Plasmid diversity among genetically related *Klebsiella pneumoniae* bla_{KPC-2} and bla_{KPC-3} isolates collected in the Dutch national surveillance

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Running title: Plasmids of *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3}.

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Abstract

Carbapenemase-producing *Klebsiella pneumoniae* emerged over the past decades as an important pathogen causing morbidity and mortality in hospitalized patients. For infection prevention and control, it is important to track the spread of bacterial strains in humans including the plasmids they contain. However, little is known concerning the plasmid repertoire among *K. pneumoniae* strains. Therefore, the major aim was to recapitulate the size, contents and diversity of the plasmids of genetically related *K. pneumoniae* strains harboring the beta-lactamase gene bla_{KPC-2} or bla_{KPC-3} to determine their dissemination in the Netherlands and the former Dutch Caribbean islands from 2014-2019. Next-generation sequencing was combined with long-read third-generation sequencing to reconstruct 18 plasmids of *K. pneumoniae*. wgMLST revealed five genetic clusters (termed KpnClusters) comprised of *K. pneumoniae* bla_{KPC-2} isolates and four clusters consisted of bla_{KPC-3} isolates. Each cluster was characterized by a distinct resistome and plasmidome. KpnCluster-019 bla_{KPC-2} isolates were found both in the Netherlands and the Caribbean islands. *K. pneumoniae* bla_{KPC-3} isolates were found in the collection of the Netherlands. The 18 plasmids were mostly unrelated and varied between *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3} clusters. However, the large and medium sized plasmids contained a variety of antibiotic resistance genes, transposons, insertion sequence elements, conjugal transfer systems, cation transport systems, toxin/antitoxin systems, and prophage-related sequence elements. The small plasmids carried genes implicated in virulence. Thus, implementing long-read plasmid sequencing analysis for *K. pneumoniae* surveillance provided important insights in the success and understanding of transmission of a KpnCluster-019 bla_{KPC-2} strain between the Netherlands and the Caribbean.

Importance

Carbapenemase-producing *Klebsiella pneumoniae* has spread globally and is of great concern for debilitated patients. *K. pneumoniae* is notorious for spreading antimicrobial resistance genes by plasmids among *Enterobacterales*. Combining short and long read sequencing enables reconstruction of plasmids containing antibiotic resistance genes, conjugation machinery, transposons, toxins and/or virulence determinants and thereby enhancing international pathogen surveillance.

Introduction

Antimicrobial resistance is spreading rapidly among *Enterobacterales*, including *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter* spp. (1). Within the cell, extra-chromosomal DNA such as plasmids encode genes that confer resistance to last resort antibiotics, including carbapenems and colistin, and can transfer between *Enterobacterales* (2). Currently, carbapenemase-producing *Enterobacterales* (CPE) rank among the most problematic nosocomial pathogens with limited outlook on novel effective therapeutics (3, 4). With the current increase of multidrug-resistant infections with CPE worldwide, total healthcare costs are anticipated to increase. *K. pneumoniae* is often referred to as the "canary in the coalmine", as new antimicrobial resistance (AMR) genes have been associated with *K. pneumoniae* in the first clinical reports prior dispersal of the AMR genes among other Gram-negative bacteria (5). Most newly acquired AMR genes of *K. pneumoniae* are the result of horizontal gene transfer through conjugative plasmids (6–8). The *K. pneumoniae* carbapenemase KPC encoded by the bla_{KPC} gene is an Ambler class A serine carbapenemase, which is often located on a transmissible plasmid-associated transposon Tn*4401*, or variants hereof (9–12). Tn*4401* consists of flanking imperfect repeat sequences, a Tn*3* transposase gene, a Tn*3* resolvase gene and the IS*Kpn6* and IS*Kpn7* insertion sequences (10). The bla_{KPC-2} and bla_{KPC-3} carbapenemases are the most commonly identified variants that have spread

65 globally and provide resistance to penicillins, carbapenems, cephalosporins, cephamycins and
66 monobactams (13, 14). The KPC-2 and KPC-3 carbapenemases differ in only one amino acid as a histidine
67 at position 272 is mutated to tyrosine (H272Y) in the KPC-3 variant (15).

