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MAJOR ARTICLE

Whole Genome Sequencing of Peruvian *Klebsiella pneumoniae* Identifies Novel Plasmid Vectors Bearing Carbapenem Resistance Gene *NDM-1*

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Background. *Klebsiella pneumoniae* is a bacterial pathogen with increasing rates of resistance to carbapenem antibiotics, but the population structure and genetic drivers of carbapenem-resistant *K pneumoniae* (CRKP) remain underexplored in developing countries. Carbapenem-resistant *K pneumoniae* were recently introduced into Peru but have grown rapidly in prevalence, enabling study of this pathogen as it expands into an unaffected environment.

Methods. In this study, using whole genome sequencing, we show that 3 distinct lineages encompass almost all CRKP identified in the hospital where it was first reported in Peru.

Results. The most prevalent lineage, ST348, has not been described outside of Europe, raising concern for global dissemination. We identified metallo- β -lactamase *NDM-1* as the primary carbapenem resistance effector, which was harbored on a novel vector resulting from recombination between 2 different plasmids, pKP1-NDM-1 and pMS7884A.

Conclusions. This study is the first of its kind performed in Peru, and it furthers our understanding of the landscape of CRKP infections in Latin America.

Keywords. carbapenems; *Klebsiella pneumoniae*; Latin America; MLST; whole genome sequencing.

Klebsiella pneumoniae is considered one of the most important opportunistic pathogens in both community and nosocomial infections [1, 2]. Treatment of this organism has become complicated by an increasing rate of resistance to most antibiotics that are commonly used in clinical practice, and it is exacerbated by its ability to persist within hospital environments and propagate nosocomial transmission of highly resistant strains [3, 4]. Although carbapenem antibiotics were long considered a therapy of last resort against *K pneumoniae*, an isolate producing a metallo- β -lactamase capable of hydrolyzing those drugs was reported in Japan in 1994s [5]. Since that discovery, several other classes of carbapenemases have been found both in *K pneumoniae* and in other species of *Enterobacteriaceae* [6]. Of particular note is the broad range New Delhi metallo-β-lactamase (NDM), which was first identified in a *K pneumoniae* isolate from a patient hospitalized in India [7] and subsequently determined to represent a novel fusion of an aminoglycoside phosphotransferase and metallo- β -lactamase, creating a chimeric enzyme capable of being distributed across a wide range of species [8]. Sixteen variants of NDM alone have now been identified in a variety of *Enterobacteriaceae* species from across the world, with dissemination of the gene frequently mediated through horizontal plasmid transfer [9].

The global expansion and dissemination of NDM and other *K pneumoniae* carbapenemases has been identified in clinical, urban, and agricultural environments, but their epidemiology and virulence varies by geographical location, as does whether the genetic determinants involved are endemic or largely imported [10–12]. As such, understanding the molecular mechanisms and molecular epidemiology of carbapenem-resistant *K pneumoniae* (CRKP) in a specific environment necessitates focused investigation of strains collected from the region of interest [10]. However, CRKP are generally less well described in resource-poor nations than in industrialized countries and those having well developed antimicrobial resistance (AMR) surveillance networks [10, 11]. There consequently remain significant uncertainties about descriptive features of CRKP from developing countries, and especially such nations where carbapenemases are newly emerging.

In Latin America, CRKP were first identified in Brazil in 2003 [13] and have rapidly increased in prevalence throughout the region. This trend is especially notable in Peru, which first

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confirmed CRKP as late as 2013 [14] with the national resistance rate reaching ~8% of all *K pneumoniae* isolates by 2016 [15]. In 2017, the *NDM-1* gene was reported from multiple Peruvian *K pneumoniae* isolates in a public hospital in Lima [16], although subsequent retrospective surveys of banked carbapenem-resistant organisms date *NDM-1*'s introduction to Peru in 2013, coincident with the entry of CRKP. Yet, despite their rapidly rising prevalence in the country, thorough molecular descriptions of the strains carrying carbapenem resistance and their relationships to other *K pneumoniae* in Latin America and globally have not yet been provided. Indeed, recent reviews detailing the continent-wide prevalence of carbapenemase genes identified only 1 study where whole genome sequencing was used to interrogate the resistant isolates [15, 17].

To address this knowledge gap, in this study we performed whole genome sequencing of a retrospective collection of *K pneumoniae* isolates originating from the Peruvian hospital where *NDM-1* was first reported. We used these data to define the phylogenomic epidemiology and molecular mechanisms of carbapenem resistance in that population. In addition to addressing a currently unexplored area of regional pathogen research, the relatively recent introduction of CRKP and *NDM-1* to Peru provides opportunities to examine the expansion of the pathogen into a previously naive environment.

