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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 multiresistance presented by in *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 (NTEKPC) 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 elemgeents, a search algorithm was performed using NCBI databases, sequences were filtered, and pair-aligned to describe the *bla*KPC genetic environment. Also, a PCR-based strategy was designed to target Tn*4401* variants and NTEKPC groups and assessed in 61 Colombian clinical isolates. By the *in-silico* approach it was found 51 *bla*KPC-positive entries longer than 3kb (in the *bla*KPC upstream region), from these, 72.7% carried an NTEKPC. On the PCR assay, Tn*4401* was the most frequent element among the *P. aeruginosa* in Colombia, however NTEKPC-IIf was presented on 29.5% of the isolates, in different genetic lineages and at least in four hospitals. These results show high NTEKPC prevalence in *P. aeruginosa*.

Keywords: multiresistance; *Pseudomonas aeruginosa*; *bla*KPC; NTEKPC; carbapenemases; Tn*4401*

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1. Introduction

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 bacteria multi resistant 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 propagation of this gene, like 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 NTEKPC (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 NTEKPC 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 with information that allows elucidating the dissemination mechanisms of this resistance determinant. Subsequently, a method was designed for the identification by PCR of the Tn*4401* variants and NTEKPC 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, to establish the frequency of circulation of NTEKPC elements in this species in Bogotá, Colombia.

2.1. In Silico Phase

2.1.1. Exploration of the blaKPC 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's 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 NTEKPC and its different subgroups (I, II and III), whose classification criteria are based on the region upstream of *bla*KPC.

In case of no association with previously reported genetic environments, the entry was characterized by manual curation using 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 [18], showing identity between pairs in a window of 300-bp.

2.2. PCR Essay for Tn4401 or NTEKPC Identification

2.2.1. 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 NTEKPC subgroups. Briefly, to amplify the different upstream regions 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 it has not been reported on the NTEs. However, for the NTEKPC subgroups, the primers were design to align with the IS*Kpn27* (initially misreported like IS*Kpn8*) (NTEKPC-I), the resistance gene *bla*TEM (NTEKPC-II) and with 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 and 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 NTEKPC (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

As result of the *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 NTEKPC 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 a different behavior to that observed in *Klebsiella pneumoniae*. Also, all the sequences were associated with the *bla*KPC-2 isoform, which historically is the predominant variant in the world [8]. In *P. aeruginosa*, NTEKPC 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
NTEKPC-I *n* = 32	IS*Kpn27*	China (*n* = 30)	Chromosome (*n* = 5)
			Plasmid (*n* = 25)
		Brazil (*n* = 2)	Plasmid (*n* = 2)
NTEKPC-II *n* = 9	*bla*TEM	China (*n* = 5)	Plasmid (*n* = 5)
		Brazil (*n* = 1)	Plasmid (*n* = 1)
		Colombia (*n* = 3)	Plasmid (*n* = 3)
NTEKPC-III *n* = 0	Tn*5563* resolvase	Not reported	Not reported
NTEKPC *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 NTEKPC, which is characterized by having an IS*Kpn27* upstream *bla*KPC. However, majority of these reports (93.75%) come from China suggesting NTEKPC-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, so this genetic environment is also consider as a NTEKPC, most probably of group I [20]. Interestingly, the only subgroup of NTEKPC that was presented on the chromosome of a *P. aeruginosa* (*n* = 5) was the NTEKPC-I, which may indicate the vertical transmission of *bla*KPC through this type of element.

One entry presented upstream *bla*KPC a Tn*3* resolvase and an unknown resolvase, that did not match to the current nomenclature [7], so could not be classified as any of the

stablished groups. In other case, the upstream region showed an IS26 insertion sequence which is not related with any stablished 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. Remaining seven entries (11.36%), belonged to NTE$_{KPC}$-II, and were reported in China, Brazil and Colombia [7,21,22].

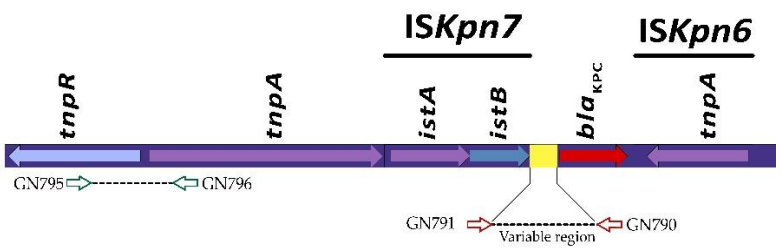
Sixteen entries reported sequence type for the *P. aeruginosa*, and from these, 6 (37.5%) were ST463 and all of them were presented on China, these isolates carried NTE$_{KPC}$-I, ST1006 was reported by two isolates (12.5%) and three isolates (18.75%) reported ST235, the rest of the STs (ST381, ST697, ST316, ST277 and ST308) were presented in just one isolate and in different countries, this also suggests dissemination of local clones. Sequence type 235, ST308 and ST1006 were found in Colombia; ST277 and ST381 in Brazil and ST463 and ST697 in China. The appearance of diverse genetic backgrounds associated with NTE$_{KPC}$ in *P. aeruginosa* suggests these type of elements may play a preponderant role to the *bla*$_{KPC}$ dissemination in this species. However, 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. Tn4401 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*4401*i	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.