68 CPE isolates including *K. pneumoniae* are routinely send to the National Institute for Public
69 Health and the Environment (RIVM) and are typed by Illumina next-generation sequencing (NGS) in the
70 Dutch National CPE Surveillance program to identify AMR genes and to determine possible transmission
71 of strains (16). NGS typically yields short sequence reads of 150 bases, thereby hampering the assembly
72 of complete chromosomes and plasmids (17). This is often due to large mobile genetic elements, such as
73 insertion sequence elements, transposons, and other repetitive sequences *e.g.* tandem repeat regions
74 of >1500 bp in size. However, combining Illumina NGS sequencing with long-read third generation
75 sequencing (TGS), which produces 1,000 to 500,000 bases or longer sequence reads, can overcome this
76 problem and enables the reconstruction of chromosomes and complete plasmids (18, 19). Currently, the
77 transmission of *K. pneumoniae* between persons in different countries and the impact hereof is not
78 thoroughly understood. It is also not clear whether plasmids of *K. pneumoniae* circulate endemically in
79 the Netherlands or that are introduced from other high-prevalence countries. While the prevalence of
80 carbapenemase producing *K. pneumoniae* and associated infections in the Netherlands is relatively low,
81 the establishment of genomic surveillance of *K. pneumoniae* using TGS is of high importance (20, 21). It
82 provides for insights in the transmission of specific strains containing plasmids with AMR genes and/or
83 virulence determinants. We therefore investigated the distribution of *K. pneumoniae* cluster isolates
84 harboring bla_{KPC-2} or bla_{KPC-3} alleles obtained from the Dutch National CPE Surveillance Program and
85 analyzed the contents of its plasmids using long read third-generation sequencing.

86

Results

Distribution and genetic relationship of *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ carrying *K. pneumoniae*

A collection of 480 carbapenemase-producing *K. pneumoniae* isolates submitted to the Dutch National CPE Surveillance program from January 1st 2014 until June 30th 2019 to the National Institute for Public Health and the Environment (RIVM) were included in this study. The study collection comprised 84 *K. pneumoniae bla*$_{KPC}$-positive isolates of which 51 contained the *bla*$_{KPC-2}$ allele and 33 harbored the *bla*$_{KPC-3}$ allele (Table 1). Sixty isolates originated from the Netherlands and 24 isolates originated from the Caribbean. Of the 24 Caribbean isolates, 22 carried the *bla*$_{KPC-2}$ allele and only two contained the *bla*$_{KPC-3}$ allele. Whole genome multi-locus sequence typing (wgMLST), using an in-house wgMLST scheme based on 4,978 genes, of the 480 carbapenemase-producing *K. pneumoniae* isolates collected in the RIVM revealed that 23 *K. pneumoniae bla*$_{KPC-2}$ isolates grouped together in five distinct genetic clusters. Fifteen *K. pneumoniae bla*$_{KPC-3}$ isolates grouped in four distinct clusters which were obtained from the Netherlands and 46 isolates were unrelated. The *K. pneumoniae* cluster isolates (termed KpnClusters) had unique wgMLST cluster types, and were not described previously (Table 1, Fig. 1). KpnCluster-003 and KpnCluster-005 were comprised of five *K. pneumoniae bla*$_{KPC-2}$ isolates that were exclusively obtained from the Netherlands, while KpnCluster-021 and KpnCluster-041 contained five isolates from the Caribbean. The majority (*n* = 10) of the KpnCluster-019 isolates were obtained from the Caribbean. However, three isolates were from the collection of the Netherlands. One person from whom a KpnCluster-019 isolate was retrieved in August 2017 in the Netherlands, lived in the Caribbean until June 2017 and migrated to the Netherlands in July, demonstrating intercontinental transmission. No epidemiological data could be retrieved from the other two Dutch KpnCluster-019 isolates. Furthermore, most genetic clusters were only distantly related to each other (Fig. 1). The genetic distance between KpnCluster-019 and KpnCluster-041 was 30 alleles and for KpnCluster-003 and KpnCluster-005 53 alleles. KpnCluster-008 differed 132 alleles from KpnCluster-005. While the allelic

difference between these clusters was low, the other genetic clusters differed 3573 to 3610 alleles from KpnCluster-005. This confirmed that most clusters were unrelated, and it is in line with the location of these genetic clusters in the minimum spanning tree.

The resistome diversity among genetic clusters

Analysis of the NGS-derived resistomes of the cluster and non-cluster isolates showed that *K. pneumoniae* harbored either the bla_{KPC-2} or the bla_{KPC-3} allele, none of the isolates carried both alleles (Fig. 2, Suppl. Fig. 1). Nearly all of the *K. pneumoniae* isolates contained the *fosA*, *oqxA* and *oqxB* genes conferring resistance to fosfomycin and fluoroquinolone antibiotics, respectively. An unweighted hierarchical clustering (UPGMA) based on the presence or absence of AMR genes revealed that most genetic cluster isolates group together per cluster, since the resistomes were more than 85% similar. In contrast to this, the resistomes of the non-cluster isolates were very diverse and less related since the resistomes of these isolates were less than 85% similar (Suppl. Fig. 1). Likewise, the resistomes of one group of *K. pneumoniae* KpnCluster-003 bla_{KPC-2} and KpnCluster-008 bla_{KPC-3} cluster isolates with 53 to 132 alleles difference were also unrelated. KpnCluster-019 isolates are unique when compared to the bla_{KPC-2} clusters KpnCluster-003, KpnCluster-005, and KpnCluster-021, in that they carried aminoglycoside (*aac(3)-IIa*), extended spectrum beta-lactams (*bla*CTX-M-15, *bla*SHV-26), fluoroquinolone (*qnrB1*) and tetracyclin (*tetA*) antimicrobial resistance (AMR) genes. KpnCluster-019 and KpnCluster-041 isolates, obtained from the Caribbean, were closely related based on wgMLST, and group together based on the resistome too. The absence of AMR genes *aph(3'')-Ib*, *aph(6)-Id* and *sul2* in five of KpnCluster-019 isolates, including the TGS sequenced isolates, indicate the absence of an AMR gene containing plasmid. In addition, the presence of three KpnCluster-019 isolates from the Netherlands with varying resistomes within the cluster suggests additional transmissions. KpnCluster-