MATERIALS AND METHODS

Samples and Phenotypic Testing

Isolate collection was conducted over 3 consecutive months from October to December 2016 in Lima, Peru at Hospital Nacional Dos de Mayo, a 600-bed public teaching hospital located in the downtown area. All *K pneumoniae* isolates that were collected during routine clinical care from both inpatients and the adjoining outpatient clinic were included. Antibiotic resistance testing was performed in the hospital microbiology laboratory as part of routine clinical care, with the majority of samples tested using BD Phoenix machine (27 samples) and the remainder with disk diffusion assays during a period when the instrument was not available. Clinical data on antibiotic resistance patterns and epidemiological information were retrospectively collected through a review of the medical records. Resistance phenotype data were not available for 10 isolates after record review. Isolates originated from blood, urine, pulmonary aspirates, sputum, and wounds and were each collected from different patients, for an initial collection of 70 unique isolates.

Sequencing and Assembly

After clinical testing was complete, deoxyribonucleic acid (DNA) was extracted from isolates using the Geneaid Presto MinigDNA Bacteria Kit and stored at −80 until sequencing was performed. Whole genome sequencing libraries were prepared as described elsewhere [18]. Sequencing was performed on the Illumina NextSeq platform using 300 cycle chemistries to a minimum average read depth of at least 20× per isolate. After sequencing, de novo draft genomes were assembled using AbySS v2.0.2 [19]. To ensure that the conventional microbiological species identification was concordant with the genomic classification, pairwise ANIb analysis was performed against a representative collection of publicly available *Klebsiella* genomes using the Pyani package (https://github.com/widdowquinn/pyani). Of the 70 total genomes analyzed, 10 matched *Klebsiella* species that were not *K pneumoniae* and 2 others evidenced polymicrobial contamination, resulting in a final set of 58 *K pneumoniae* genomes included in further analysis.

Molecular Epidemiology and Resistance Gene Identification

Multilocus sequence typing (MLST) groups were assigned using PubMLST toolkit [20]. Whole genome molecular epidemiology analysis was performed as described elsewhere [21], using a combination of all-by-all pairwise distance matrices and approximately maximum-likelihood phylogenetic trees constructed using FastTree 2.1 [22]. Variant calling was performed relative to *K pneumoniae* strain 4/1–2 (GenBank accession no. CP023839.1). Resistance genes in each isolate were identified using AMRGeneFinderPlus [23].

Plasmid Reconstruction

Contigs from de novo assemblies that bore *NDM-1* were manually closed by identification of split read pairs and single sequence reads that spanned contig endpoints, indicating their context as circular plasmids. *Basic Local Alignment Search Tool (*BLAST) [24] analysis of reconstructed plasmid sequences against the nonredundant National Center for Biotechnology Information (NCBI) sequence database was used to classify discrete genetic elements comprising the larger plasmids. Putative breakpoints between donor plasmids were initially confirmed by manual inspection of sequence reads that spanned the junctions between donor fragments. Junctions were subsequently confirmed in representative plasmid isoform 1 using polymerase chain reaction (PCR), which spanned the inferred breakpoints. The PCR primer pairs for this purpose were designed on either side of the junctions between pMS7884A and pKP1-NDM1 at position 0 (forward primer 5'-GTCTGCGCCAATATGTTCAA-3', reverse primer 5'-GACGATCAAACCGTTGGAAG-3') and position 3781 (forward primer 5'-TTTTCCACGTCAATCAACCA-3', reverse primer 5'-GGCAATTCTATGCGTTGCTA-3').

To confirm that *NDM-1*-containing elements were truly circular plasmids as well as to rule out cryptic gene duplication events, we designed outward-facing primers within the *NDM-1* gene (forward primer 5'-ACGGTTTGGCGATCTGGTTTT-3', reverse primer 5'-TGGTCGCCAGTTTCCATTTG-3'), which would enable amplification of a plasmid but would preclude amplification from material integrated into a genomic context.

Amplification of extracted DNA from strains carrying isoform 1 yielded the expected ~7-kb product, consistent with a circular context and a plasmid of the expected size without measurable gene duplication events. Open reading frames within reconstructed plasmid sequences were identified using SnapGene, and their functional roles were evaluated by BLAST searches.

