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Insilico Comparative Genome Analysis On Resistome Determinants And Mobile Elements Of Ndm Resistance

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Abstract:
Objective: The study focuses on a genome wide comparison of NDM carriers in view to analyze the Insertion Sequences (IS) along with other associated antibiotic resistance genes that might accord with the virulence and dissemination of the blaNDM gene.
Material and Methods: A total of 380 plasmids from NCBI were taken for analysis. Out of 380 plasmids analyzed; Resfinder tool sorted 101 plasmids of varying sizes (3kb to 350kb) carrying blaNDM gene from different organism. ISSaga tool annotated ISs (ISAba125, IS26, ISEc33, ISSen4 etc) for the 101 plasmids taken for analysis. Phylogenetic analysis of 101 plasmids using Mafft were clustered based on the IS type and NDM variant.
Results: Aminoglycoside resistance was found among 70 plasmids (69%) followed by sulfonamide resistance (56%) and other beta lactamase resistant genes (39.6%) in addition with blaNDM gene. ISAba125 (34%) is found to be the most prevalent IS followed by IS5D and ISVsa3. ORF sequences analyzed projects on the dispersal capability of these IS to become potential composite/conjugative transposons and hence facilitate transfer of NDM resistance. Phylogenetic tree generated forecasted the intimacy of different plasmids mobilized at global level.
Conclusion: The study gives a predisposing significance on plasmid-mediated antimicrobial resistance spread both locally and globally influenced of mobile elements. Hence, understanding the genome plasticity and dynamics of transmission of NDM resistance would throw light on the evolutionary pathway of multidrug resistant superbugs.

Keywords: NDM, Insertion Sequence, Antibiotic Resistance, Genome wide analysis

INTRODUCTION

Antibiotic resistance is a serious concern to public health, undermining our ability to treat common illnesses that are spreading throughout the world. The global emergence of Carbapenem-resistant Enterobacteriaceae has recently been linked to increased morbidity and death [1]. The Centers for Disease Control and Prevention (CDC) in the United States regards them as a serious hazard to human health.
The enzymatic degradation of β-lactamases is the primary cause of -lactam resistance among Enterobacteriaceae, including carbapenem resistance. The most prevalent carbapenamase, New Delhi metallo lactamase (NDM), poses a great danger to public health on a worldwide scale, having been discovered in more than 70 countries since 2009, following a Swedish resident's medical treatment in India. NDM is the subtype of the Class B MBL, which can inactivate most antimicrobial drugs but is still sensitive to colistin and tigecycline [2]. Through site-specific recombination of the NDM gene, these bacteria frequently contain several resistance genes. So far, 31 variants of blaNDM gene (NDM1-NDM31) have been identified from various bacterial species in various countries as per Betalactamase database

(http://bldb.eu/).The development of unique unrelated clones has been reported in recent reports of NDM infections arising in newer geographical areas [3]. As a result, it is critical to comprehend the mechanisms of resistance development and transfer.
NDM variations were discovered to be linked with all other classes of antibiotic resistance enzyme producing genes, such as AmpC, IMP, TEM, bleomycin resistance, sul, aad, and so on. Under the regulation of ISAba125, the bleMBL and blaNDM genes are usually co-expressed (Tn125). The development of resistance gene clusters in close proximity is linked to mobile elements, resulting in a multidrug resistant phenotype [4]. In bacterial genomes, insertion sequences (ISs) are the smallest transposable mobile genetic elements that are extensively dispersed. ISs elements carry the gene sequences encoding a transposase, allowing them to move across the bacterial genome. The exchange of resistance genes between bacterial chromosome / plasmid and their integration into particular genetic elements, called integrons, play a major role in acquisition and dissemination of resistance genes [5].
Hence, the objective of this study is to perform an Insilico analysis on plasmid genomes carrying NDM resistance in order to scrutinize the Insertion Sequences along with other antibiotic resistance genes that might accord with the virulence and dissemination of the NDM resistance.