025 bla_{KPC-3} isolates contained the aminoglycoside (*aac(3)-IIa*) and beta-lactam AMR genes (*bla*SHV-28), while the other Kpn bla_{KPC-3} clusters did not. Notably, *mcr* genes conferring resistance to colistin were not detected in the 84 isolates analyzed. The majority of the *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3} isolates were resistant to meropenem (47/84; 56%). More specifically, seven of the 23 *K. pneumoniae* bla_{KPC-2} cluster isolates (30%) and 13 of the 15 bla_{KPC-3} cluster isolates (87%) were resistant to meropenem. The remainders of the cluster and non-cluster isolates were intermediate resistant or sensitive for meropenem (Table 1).

Antibiotic resistance genes among the genomic elements of the distinct genetic clusters

Long-read sequencing of seven isolates from six of the nine genetic *K. pneumoniae* bla_{KPC} clusters, revealed 18 plasmids with varying sizes (Fig. 3). Plasmids containing either the bla_{KPC-2} or bla_{KPC-3} allele were diverse in size. The large (≥150-250 kb) and medium (≥50-150 kb) sized plasmids contained one or two replicons from the incompatibility group IncFIB(K) and IncFII(K), IncHI2 and IncHI2a, or IncFIB(pQil) (Fig. 3). The small plasmids (<50 kb) contained ColRNAI or IncX3/IncL/IncP6 type of replicons. The chromosomes of the analyzed isolates contained on average five acquired AMR genes, while the plasmids contained on average nine AMR genes. Fourteen of the 18 plasmids contained AMR genes from various classes and four plasmids from the isolate of KpnCluster-021 did not. The AMR genes conferring resistance to phenicol, trimethoprim and macrolide antibiotics were located only on medium or large sized plasmids. The small plasmids had one or two AMR genes conferring resistance to aminoglycosides or beta-lactams. Resistance genes for fosfomycin (*fosA*) and fluoroquinolones (*oqxA* and *oqxB*) were exclusively located on the chromosomes of the seven cluster isolates. KpnCluster-019 and KpnCluster-021 associated with the Caribbean contained plasmids encoding genes for phenicol and tetracyclin resistance. The KpnCluster-019 and KpnCluster-021 plasmids were not found in non-cluster

157 isolates, whereas the plasmids of the other clusters were detected in a subset non-cluster isolates (Fig.
158 3). The plasmids of KpnCluster-003 and KpnCluster-005 were present in each of its cluster isolates,
159 however, in isolates of the other clusters occasionally plasmids were lost, thereby impacting the
160 composition of the resistome (Fig. 2 and 3).

161 The *bla*$_{KPC-2}$ KpnCluster-019 isolates were obtained from both the Caribbean and the
162 Netherlands, while *bla*$_{KPC-2}$ KpnCluster-021 isolates originated only from the Caribbean (Table 1, Fig. 3).
163 In the KpnCluster-019 isolate RIVM_C014906, three copies of the bla$_{KPC-2}$ gene were present, while other
164 cluster isolates had only one *bla*$_{KPC}$ copy. One copy was located in the chromosome, one copy in the 200
165 kb plasmid pRIVM_C014906_1 and a third copy on the 16 kb plasmid pRIVM_C014906_3. All these three
166 *bla*$_{KPC-2}$ copies were located on a highly similar *Tn4401*a-derived Δ*Tn4401*a-like transposon of 5.6 kb in
167 this strain. The chromosomes contained this Δ*Tn4401*a-like transposon in the exact same region.
168 KpnCluster-003, KpnCluster-005, KpnCluster-008 and KpnCluster-025 consist of isolates that were
169 obtained in the Netherlands and in these isolates the *bla*$_{KPC}$ allele was located on a Tn*4401a* transposon
170 of 10 kb.

171

172 **Comparison of the *K. pneumoniae* plasmid content**

173 An UPGMA clustering based on the DNA sequence of the 18 plasmids revealed that the majority
174 of the plasmids were unrelated (Fig. 4). The largest two plasmids pRIVM_C008981_1 and
175 pRIVM_C014947_1 carried the largest number of genes and this number decreased by the decreasing
176 size of the plasmids. Most the plasmid located genes had unknown function. The large and medium
177 sized plasmids contained the *klcA* gene, encoding an antirestriction protein implicated in the facilitation
178 of *bla*$_{KPC}$ allele transfer (22). None of the plasmids contained known virulence determinants such as
179 *rmpA*, *rmpA2*, *iroBC*, or *iucABC* implicated in hypervirulence (23, 24). Comparison of the large plasmids