Data Availability

Sequence data generated for this study are available from the NCBI Sequence Read Archive ([SRA] http://www.ncbi.nlm.nih.gov/sra) under study accession number PRJNA592157. Assembled plasmid sequences are available from NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank) under accession numbers MN816229–MN816233.

RESULTS

Molecular Epidemiology of Carbapenem-Resistant *Klebsiella pneumoniae* in Peru

In 2015, the proportion of *K pneumoniae* that were CRKP in the study hospital was 11% (internal tracking data), whereas sample collection for this study revealed that 19 of 47 *K pneumoniae* isolates (42.5%) with available carbapenem susceptibility data had a resistant phenotype, representing an approximately 4-fold increase in frequency within 1 year. Fifty-five isolates were distributed among 28 distinct MLSTs (Figure 1). Two other isolates displayed unique and previously unreported MLST, and the remaining isolate did not have sufficient data for a MLST to be confidently assigned. Five MLST groups contained 3 or more isolates each, but, notably, 3 groups encompassed 90% of all phenotypically confirmed carbapenem-resistant isolates: ST348 (10 isolates), ST147 (5 isolates), and ST11 (4 isolates).

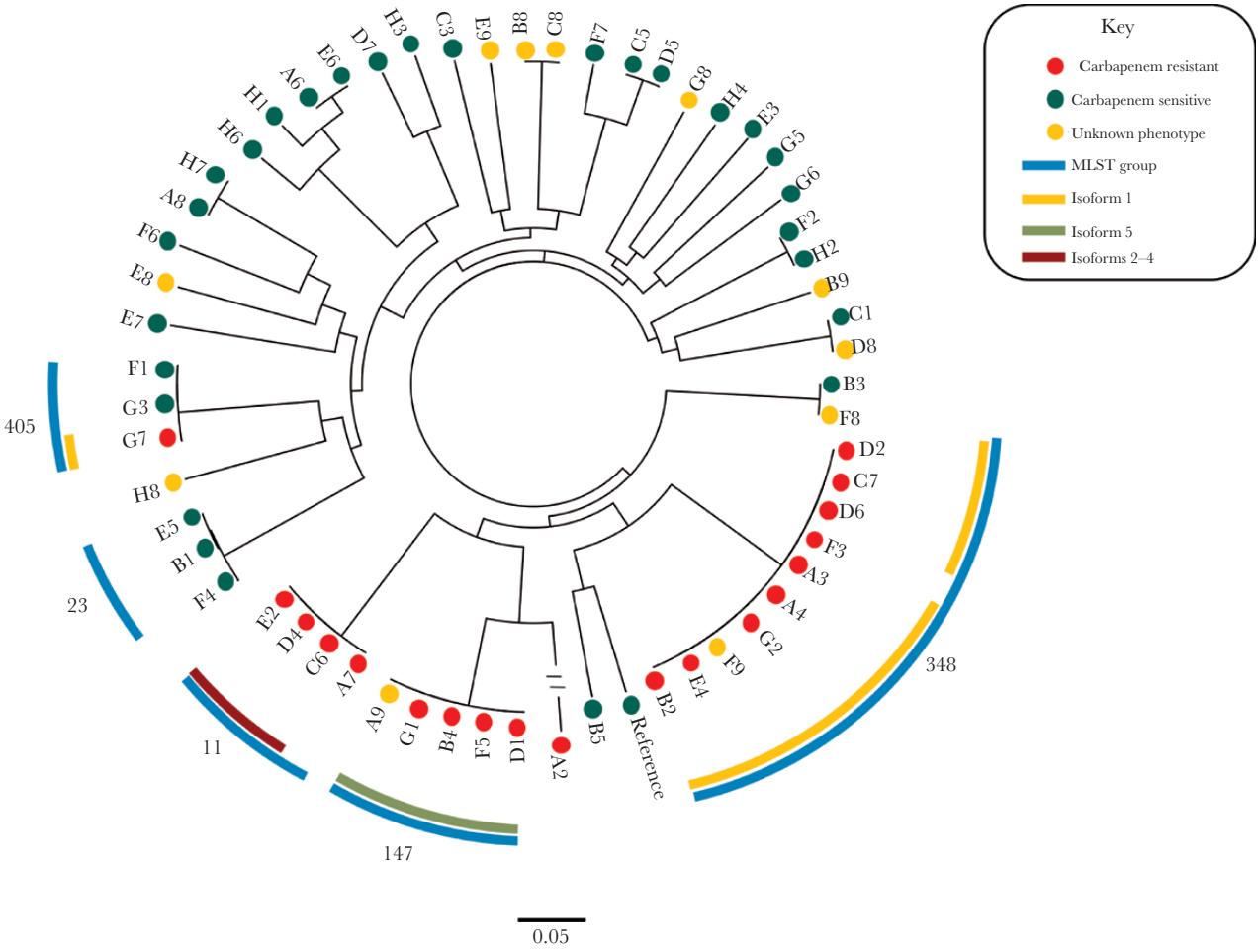

Figure 1. Phylogenomic tree of *Klebsiella pneumoniae* isolates. Approximate maximum likelihood phylogeny reconstruction of the population structure of *K pneumoniae* from whole genome data. Variants were called relative to *K pneumoniae* strain 4/1–2 (GenBank accession no. CP023839.1). Scale bar indicates the number of changes per site. Multilocus sequence typing (MLST) groups with 3 or more representative isolates are indicated by the outermost blue bar with the associated MLST classification indicated. The innermost colored bar represents the plasmid isoforms present in the underlying isolate. Terminal node colors indicate the carbapenem resistance phenotypes of individual isolates. The broken line leading to isolate A2 has been truncated to fit the figure dimensions, with a true distance of 0.81.

To assess the possibility of patient-to-patient transmission, we next quantitated the number of single-nucleotide polymorphisms (SNPs) that distinguished each possible pairwise combination of isolates and searched for highly similar clones that were obtained from different patients. The average number of SNPs differentiating any 2 isolates from the study population was 29044.2 (standard deviation [SD] = 6423.4), indicating substantial genetic diversity across the population [21]. Within each carbapenem-resistant MLST group, the average number of SNPs distinguishing isolate pairs was considerably smaller, with ST348 having an average of 182.5 (SD = 59.7) pairwise differences, ST147 with 530.7 variants (SD = 240.3), and ST11 with 536.3 (175.6). This degree of genetic relatedness provides evidence for a limited number of genetically related, endemic CRKP strains. However, the isolate pair having the smallest number of pairwise distances was separated by 110 variants, indicating that no groups in this analysis can be confidently shown to result from direct transmission [10].