METHODOLOGY:

Retrieval of Complete Plasmid sequences:

A total of 380 plasmid sequences from the NCBI plasmid database (Nov 2017) were retrieved for analysis.

Antibiotic Resistant gene identification:

The antibiotic resistant genes in the plasmids were found using the tool, Resfinder(https://cge.cbs.dtu.dk/services/ResFinder).

Insertion Sequence Analysis:

Genetic features and properties of each Insertion Sequences present in the sorted plasmids were identified using the ISFinder database (http://issaga.biotoul.fr/issaga_index.php). ISsaga (IS Semi-automatic Genomic Annotation) provides general prediction and annotation around the genome of interest which works under the platform of ISFinder [6]. ISsaga identified the ISs associated with Open Reading frames (ORF).

ORF Analysis:

Transposases are the enzymes which catalyze IS movement. The genes present in ORF region of ISs which helps in their mobility were found using Conserved Domain Database (CDD) – NCBI.

Phylogenetic Tree Construction:

Phylogenic analysis was also performed using MAFFT [7] by considering only blaNDM gene and its surrounding IS sequences (Approximately 4000bp) among 101 plasmids.

RESULT AND DISCUSSION:

The genome analysis (Insilico) was conducted by analyzing 380 complete plasmid sequences (3kb to 350kb) retrieved from NCBI database for the query "NDM and Complete Genome". Based on the annotation, 101 plasmid sequences were identified to be blaNDM-positive out of 380. *Klebisella spp*. (42%), *E. coli* (36%) and *Acinetobacter spp*. (11%), and other bacteria are the most common carriers. Out of 101, 71 plasmids were from clinical samples, 4 from environmental samples and others are undetermined.

Occurrence of NDM resistance gene(s)

Plasmid sequences were taken from 6 continents over a period of 6 years (2011-2017). Asia is the major reservoir (47.52%) of NDM producers. China contributes to 26.7% followed by Myanmur (9%) in accordance to the available plasmid database from Asia. North American continent ranks second largest, with 32.65% NDM abundance. Among the major countries, USA serves as the major reservoir (30.67%) followed by China (26.7%).

Among the 101 plasmid sequences examined, seven variations (NDM 1, 4, 5, 6, 7, 9, and 16) were discovered (Figure 1). NDM-1 is the most common variation (found in 68 plasmids), followed by NDM-5 (18 plasmids), and NDM-6 and NDM-16, each found in a single plasmid. According to the data used in the research, the United States and China are the primary sources of NDM-1. NDM-5 was established in the United States, China, Japan, and Australia. The two variations come from the replacement of Valine by Leucine at position 88 and Methionine by Leucine at position 154, which was first identified in E.coli in the United Kingdom in 2011. NDM-5 has higher hydrolytic activity against carbapenems, cefotaxime, cephalotin, and ceftazidime than NDM-1 [8].

NDM 7 has been reported in the United States, Canada, France, Japan, and Switzerland, and also NDM-4 (Japan) and NDM 9 was found in just three plasmids (South Korea and China) according to this study data. In E. coli from Canada, variant NDM 7 with alterations at positions 130 (Aspartate to Asparagine) and 154 (Methionine to Leucine) was discovered for the first time (2013). A single amino acid (E152K) distinguishes NDM 9 from NDM-1 in K. pneumoniae from China whereas the NDM-4 variation has an amino acid change from Methionine to Leucine at position 154 [9].

In the plasmid used for the study, NDM 6 (USA) and NDM 16 (Colombia) are unusual occurrences.NDM 6 was isolated from E. coli (USA, 2011) with an Alanine to Valine substitution at position 233. NDM 16, which was newly discovered in the United States from K. pneumonia, contains a 264th position substitution of Arginine to Histidine [10].