Also, 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, it is generated specific products for the NTEKPC-I, NTEKPC-II, and NTEKPC-III elements (Figure 2). For NTEKPC-III amplification, the primer was designed to target the *tnpR* present on Tn*5563*, unique to this element. For the differential amplification of NTEKPC-II, the primer targets the *bla*TEM resistance gene and for NTEKPC-I targets the *tnpA* of IS*Kpn27* (Figure 2). The IS*Kpn27 tnpA* gene is found in both NTEKPC-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 differentiation between them.

This strategy is the first reported that allows to identify and differentiate groups of NTEKPC 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 in research, to contribute to the surveillance of this resistance gene.

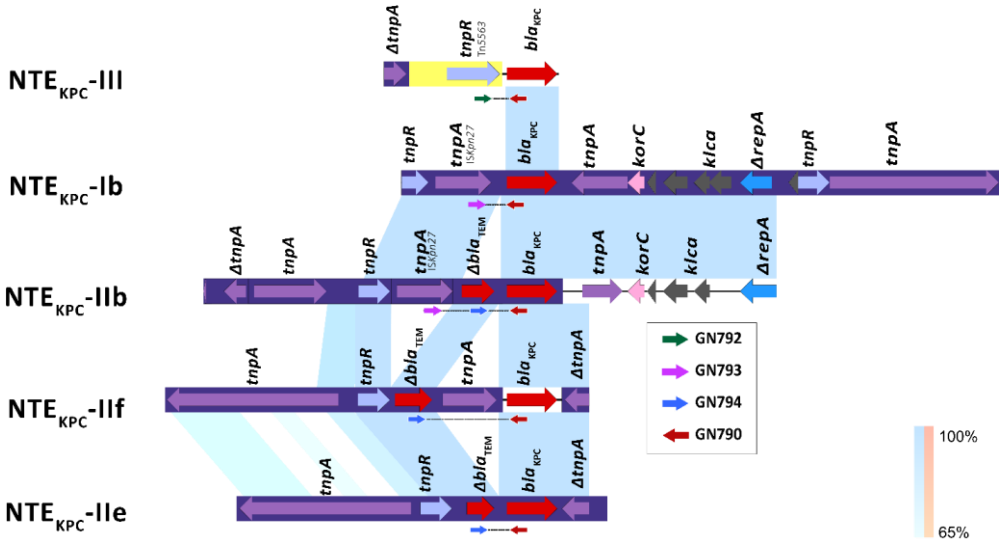


Figure 2. Schematic representation of the location of primer GN790 in conjunction with GN792, GN793 and GN794 for the differential amplification of NTEKPC-III, NTEKPC-II and NTEKPC-I, respectively. Light blue bars between sequences indicate areas of identity. White arrows with colored outlines represent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

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, the Tn*4401* (n = 37, 60.7%) and the NTEKPC-II (n = 19, 31.1%), also five (8.2%) isolates did not amplify for any of the PCRs (Figure 3). Although the primers were designed to determine the NTEKPC group (either I, II or III), but not the specific variants among them (the product generated for the NTEKPC-II variants a, b, c and e is the same, 371 bp), it is possible to differentiate the NTEKPC-IIf, since this variant has an insertion of a *TnpA* gene, between *bla*TEM and *bla*KPC (Figure 2). Here, of 19 NTEKPC-II positive isolates identified, 18 (94.7%) harbored NTEKPC-IIf elements, whilst the remaining isolate amplified the 371 bp product.

The main platform associated with *bla*KPC was the Tn*4401* (60.7%). Although 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 NTEKPC-IIf positive isolates, and in

three of these, 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 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. 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 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].

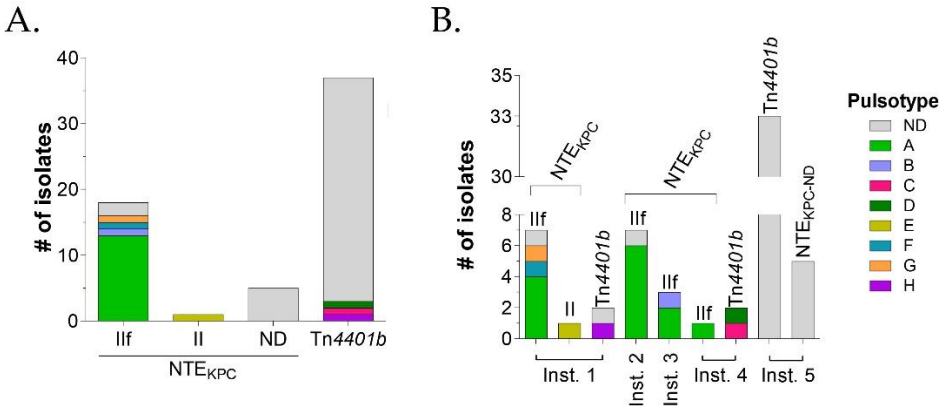


Figure 3. Genetic platforms associated with *bla*$_{KPC}$ in five hospitals in Bogota, Colombia. (**A**) Distribution of the NTE$_{KPC}$ and Tn*4401* elements, in a cohort of 61 clinical isolates. (**B**) Frequency of the elements associated with *bla*$_{KPC}$ informed by institution. PFGE pulsotypes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

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 thought 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 amplified for 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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