Molecular Mechanisms of Antimicrobial Resistance

Although not all samples were tested against all drug classes, clinical antimicrobial susceptibility testing data were available for 48 isolates (Figure 2). The population showed high rates of resistance across 5 of the 6 drug classes tested, with the highest prevalence of resistance to cotrimoxazole (86.7%, 39 of 45 isolates) and the lowest to amikacin (4.1%, 2 of 48 isolates). The multidrug resistance phenotypes of our sample collection were also supported by AMR genotype analysis, which identified an average of 15 (SD = 7.5) different AMR genes per isolate (Figure 3). Thirty-one AMR genes were recurrently identified in at least 10% of the total population, with the most frequently occurring AMR being *fosA* (100%) and *oqxA* (87.9%), which are considered intrinsic *to K pneumoniae* [25, 26], followed by *blaOXA-1* (69.0%), *catB3* (67.2%), and *blaCTX-M-15* (67.2%).

The *NDM-1* gene was found in 17 of 20 (85%) isolates having a carbapenem resistance phenotype. In 2 other carbapenem-resistant isolates, F3 and A7, *blaKPC-2* was identified, and 1 isolate, A3, carried both *NDM-1* and *blaKPC-2*. In all 3 of the isolates carrying *blaKPC-2*, the gene was located on plasmid pKPC_CAV1042-89 (GenBank accession no. NZ_CP018669), which is a well established vector of *blaKPC-2* in *K pneumoniae* [27]. Although various other extended-spectrum β-lactamase (ESBL) genes were concurrently found in the carbapenem-resistant isolates, *NDM-1* and *blaKPC-2* are the only factors having a well defined capacity to confer carbapenem resistance [6]. A single isolate showed discordance between genotype and reported phenotype: isolate A2 was carbapenem resistant but did not carry identifiable sequence from *NDM-1* or *blaKPC-2*. However, this isolate was phylogenomically distant from the others included in the study (Figure 1) [28]. Pairwise ANIb analysis comparing the genome of isolate A2 to the other species comprising the *Klebsiella* species complex and to the closet matching genomes available from NCBI demonstrated that by homology A2 qualifies as a novel genomospecies that is distinct from, but most closely related to, *K pneumoniae* [18, 25, 28–31]. It could thereby plausibly possess a distinct carbapenem resistance mechanism from the other isolates, or, alternatively, the discordance could represent a false phenotype call.