Clinical relevance of NDM and its Co resistance

Co-resistance is a phenomenon most commonly found in *Enterobactericeae* that includes the transfer of several genetic elements into the same bacterial isolate and/or the acquisition of mutations in distinct genetic loci, resulting in resistance to multiple antibacterial agents. Seventeen of the plasmids analyzed carry genes encoding aminoglycoside resistance (69%) followed by sulfonamide resistance (58%). The resistance to beta lactamase genes was identified in 39.6% of plasmids. This is consistent with the findings of Jain et al., 2014 [11]. NDM variations have also been shown to coexist with methyltransferase (32%), dihydrofolatereductase (32%), macrolide phosphatransferase (25%), and other aminoglycoside resistance genes such as (aadA2, adc (6') lb-cr, aph(3')-VIa (Figure 2). The

current investigation indicates that *Klebsiella spp* is resistant to the majority of third-generation cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems, followed by E.coli, implying that the development of XDR is in concern. The clinical isolates of *Acinetobacter spp*., have been found to exhibit high levels of qac gene carriage along with VIM and NDM, [12].

NDM variants coexisted in plasmid genomes comprising Extended Spectrum Beta Lactamase (ESBL) genes such as blaTEM, blaOXA, blaCTX, blaCMY, blaSHV, and blaDHA in 18, 16, 12, 10, 4, and 3 plasmids, respectively. According to Biedenbach et al. (2014), 106 NDM-positive *Enterobacteriaceae* isolates carried genes encoding ESBLs, 99 (93.4 percent) carried blaCTX-M, and 93 (93.9%) carried CTX-M-15 [13]. Additional than NDM, plasmids producing ESBLs (analyzed in this work) did not encode any other MBL genes. According to Mahalingam et al. (2018), NDM variants such as NDM1, NDM4, NDM5, NDM6, and NDM7 were found in E.coli isolates as well as TEM and OXA from a tertiary care hospital in South India [14].But the data analyzed in our study showed plasmids of size below 50kb possessed only blaNDM gene in their genome (16/20 plasmids of size below 50 kb).

The bleMBL gene, which confers resistance to the anti-tumor glycopeptide chemical bleomycin, was found to be present downstream of the blaNDM-1 gene in 80% of other plasmids. Only a few of the plasmids included genes that conferred resistance to sulfonamide and methylases. Tetracycline resistance was discovered in 9 of the plasmids studied. This study also reveals sensitivity to antibiotics like colistin, glycopeptides, fusidic acid, nitroimidazole and oxazolidinone as per the data analysed. A study by Porretta et al, 2020 described that Covid – 19 patients have colonization of NDM resistance in Italy. NDM acquisitionin Covid infected patients increased the hospital day duration (32.9 vs. 15.8 days) [15].

Correlation of GC contentwith Antibiotic Resistance

The Figure 3 illustrates the correlation between GC content amongst various Antibiotic resistance genes reported. Kubasovaet al, 2016 has accounted for elevated GC richness in antibiotic resistance determinants [16]. The analysis states a GC range of 30% to 68% for the varying antibiotic resistance genes occurring in the plasmids. When compared to smaller plasmids (KP178355 – 3205bp), larger plasmids had reduced GC content (CP021699 - 354705 bp). According to Klein et al. (2018), pathogenicity islands with reduced GC content came from a foreign source or were recently introduced into this genome [17]. The present study iterates the above finding to the occurrence/distribution of IS sequences (in the data set taken for analysis) is observed to be an influencing factor for HGT of antibiotic resistance factors.

Insertion sequences and HGT

The remarkable ability of bacteria to exchange their genetic resources via Horizontal Gene Transfer is the most significant element in the evolution of antibiotic resistance (HGT). A number of genetic components, including integrons, transposons, integrative conjugative elements (ICEs), plasmids, and genomic islands, aid in the transfer of resistance genes [18].The blaNDM gene appears to propagate efficiently across Enterobacteriaceae, owing to the interaction of mobile elements such as Insertion Sequences and Transposases. IS are small genomic elements that typically include one or two ORFs coding for a transposase.