revealed that pRIVM_C008981_1 and pRIVM_C015139_1 from KpnCluster-003 and KpnCluster-005 displayed 90% similarity (Fig. 4). Plasmid pRIVM_C014947_1 was not related to any other of the large plasmids. Despite the low similarity, these large plasmids shared important clusters of genes among them. They all contained the *silE* and *silP* genes encoding a silver-binding protein and a silver exporting ATPase, *cusSRCFB* genes implicated in cation efflux, the *copABCD-pcoE* genes involved in copper resistance and the *arsHACBAD* arsenic resistance gene cluster. These large plasmids also contained *fecIRABCDE* implicated in Fe(3+)-dicitrate transport, the *traIDSQCVAJM-ylpA* plasmid conjugation gene cluster, and the *higA-higA1* antitoxins, except pRIVM_C014947_1 and pRIVM_C014947_2. In addition, the large plasmids also contained a proportion of plasmid-specific and thus *K. pneumoniae* cluster specific content (Suppl. Fig. 2).

The medium-sized plasmids contained the *virB* virulence regulon transcriptional activator and the *merAC* mercuric reductase and transport protein. While pRIVM_C015274_1 and pRIVM_C015451_1 contained a plasmid conjugation gene cluster, pRIVM_C014906_2 and pRIVM_C015139_2 contained truncated versions hereof. The more distantly related pRIVM_C014906_2 plasmid from KpnCluster-019 had in addition to the *higA-higA1* antitoxins also a *ccdA-ccdB* toxin-antitoxin system. The small plasmids (<50 kb) contained genes implicated in virulence. Plasmids pRIVM_C015139_3 and pRIVM_C015274_2 displayed 99% similarity and carried the *virD4-B9-B8-B4-ptlH* Type IV secretion system. pRIVM_C014947_4 contained a *merPT* mercuric transport system, while pRIVM_C014906_3 and pRIVM_C015274_3 carried a *ceaC* colicin-E3. The plasmid pRIVM_C014947_5 contained the *bdlA* gene encoding a biofilm dispersion protein.

Transposable elements in *K. pneumoniae* plasmids

The large and medium sized plasmids contained the most transposase sequences, and each plasmid had its unique transposon signature (Fig. 5). The IS1 and IS3 transposase families dominated in the *K. pneumonia*e plasmids. The IS1 family transposase was found most frequently among the plasmids and in most copies within plasmids. In the large and medium sized plasmids, the bla_{KPC} allele was located on a Tn*4401a* transposon, except in pRIVM_C014906_1. In the small plasmids carrying a bla_{KPC}, the carbapenemase allele was located on a Δ*Tn4401*a-like transposon. The large plasmids pRIVM_C008981_1, pRIVM_C015139_1 and pRIVM_C014906_1 harbored 37, 32 and 31 annotated tranposases, respectively. In contrast, the largest plasmid pRIVM_C014947_1 of 250.6 kb from KpnCluster-021 contained only 12 transposons. The remainder of the plasmids from KpnCluster-021 also contained very few transposase sequences, in contrast to the other plasmids from the different clusters. The highly related pRIVM_C015139_3 and pRIVM_C015274_2 plasmids (99% similarity) had identical transposons. While IS66 and IS110 family transposase sequences also dominate in the large plasmids, the medium sized plasmids contained IS3 family type of transposases. The medium sized plasmids contained eleven to 23 transposases, and the small plasmids less than ten.

Similarity with previously reported plasmids

BLAST analysis of the *K. pneumoniae* plasmids identified in this study showed that 8 of the 18 plasmids were similar to previously reported plasmids in the NCBI sequence database (Table 2). These plasmids covered five distinct genetic clusters, except pRIVM_C008981_1 from KpnCluster-003. To date, none of these plasmids were reported to be implicated in healthcare-associated outbreaks. Plasmids pRIVM_C008981_1, pRIVM_C014906_1, pRIVM_C014906_3 containing bla_{KPC-2} and pRIVM_C015274_1 harboring bla_{KPC-3} from distinct genetic clusters only had low sequence coverage 35-87% with plasmids present in the NCBI sequence database. The other bla_{KPC-2} and bla_{KPC-3} plasmids had high (93-99%)

sequence coverage, indicating that these similar plasmids were detected previously by other researchers. Plasmids pRIVM_C014906_2, pRIVM_C015139_1, pRIVM_C015274_2 and pRIVM_C015274_3, not carrying a bla_{KPC} allele, displayed 97-100% sequence coverage and 99-100% identity to plasmids isolated from *K. pneumoniae* from different countries (Table 2). Plasmids pRIVM_C014947_5 and pRIVM_C014947_6 from KpnCluster-021 had 100% sequence coverage with 92.18 to 99.99% identity with plasmids isolated from *Enterobacter hormaechei*. Plasmids similar to pRIVM_C014947_1, pRIVM_C014947_3, pRIVM_C014947_5 and pRIVM_C014947_6 from KpnCluster-021 were detected previously in a variety of hosts, *e.g. Salmonella enterica*, *K. pneumoniae*, and *E. hormaechei*, suggesting these plasmids are broad-host range.