Isolates carrying the *NDM-1* gene also had a greater number of additional resistance factors than strains lacking *NDM-1*, possessing an average of 22.2 (SD = 2.3) AMR genes compared with 12.0 (SD = 6.8) resistance genes for isolates lacking that factor ($P = 1.8 \times 10^{-11}$, Student's *t* test) (Figure 3).

Novel Plasmids Carry *NDM-1*

In all cases that *NDM-1* was detected, sequencing indicated that it was incorporated into a novel plasmid derived from 2 distinct, previously described resistance vectors. The first donor plasmid, pMS7884A (GenBank accession no. NZ_CP022533), is a widely dispersed, 330-kb IncHI2 class vector supported by diverse *Enterobacteriaceae* species and known to harbor multiple AMR genes [32]. The second is the 139-kb IncC class plasmid designated pKP1-NDM-1 (GenBank accession no. KF992018), which bears the *NDM-1* gene and has previously been identified in carbapenem-resistant *Enterobacteriaceae* from Latin America [33, 34]. Across the strain collection there were 5 distinguishable variants of this fusion resistance vector (reported here as isoforms 1–5, in order of increasing size). These isoforms associated closely with particular *K pneumoniae* phylogroups, suggesting inheritance by descent (Figure 1).

In all isoforms, the pMS7884A donor plasmid imparted a ~3.2-kb segment containing a Tn3 family transposase gene, whereas the pKP1-NDM-1 donor plasmid provided ~3.8 kb of DNA including the *NDM-1* resistance element (Figure 4). Isoform 1 was composed only of these 2 fragments, whereas remaining isoforms were characterized by the insertion of a second, ISKpn26 family transposase derived from plasmid pKPN-edaa [35] (GenBank accession no. 026398) into the IncHI2 backbone. Isoform 5 incorporated a full-length coding sequence of that transposase, whereas isoforms 2–4 had lost varying portions of the element at slightly differing breakpoints, likely reflecting independent transposase excision events and resulting in plasmids of varying size.

Isoform 1 was found in 9 ST348 isolates and in 1 isolate from the distantly related ST405 phylogroup, consistent with a horizontal transmission event (Figure 1). Isoforms 2–4 were present in a single isolate each, but all belonged to phylogroup ST11. Isoform 5 was carried by 5 isolates from phylogroup ST147, 3 of which could be fully closed using available sequence data.

DISCUSSION

The *NDM-1* gene was first identified in Latin American *Enterobacteriaceae* from Guatemala in 2011 [36], and it has