The ISs most commonly associated with the blaNDM genes are ISAba1, ISAba2, ISAba3, ISAba4, ISAba125, ISAba14, IS26, ISEc33, ISPa7 and ISSen4 [19 – 23]. IS elements are susceptible to HGT due of their connection with antibiotic resistance genes. This analysis discovered 16 distinct Insertion sequences (from 9 different families) surrounding the blaNDM gene (Figure 4). ISs of various length (750-4000 kb) and truncated/partial were common among the plasmids studied.The most abundant being ISAba125 (34%) associated with NDM resistance. Poirel*et al*, 2012 showed that the blaNDM-1gene was bracketed by two novel insertion sequences, namely, ISEc33 and ISSen4, belonging to the IS*630* and IS*3* families, respectively. He stated that these ISs were commonly found either in combination or as truncated sequence of ISAba125[24]. The most striking aspect of this study is the presence of ISAba125 as a truncated or incomplete IS in 28 plasmids, either upstream or downstream of IS5 (IS5D) and IS630 (ISSup2 and ISEc33) family sequences. A study by Ying et al, 2012 concluded that the ISAba125 played a key role in the mobilisation of blaNDM, and subsequent transfer events between *Acinetobacter spp*. and *Enterobacteriaceae* were mediated by additional insertion elements such as ISEc33, ISSen4 and IS26 [25]. According to the study, the most frequent ISs after ISAba125 are IS5D (16%) and ISVsa3.

i)IS association with antibiotic resistance genes

Mobile genetic elements are well recognized for the role they have played in the dissemination of antimicrobial resistance genes in Gram-negative bacteria and in the rise of multi-drug resistance in several human pathogens. Different ISs predicted to have some pattern in association with other antibiotic resistant genes. In the current research, ISVsa3 from the IS91 family was revealed in 12 plasmids with just the 16s rRNA methyl transferase and sulfonamide genes in their genome. Twelve plasmids with IS5D were confirmed to carry just the blaNDM gene; no additional antibiotic resistance genes were detected in their genome. ISAba125 is present on several plasmids with various antibiotic resistance genes, but no comparable patters have been discovered. In most of the *Enterobacteriaceae*, blaNDM-1 has been detected between a truncated IS*Aba125* located upstream and bleMBL at the downstream. Bleomycin used for cancer therapy underlines the development and dissemination of bleMBL genes amongst other pathogenic bacteria.

ii)IS sequences towards dissemination of resistance genes

ISs have one or two ORF gene products that are required for their motility. Transposases are classified as DDE, DEDD, HUH (Y1), Tyrosine (Y), and Serine (S) based on the amino acids found in their catalytic site [26]. The majority of ISs examined in the study are members of the DDE transposases family.DDE transposons are short (0.7–2.5 kb long) genetically compact DNA segments with one or two open reading frames (called after a conserved amino acid trio, Asp, Asp, Glu, the active site). DDE transposon has either tnpA or tnpB gene in their ORF region in analyzed IS sequences which are responsible for DNA integration, recombination and transposition. Some ISs like IS5D, ISKPn14 has Ins family of proteins in their ORF region which is also responsible for DNA binding and transposase activity.

The most common IS of the study, ISAba125is a 1,087-bp element belonging to the IS30 family, containing a single open reading frame corresponding to the 322-amino-acid-long transposons and the genes were located inside the composite transposon, named Tn125. The

target site duplication enclosing Tn*125* corresponding to CTG sequence represents a hot spot of transposition for ISAba*125* and consequently for transposition of Tn*125*.In A.baumannii, ISAba125 translocation has been linked to CarO protein changes, which modify multidrug resistance in the genome [27].

ISCR elements from the IS91 family have been identified as potent antibiotic resistance gene captures capable of forming large clusters of antibiotic resistance genes on the genome [26].ISVsa3-like elements have been identified as important players in the evolution of antimicrobial resistance in IncA/C plasmids. The degree of diversity in copy number of these ISs varies based on the frequency of occurrences within and across plasmid genomes for each element (IS2, IS3, IS5, and IS30). Variations in copy number, on the other hand, were linked to high transposition levels [27].