Prophage sequences in the *K. pneumoniae* cluster genomes

PHASTER analysis revealed that the majority of the large and medium-sized plasmids from different genetic clusters with IncFIB(K) or IncFIB(pQil) and IncFII(K) replicons contained one to four regions with prophage-related sequences *e.g.* genes encoding putative phage integrase, phage-like proteins, coat proteins, and/or tail shaft proteins (Table 3). The size of the prophage sequence regions varied per plasmid. The most commonly found prophage-related sequence in large and medium-sized plasmids of cluster isolates was an *Escherichia* phage RCS47 (Table 3). This sequence entails the 14.2 kb *ygbMLKJI-bla*SHV-*recF-lacY* region flanked by IS26 elements and representing 12% of the RCS47 prophage genome. The small plasmids of <50 kb lacked phage-related sequences. In contrast, the chromosomes of cluster isolates carried at least three to nine phage sequence regions covering 10-50% of the phage genome. These phage sequence regions covered a wide variety of distinct phages, including prophage sequences from *Salmonella*, *Klebsiella*, *Cronobacter*, *Enterobacteria* phages (Suppl. Table 2). The most commonly found prophage sequence in *Klebsiella* chromosomes was the Enterobacteria phage P4.

Discussion

We showed that a *K. pneumoniae* strain carrying bla_{KPC-2} was transmitted between the Netherlands and the Caribbean. This is based on the high genetic relatedness of the 13 isolates from KpnCluster-019 as assessed by wgMLST and their highly similar resistome and plasmidome. We found that one person lived in the Caribbean and migrated to the Netherlands. After migration, a KpnCluster-019 isolate was obtained from this person in a Dutch hospital. Possibly other transmissions by other persons could have occurred, but these were not confirmed in this study. By combining short-read with long-read sequencing data, we identified 18 plasmids of seven *K. pneumoniae* isolates from six distinct genetic clusters found in the Netherlands and the Caribbean and analyzed these plasmids for its AMR gene profile, bla_{KPC} transposons, replicons, transposon families, and gene content. The plasmid composition varied among the genetic clusters. The cluster isolates had unique wgMLST cluster types which were not published previously and differ from globally circulating extensively drug-resistant (XDR) and highly virulent (hvKp) *K. pneumoniae* strains (23, 24). KpnCluster-019 is unique compared to the other cluster isolates analyzed in this study for the following reasons. First, KpnCluster-019 harbors a unique and extensive set of AMR genes on the chromosome and in its plasmids. Secondly, KpnCluster-019 isolates were the only to contain three copies of the bla_{KPC-2} allele, two on two different plasmids and one in the chromosome. The localization of bla_{KPC-2} on the chromosome and additional bla_{KPC-2} copies have been reported previously and is further complicating the understanding of transmission of multidrug-resistant *K. pneumoniae* (25, 26). Thirdly, KpnCluster-019 and also KpnCluster-021 isolates from the Caribbean harbored the bla_{KPC-2} allele on a 5.6 kb Δ*Tn4401*a-like transposon, while the other isolates from the other genetic clusters from the Netherlands contained bla_{KPC} on a 10kb Tn*4401a* transposon. Most global descriptions of *K. pneumoniae* bla_{KPC} the past decade have been associated with Tn*4401a* or isoforms hereof (9). The traditional association of bla_{KPC} with the Tn*4401a* transposon has possibly been eroded in *K. pneumoniae* isolates from the Caribbean to a smaller variant. This is the first report of identification of

a 5.6 kb Δ*Tn4401*a-like *bla*KPC-2 transposon of *K. pneumoniae* in the Netherlands. Preliminary surveillance data analysis revealed that the Δ*Tn4401*a-like element carrying bla_{KPC-2} and smaller variants disseminated among *E. cloacae*, *Serratia marcesens*, *K. oxytoca* and *E. coli* in the Netherlands (unpublished data). Future work will seek to understand the dissemination of the Δ*Tn4401*a-like bla_{KPC-2} element among CPE in the Netherlands. Lastly, the plasmids of KpnCluster-019 isolates contained also unique plasmid content, including a distinct transposon signature, two toxin-antitoxin systems and a *ceaC* colicin which possibly contribute to the success in survival, niche adaptation or transmission of this strain.

The *K. pneumoniae* bla_{KPC-3} isolates had higher MICs for meropenem than the *K. pneumoniae* *bla*KPC-2 isolates, which is in line with a previous study (21). The KPC-2 enzyme differs in a single amino acid substitution (Histidine 272 to Tyrosine) from KPC-3. Additional changes in KPC-3 can lead to increased resistance for ceftazidime and cephamycin (27). The increase in meropenem resistance observed in our study is possibly correlated with improved ability of KPC-3 enzymes to hydrolyze the meropenem antibiotic (15). Alternatively, additional beta-lactamase genes such as bla_{OXA-1}, bla_{OXA-9} or bla_{TEM-1A} may contribute to increased resistance for meropenem (28).