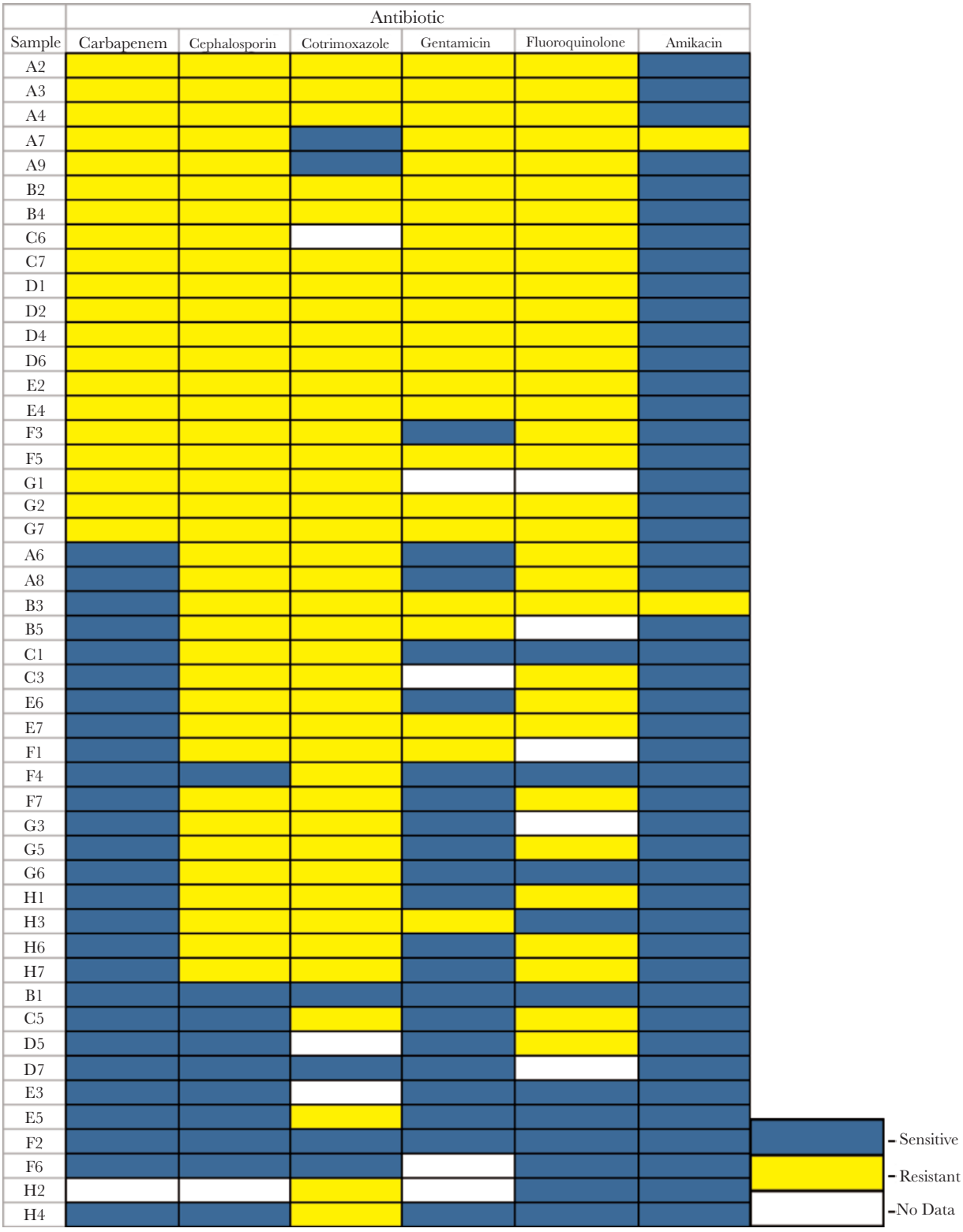

Figure 2. *Klebsiella pneumoniae* phenotypic resistance by drug class. Each row corresponds to an isolate and columns indicate antibiotic class or individual antibiotic tested.

subsequently been recovered from several other countries [31, 37]. However, its presence in Peru was reported more recently, when in 2016 CRKP isolates from a single hospital, Hospital Nacional Dos de Mayo in Lima, were noted to carry the gene [16]. In this study, we performed retrospective genomic analysis of isolates originating from this Peruvian index hospital to