Phylogenetic classification of NDM Plasmids influenced by IS elements

Phylogenetic analysis was done to the blaNDM gene along with the flanking IS regions. The plasmids were grouped into 5 clusters (Figure 5). Cluster 1 and 5 are major clusters and Clusters 2, 3 and 4 were minor clusters. Cluster 1 and Cluster 5 has 5% genetic variation. Cluster 5 has only blaNDM1 gene comprising 26 plasmids from different countries. Cluster 1 comprised of different IS and different NDM variants from varied geographical location but predominated with blaNDM1 gene.

Cluster 2 comprises just the blaNDM5 gene and two ISs (ISApl1 and ISAba125), both of which are members of the IS30 family. Cluster 3 consists of ten plasmids with IS5D insertion sequences including three NDM variants (NDM4, 5, and 7), each of which is caused by Met154 to Leu amino acid changes.Cluster 4 is split into two subgroups: the first has just the blaNDM5 gene along IS5D, while the second has NDM7 bordered by additional IS elements. Stoppe et al, 2018 stated that meta-analysis of 39 different studies from 24 countries showed no sub structuring patterns of phylogroups denotes there is no correlation between phylogeny and geographical location [28]. But the results envisage that, types and number of IS elements occurring within the plasmids have an influence on the NDM resistance profile over wide range of geographical locations.

CONCLUSION

Horizontal gene transfer is a predisposing factor towards rapid spread of potential drug resistance factors. Association of Insertion sequence with resistance determinants in pathogenic bacteria proposes a concern with respect to its potential for dissemination within groups and also other closely related pathogens. The present insilico analysis based on sequence information retrieved from NCBI discloses the propensity of these clinically relevant bacteria to create grave outbreak incidences due to the abundance of insertion sequences in close proximity in a highly fluidic plasmid genome. The study reveals an important consideration that NDM resistant *Enterobacteriaceae* is crucial for the hospital infection management and therapeutic options across the globe. The results though based on sequences submitted to NCBI, throws light on the abundance of NDM resistance in different continents, present amidst adequate genetic elements to keep them mobile and precarious. Finally, this study gives a predisposing significance on plasmid-mediated antimicrobial resistance spread both locally and globally influenced of mobile elements.

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STATEMENT OF ETHICS
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DISCLOSURE STATEMENT
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metallo-β-lactamase genes, and of common insertion sequences, in epidemic clones of *Acinetobacter baumannii* from Spain. *Journal of Antimicrobial Chemotherapy*, March 2013; 68(3): 550–553.

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Figure 1: Frequency of NDM Variants in 101 Plasmids