Despite the limited number of long-read sequenced isolates, we have highlighted important new insights in the genomic surveillance of a notorious multi-antibiotic resistant nosocomial pathogen. In some clusters, the plasmidome varied as this was likely due to loss of a plasmid. Also, the resistome data suggest the presence of other plasmids in cluster isolates that were not present in the isolates that were sequenced using TGS. To overcome this limitation, all isolates used in this study should have been sequenced using long-read third generation sequencing. Nevertheless, we identified plasmids in *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3} cluster isolates which vary in size from large, medium and small. The large and medium sized plasmids were enriched for a variety of transposons, conjugation transfer

295 systems, cation efflux systems including Fe(3+)-dicitrate transport, and genes encoding for silver, copper
296 and arsenic resistance. The small plasmids contained putative virulence determinants. The presence of
297 these systems may contribute to the success of transmission of specific *K. pneumoniae* strains in the
298 hospital setting or the community (13, 29, 30). Escherichia RCS47 prophage sequences were found on
299 medium and large plasmids in the cluster isolates analyzed. In contrast, the chromosomes contained a
300 variety of prophage-related sequences. RCS47 is a P1-like bacteriophage carrying the ESBL-encoding
301 *bla*SHV-2 gene was isolated from a clinical *Escherichia coli* strain (31). The prevalence of P1-like
302 prophages in animal and human *E. coli* strain collections was 12.6% (31). The presence of P1-like phage
303 sequences in plasmids of a snapshot of the *K. pneumoniae* population in the Netherlands and the
304 Caribbean suggest that the role of P1-like phages in disseminating antibiotic resistance may be
305 underestimated (32).

306 In conclusion, long-read sequencing contributed to the understanding of the successful
307 transmission of the KpnCluster-019 *K. pneumoniae* bla_{KPC-2} strain. Plasmid content such as conjugation
308 machinery, transposons, virulence determinants and phages may contribute to diversification, and
309 dissemination of plasmids containing AMR genes, and therefore represent important plasmid features
310 that warrants future investigation. More long-read plasmid sequencing efforts of CPE and *K.*
311 *pneumoniae* in particular are required to identify the complete plasmid reservoir involved in the spread
312 of antibiotic resistance determinants in the Netherlands and the Caribbean islands.

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Materials and methods

Bacterial isolates. For the Dutch National carbapenemase-producing Enterobacterales (CPE) Surveillance program, medical microbiology laboratories from the Netherlands andthe Caribbean routinely send CPE isolates with a meropenem minimum inhibitory concentration (MIC) of ≥0.25 µg/ml and/or an imipenem MIC of ≥1 µg/ml or phenotypic (CIM-test) or genotypical evidence of carbapenemase production to the National Institute of Public Health and the Environment (RIVM) (16). For this study, 84 carbapenemase-producing *K. pneumoniae* isolates carrying either the bla_{KPC-2} allele or the bla_{KPC-3} allele were included and collected in the period from January 1st 2014 until June 30th 2019. Only the first *K. pneumoniae* isolate per patient in this study period was selected. The 84 isolates were obtained from 84 persons and from various isolation sites, *i.e*. rectum/perineum (*n* = 43), throat (*n*= 11), pus (*n* = 2), sputum (*n* = 4), urine (*n* = 10), wound (*n*= 5) and nine were from miscellaneous isolation sites. All bacterial strains were grown aerobically at 37°C on Columbia sheep blood agar plates.

Antimicrobial susceptibility testing. Resistance to carbapenem was confirmed by assessing the MICs for meropenem for all the 84 isolates using an Etest (bioMérieux Inc., Marcy l'Etoile, France). Based on the clinical breakpoints according to EUCAST, the *K. pneumoniae* isolates were classified as sensitive (≤2 mgl/L), intermediate (>2 mg/L and ≤8 mg/L) and resistant (>8 mg/L) to meropenem. In addition, all isolates were analyzed for the production of carbapenemase using the carbapenem inactivation method (CIM) as described previously (33).

338 **Next-generation sequencing and wgMLST.** All 84 *K. pneumoniae* isolates were subjected to next-
339 generation sequencing (NGS) using the Illumina HiSeq 2500 (BaseClear, Leiden, the Netherlands). The
340 NGS data of the *K. pneumoniae* isolates were used for wgMLST analyses using the in-house wgMLST
341 scheme in SeqSphere software version 6.0.2 (Ridom GmbH, Münster, Germany). Ridom wgMLST cluster
342 nomenclature were depicted in Table 1. The resulting data was imported into Bionumerics version 7.6.3
343 for subsequent comparative analyses (Applied Maths, Sint-Martens-Latem, Belgium). The antibiotic
344 resistance gene profile and plasmid replicon compositions in all of the isolates were determined by
345 interrogating the online ResFinder (version 3.1.0) and PlasmidFinder (version 2.0.2) databases available
346 at the Center for Genomic Epidemiology website (https://cge.cbs.dtu.dk/services/) (34, 35). For
347 ResFinder, a 90% identity threshold and a minimum length of 60% were used as criteria, whereas for
348 PlasmidFinder, an identity of 95% was utilized.