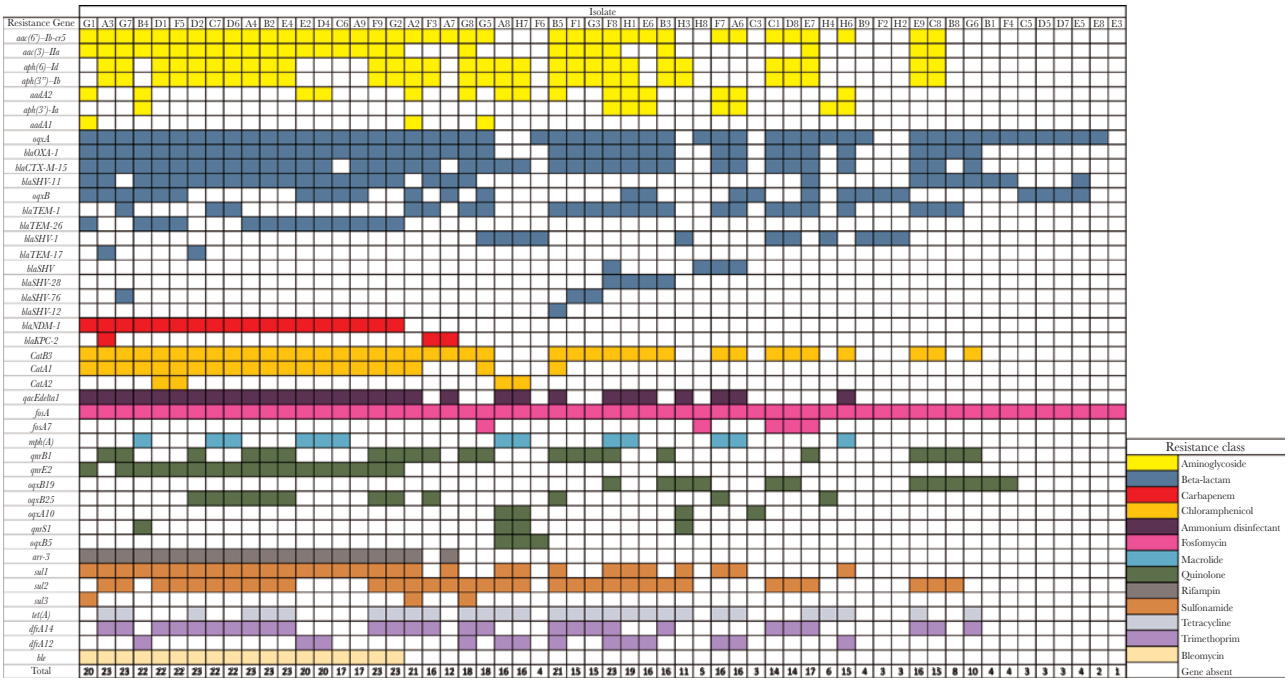

Figure 3. Presence of known antibiotic resistance genes in sequenced isolates. Each row corresponds to an antibiotic resistance gene and the columns indicate individual isolates. The rows are organized and color coded by antibiotic resistance class, and the carbapenem resistance genes *NDM-1* and *KPC-2* are highlighted in red. The total number of unique resistance genes that each isolate has is enumerated in the bottom row.

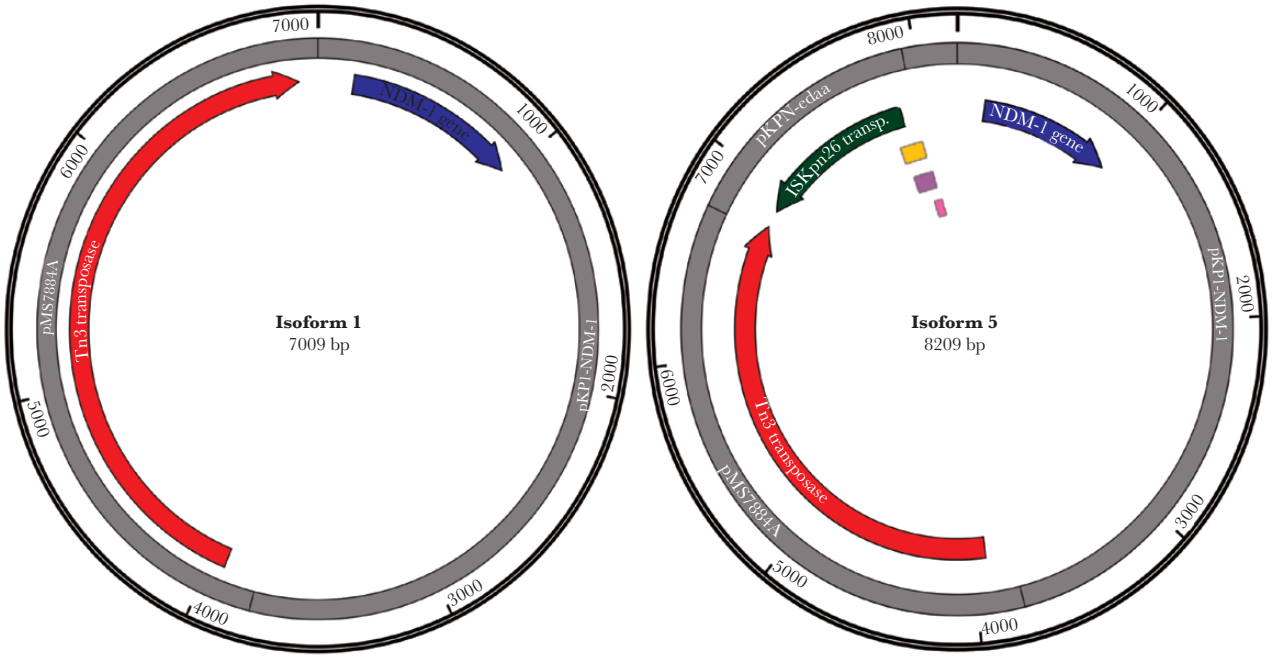

Figure 4. Sequence maps of novel *NDM-1* resistance plasmids. Outermost rings indicate base pair position, interior gray rings depict donor plasmid components, and colored arrows denote the functional plasmid open reading frames identified via BLAST search. Isoform 1 is 7009-base pairs long and composed of 2 parental plasmids, the IncC class plasmid pKP1-NDM-1 bearing the *NDM-1* gene (blue arrow), and the IncHI2 class plasmid pMS7884A contributing Tn3 transposase (red arrow). Isoforms 2–5 match the components of isoform 1 with the addition of a fragment of plasmid pKPN-edaa, which encodes an ISKpn26 transposase, into pMS7884A. Isoform 5 has a full-length ISKpn26 transposase, whereas isoforms 2–4 have partial deletions of that element, and their remaining segments are represented by the innermost colored bars within the Isoform 5 sequence map.

investigate several outstanding questions about the origins and molecular content of those organisms.