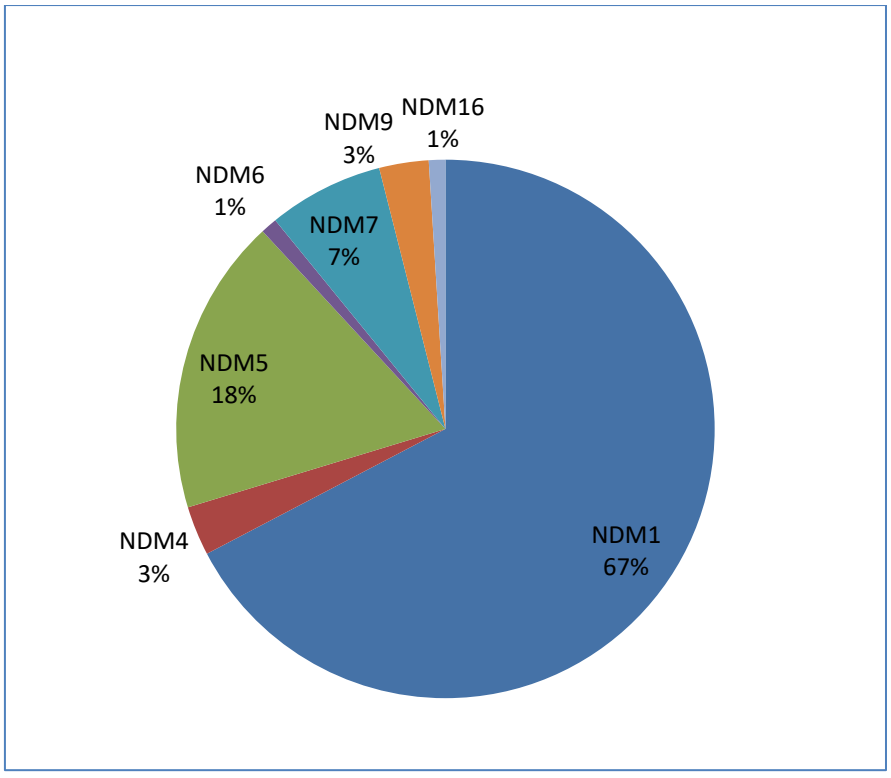

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Figure 2: Coexistence of Antibiotic resistance genes