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350 **Long-read third-generation sequencing.** One *K. pneumoniae* isolate per genetic KpnCluster was
351 sequenced using long-read third-generation Nanopore sequencing (18, 36). High molecular weight DNA
352 was isolated using an in-house developed protocol. Bacteria were grown overnight in 1.5 ml Brain heart
353 infusion broth and culture was spun down at 13,000 x *g* for 2 minutes. The pellet was washed and
354 resuspended in 500 µl of 150 mM NaCl. The suspension was spun down at 5,000 x *g* for 5 minutes and
355 the pellet was resuspended in 100 µl of QuickExtract™ DNA Extraction Solution (Lucigen) and 0.1 µl
356 Ready-Lyse™ Lysozyme solution (Epicentre) and incubated for 1 hour at 37°C. Subsequently, 85 µl 10
357 mM Tris 1 mM EDTA pH = 8 (1x TE), 10 µl proteinase K (>600 mAU/mL, Qiagen) and 5 µl 20% sodium
358 dodecyl sulfate solution were added, and the mixture was incubated at 56°C for 30 minutes. DNA was
359 precipitated overnight at -20°C by adding 0.1x volume 3M sodium acetate pH = 5.2 and 2.5x volume ice
360 cold 100% ethanol. Precipitated DNA was spun down at 13,000 x *g* for 15 minutes and pellets were

361 washed with 1 ml 70% ethanol followed by another centrifugation at 13,000 x *g* for 5 minutes. After
362 drying, the pellet was dissolved in 200 µl 1x TE and diluted to 1 µg with Nuclease-free water.

363 The Oxford Nanopore protocol SQK-LSK108 (https://community.nanoporetech.com) and the
364 expansion kit for native barcoding EXP-NBD104 was used. Briefly, a shearing step was performed using
365 g-TUBE's™ (Covaris) to obtain an average DNA fragment size of 8 kb. The DNA was repaired using FFPE
366 and end-repair kits (New England BioLabs) followed by ligation of barcodes with bead clean up using
367 AMPure XP (Beckman Coulter) after each step. Barcoded isolates were pooled and sequencing adapters
368 were added by ligation. The final library was loaded onto a MinION flow cell (MIN-106 R9.4.1). The 48-
369 hour sequence run was started without live base calling enabled on a MinION device connected to a
370 desktop computer. After the sequence run, base calling and de-multiplexing was performed using
371 Albacore 2.3.1 and a single FASTA file per isolate was extracted from the FAST5 files using Poretools
372 0.5.1 (37). Illumina and Nanopore data were used in a hybrid assembly performed by Unicycler v0.4.4
373 (38). The resulting contig files were annotated using Prokka and were subsequently loaded into
374 BioNumerics for further analyses (39).

375

376 **Minimum Spanning Tree and UPGMA analyses.** The BioNumerics software was used to generate a
377 minimum spanning tree (MST) or an UPGMA hierarchical clustering as described previously (16). The
378 MST was based on an in-house *K. pneumoniae* wgMLST scheme. The categorical coefficient was used to
379 calculate the MST. wgMLST clusters were defined as a minimum of two isolates of which the genetic
380 distance between the two isolates was ≤20 genes. An UPGMA clustering of *K. pneumoniae* bla_{KPC-2} and
381 bla_{KPC-3} isolates was performed based on the presence and/or absence of antibiotic resistance genes per
382 isolate.

383 **Plasmid reconstruction by read mapping.** The CLC Genomics Workbench version 12.0 software
384 (www.qiagenbioinformatics.com) was used to reconstruct plasmids. For this, complete plasmids
385 obtained by TGS were used as a scaffold to map the trimmed NGS reads of isolates that were from the
386 same genetic wgMLST cluster. A plasmid was scored "present" in an isolate if reads mapped to a
387 reference plasmid of interest and ≥85% of the consensus sequence size in kilo bases was reconstructed.
388 Linear DNA fragments < 5kb were omitted in this study. Nucleotide BLAST analyses on plasmid
389 sequences were performed using the https://blast.ncbi.nlm.nih.gov website and date from October
390 2019.
391
392 **Plasmid content analysis.** Bionumerics was used to extract and analyze annotated genes and
393 tranposases in the 18 different plasmids. The data was plotted in Excel. Phaster, the PHAge Search Tool
394 Enhanced Release website (http://phaster.ca/) was used to determine the presence of phage sequences
395 in the plasmids and searches date from October 2019 (40).
396
397 **NGS, TGS and plasmid data availability.** The Illumina (NGS), Nanopore (TGS) and plasmid sequence data
398 sets generated and analyzed in this study are available in NCBI in the European Nucleotide Archive (ENA)
399 under project number xxx and sequence repositories under Genbank accession numbers xxx. All data
400 supporting the findings of this study are available in this article and its supplementary information files
401 are available upon request.
402

