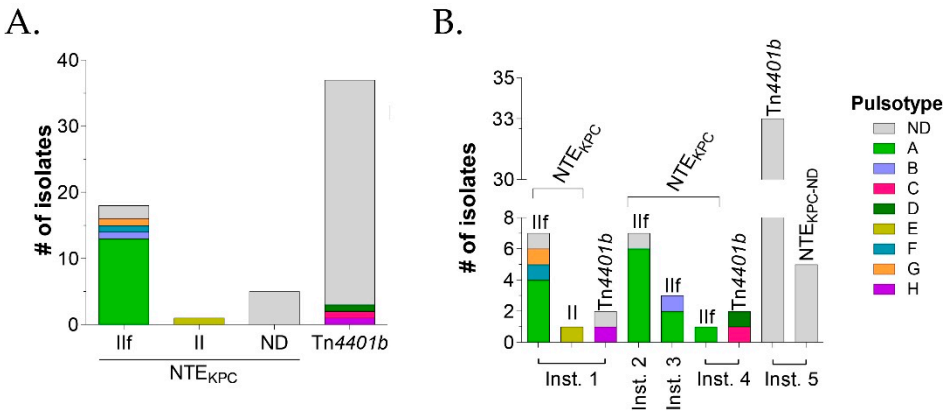


Figure 3. Genetic platforms associated with *bla*$_{KPC}$ in five hospitals in Bogota, Colombia. (**A**) Distribution of the NTE$_{KPC}$ and Tn*4401* elements, in a cohort of 61 clinical isolates. (**B**) Frequency of the elements associated with *bla*$_{KPC}$ informed by institution. PFGE pulsotypes of the isolates are shown in different colors. ND = Not determined, for the pulsotype.

The main platform associated with *bla*$_{KPC}$ was the Tn*4401* (60.7%). However, most of the isolates harboring this element came from one institution (86.8%, *n* = 33), suggesting a local spread. In four out of five institutions, there were NTE$_{KPC}$-IIf-positive isolates, and in three of these, it was the most predominant element, with 70% (*n* = 7), 100 (*n* = 7) and 100% (*n* = 3), for institutions one, two and three, respectively. Interestingly, NTE$_{KPC}$-IIf circulated among different and unrelated PFGE pulsotypes (Figure 3).

Two representative isolates of the most frequent pulsotype (A), were sequenced with a long-reads strategy to obtain a complete assembly. The results confirmed the presence of the NTE$_{KPC}$-IIf. Additionally, with the complete genome of these isolates, we performed an MLST analysis, which showed that both correspond to ST235, a globally dispersed clone [23], which has shown a high capacity to acquire antibiotic resistance genes [24]. This clone has been previously described transporting *bla*$_{KPC}$ within the classic Tn*4401* transposon [10,25]. However, to the best of our knowledge, this is the first report of the high-risk ST235 clone and fourth report of *P. aeruginosa* isolates carrying *bla*$_{KPC}$ embedded in these novel NTE$_{KPC}$ elements [2].

4. Conclusions

According to the information from the GenBank, the dissemination of the *bla*$_{KPC}$ resistance gene is mainly due to NTE$_{KPC}$ non-conventional elements. In Colombia, although the Tn*4401* was abundant (mostly in one institution), a high frequency of NTE$_{KPC}$-II elements could be evidenced, in four different institutions, and even though this genetic environment had not been previously reported in our region, it seems to be endemic to these institutions. Additionally, we found a set of isolates that did not amplify any of the designed PCRs, which indicates that these isolates do not present a Tn*4401*, but also suggests the presence of a new NTE$_{KPC}$ variant. However, additional studies are required to determine the characteristics of this region in these isolates.

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