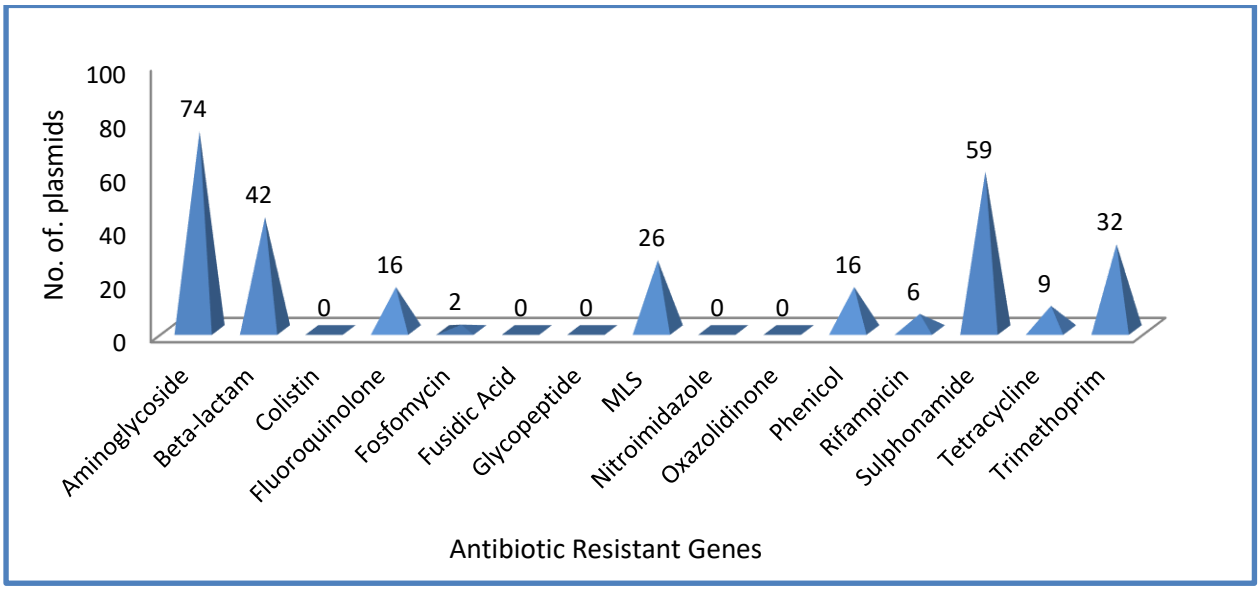

Figure 3: Percentage of GC content in Resistant Genes

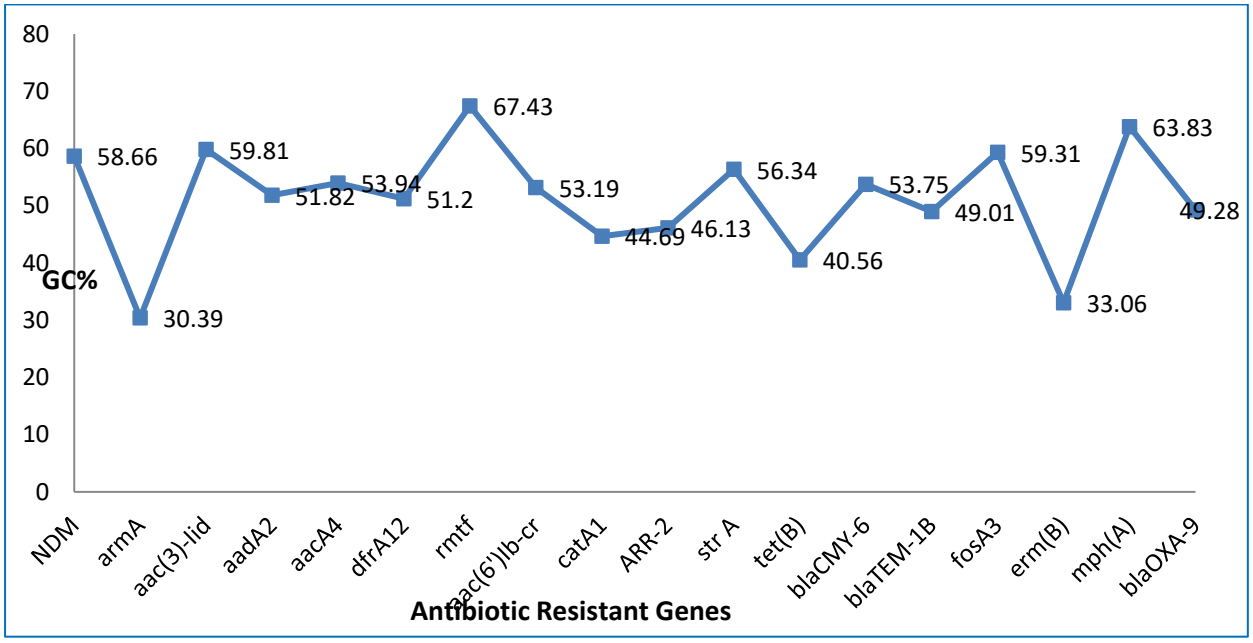

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Figure 4: IS occurrence in Plasmids analyzed