The CRKP isolates causing infections within the hospital were predominantly from 3 MLST groups. Despite the limited number of lineages recovered, we found no evidence for direct, patient-to-patient transmission based on measured pairwise genomic differences [10]. Our results are more consistent with the existence of a limited number of genetically related endemic strains, which constitute a reservoir from which patients become infected, either within the hospital or the larger community. Two of these lineages, ST147 and ST11, are well recognized and globally pervasive CRKP strains [38–41]. It was surprising that the third and most prevalent MLST, ST348 (17% of isolates), has previously been reported only in Portugal and Italy as a cause of carbapenem-resistant infections in both humans and in horses [12, 42, 43], but it has otherwise not been described outside these 2 countries. The recovery of a large proportion of MLST ST348 isolates from Peru is concerning as a potential indicator for the global dissemination of ST348 as a strain of worldwide epidemiologic importance given its carbapenem-resistant phenotype. This finding highlights the need for better surveillance within Latin America and presents an opportunity to contain the spread of this lineage by targeted intervention.

The large majority of carbapenem-resistant isolates in the study population carried *NDM-1*, and sequencing identified a novel group of related plasmids with several distinct isoforms that served as the vehicle for that resistance gene. Depending on the isoform, these plasmids have arisen from a recombination between 2 or 3 distinct *Enterobacteriaceae* donor vectors, likely mediated by encoded transposase activity. A recent study utilizing whole genome sequencing to analyze the dynamics of carbapenem resistance transmission in clinical environments also found plasmid backbone evolution mediated by transposases [35], supporting this hypothesis. More importantly, the *NDM-1* bearing pKP1-NDM-1 plasmid was recently detected within Latin America [34], independently confirming this donor plasmid's presence within the geographical region and providing a plausible background from which a recombinant plasmid could arise. Given the contemporary introduction of the requisite parental plasmid to the region, our study may capture the molecular signature of early, local dissemination of these new vectors.

The novel plasmid family is considerably smaller than any of the donor plasmids, potentially increasing their transmissibility [44]. Although they do not apparently possess the replication machinery necessary for conjugative plasmid transfer, nonconjugative plasmids bearing Tn3 transposases have been shown to form cointegrate complexes in the presence of conjugative plasmids, enabling horizontal transfer at low levels [45]. Indeed, we found that one plasmid isoform is present in 2 distantly related *K pneumoniae* strains from our population, consistent with a horizontal transmission event. Further regional surveys for detecting *NDM-1* and examining its genomic context will be necessary to assess the wider prevalence of these plasmids.

Owing to its retrospective nature, our study is limited in several respects. Live, cryopreserved bacterial specimens were not available at the time of whole genome analysis, making it challenging to verify genomic findings by functional or orthologous molecular methods. Relatedly, antibiotic susceptibility data were limited to that recorded after initial clinical testing by the hospital's clinical microbiology laboratory. Phenotypic data were derived from 2 different methodologies for resistance typing, and susceptibility data were unavailable for a small fraction of isolates that were sequenced. However, despite a lack of directly relatable quantitative information between the 2 susceptibility testing techniques used, there was near-perfect correlation of genotype and phenotype in our study. Finally, although our effort represents the largest whole genome study of CRKP to date in Peru, its scope was still relatively proscribed. The small absolute size of our population, the relatively short 3-month collection period, and the geographic origin of our samples from a single institution limit the generalizability of the study.

CONCLUSIONS

To counter the rising global threat of CRKP, focused investigations on the molecular epidemiology of these bacteria must be undertaken in the developing world, where the impact of disease is often highest [46]. In summary, our data provide new insights that will be of value to efforts to mitigate the spread of CRKP in Latin America. We find that there are a limited number of closely related *K pneumoniae* strains causing the greatest burden of carbapenem resistance, and we point to ST348 as an emerging lineage in that region. We also found that the *NDM-1* carbapenemase gene within these isolates is being disseminated on a novel vector. This study represents an important step toward understanding the landscape of CRKP in Peru, and future, longitudinal genomic surveys of larger numbers of resistant *K pneumoniae* obtained from other hospitals and other Latin American nations will help to illuminate the transmission of resistance genes and dissemination of these organisms across South America.

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