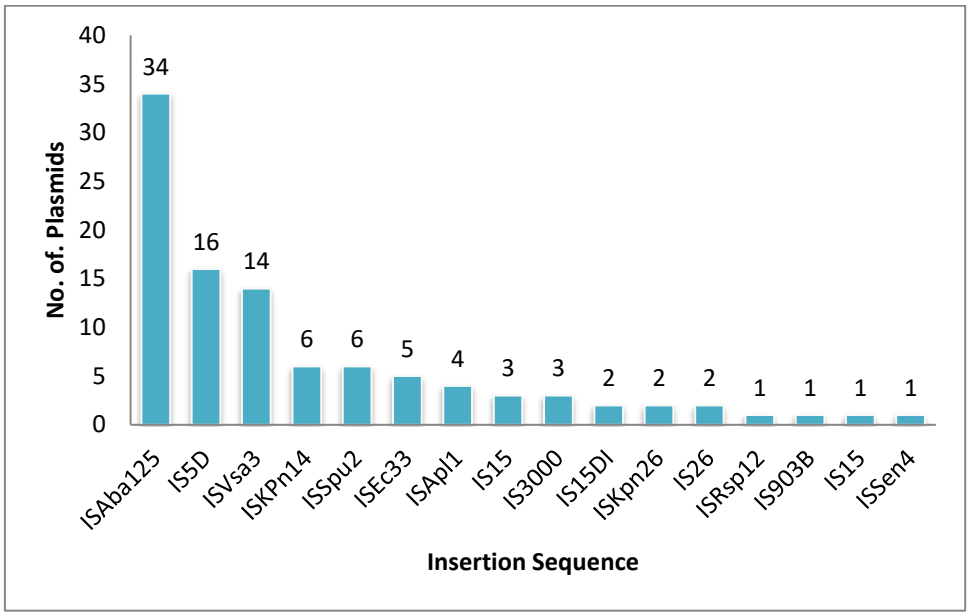
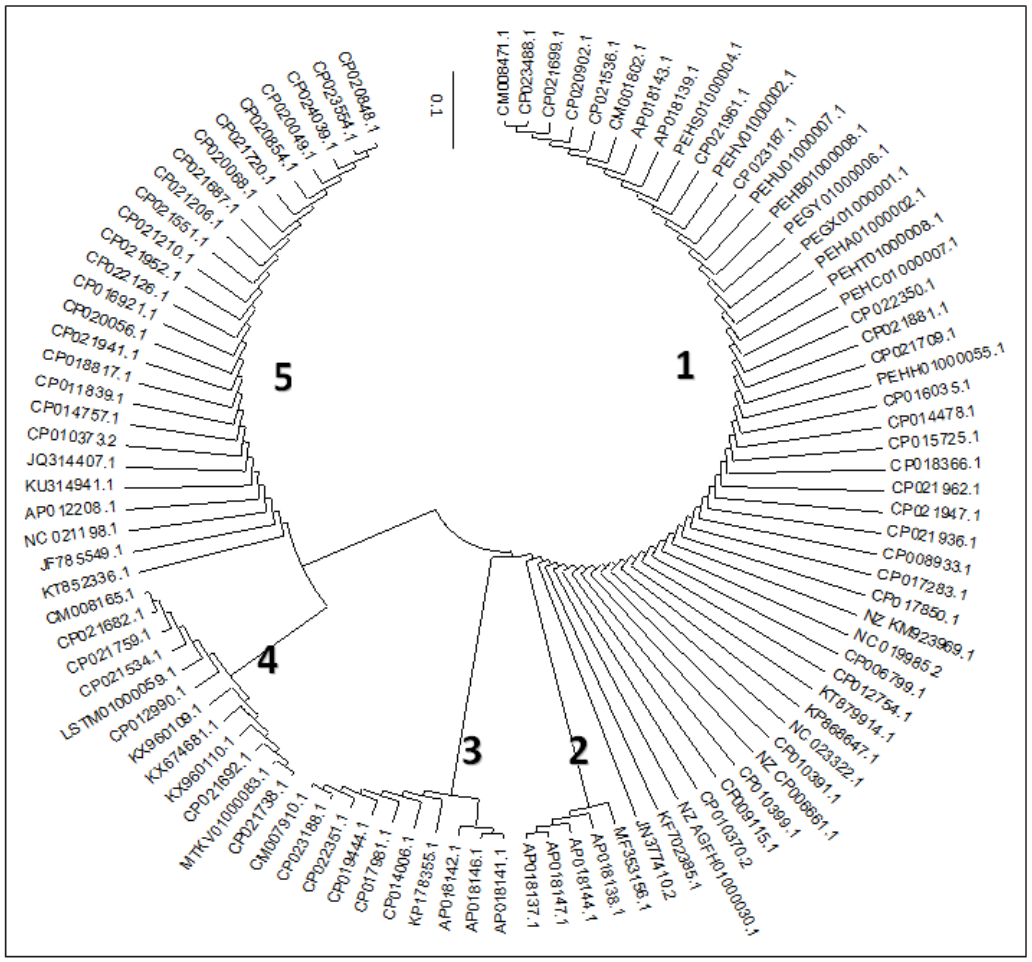


Figure 5: Phylogenetic construction for NDM gene



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