403 **Figure legends**

404

405 **Fig. 1. Minimum spanning tree based on wgMLST of 480 sequenced *K. pneumoniae* isolates.** Circles
406 represent *K. pneumoniae* isolates, and the sizes of the circles indicate the numbers of isolates. Lines
407 connecting the circles represent the genetic distance in numbers of alleles; the longer the connecting
408 line, the larger the genetic distance. *K. pneumoniae* bla_{KPC-2} isolates were marked blue and *K.*
409 *pneumoniae* bla_{KPC-3} were marked magenta. *K. pneumoniae* bla_{KPC-2} or bla_{KPC-3} cluster isolates that were
410 sequenced with TGS were marked green. Genetic clusters were indicated with either a blue or a
411 magenta halo around the circles, if two or more isolates differ ≤ 20 alleles. A categorical coefficient was
412 used for the clustering. Cluster names are indicated. Inset: genetic distance between the KpnClusters in
413 which the allelic difference is indicated by numbers.

414

415 **Fig. 2. Resistome of *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3} cluster isolates.** *K. pneumoniae* bla_{KPC-2} and
416 bla_{KPC-3} cluster isolates were indicated on the y-axis and AMR genes on the x-axis. Antibiotic classes are
417 indicated above the AMR genes in different colors. The clustering was based on the presence (squares)
418 and absence of AMR genes. Resistance genes in *K. pneumoniae* bla_{KPC-2} or bla_{KPC-3} cluster isolates that
419 were sequenced with TGS were marked with green squares. Genetic relatedness was depicted in an
420 UPGMA tree in which *K. pneumoniae* bla_{KPC-2} isolates were marked with blue branches, and *K.*
421 *pneumoniae* bla_{KPC-3} were marked magenta. Dutch KpnCluster-019 isolates were marked with an *. A
422 dotted line marks the 85% cut off.

423

424 **Fig. 3. Antimicrobial resistance genes on chromosomes and plasmids.** The presence (black squares) and
425 absence of AMR genes among the chromosomes and 18 plasmids of seven TGS sequenced isolates

(green). Chromosomes (cRIVM_C0xxxx) and plasmids (pRIVM_C0xxxx) are depicted on the Y-axis, and AMR genes on the x-axis. Antibiotic classes are indicated above the AMR genes in different colors.

Fig. 4. *K. pneumoniae* plasmid gene content. An UPGMA clustering was performed based on the plasmid DNA sequence for the determination of the genetic relation among the 18 plasmids. Similarity is indicated on the y-axis using a scale from 0 (not similar) to 100% (identical). A similarity of ≥85 to 100% is regarded as the same plasmid. The plasmids are indicated on the x-axis. The presence (black squares) and absence is indicated of annotated genes among the 18 plasmids of seven TGS sequenced isolates. If a gene was present twice, blue squares were used and more than 2, red squared were used. Colors indicated different groups of genes with a specific function.

Fig. 5. *K. pneumoniae* plasmid-localized transposases. The presence (black squares) and absence is indicated of annotated transposases among the 18 plasmids of six TGS sequenced isolates. The plasmids are indicated on the x-axis. If a transposon was present twice, blue squares were used and more than 2, red squared were used. The light grey area indicates specific transposons found in only one plasmid.

Table 1. Distribution of *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3} isolates and resistance to meropenem.

Based on the clinical breakpoints according to EUCAST, the isolates were classified as sensitive (S; <2 mgl/L), intermediate (I; ≥2 to 8 mg/L) and resistant (R; >8 mg/L).

Table 2. BLAST similarity analysis of *K. pneumoniae* plasmids.

Table 3. Predicted prophage sequences among *K. pneumoniae* plasmids.

Supplemental Figure 1. Resistome of *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3} non-cluster isolates. *K. pneumoniae* bla_{KPC-2} isolates were marked blue, and *K. pneumoniae* bla_{KPC-3} were marked magenta. *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3} cluster isolates were indicated on the y-axis and AMR genes on the x-axis. The UPGMA clustering was based on the presence (black squares) and absence of AMR genes. Antibiotic classes are indicated above the AMR genes with different colors. A dotted line marks the 85% cut off.

Supplemental Figure 2. *K. pneumoniae* plasmid gene content (continued). An UPGMA clustering was performed based on the plasmid DNA sequence for the determination of the genetic relation among the 18 plasmids. Similarity is indicated on the y-axis using a scale from 0 (not similar) to 100% (identical). A similarity of ≥85 to 100% is regarded as the same plasmid. The plasmids are indicated on the x-axis. The presence (black squares) and absence is indicated of annotated genes among the 18 plasmids of six TGS sequenced isolates. If a gene was present twice, blue squares were used and more than two, red squared were used. Colors indicated different clusters of genes with a specific function. The light grey area indicates gene specific content found in only one plasmid.

Supplemental table 1. Predicted prophage sequences among *K. pneumoniae* chromosomes.

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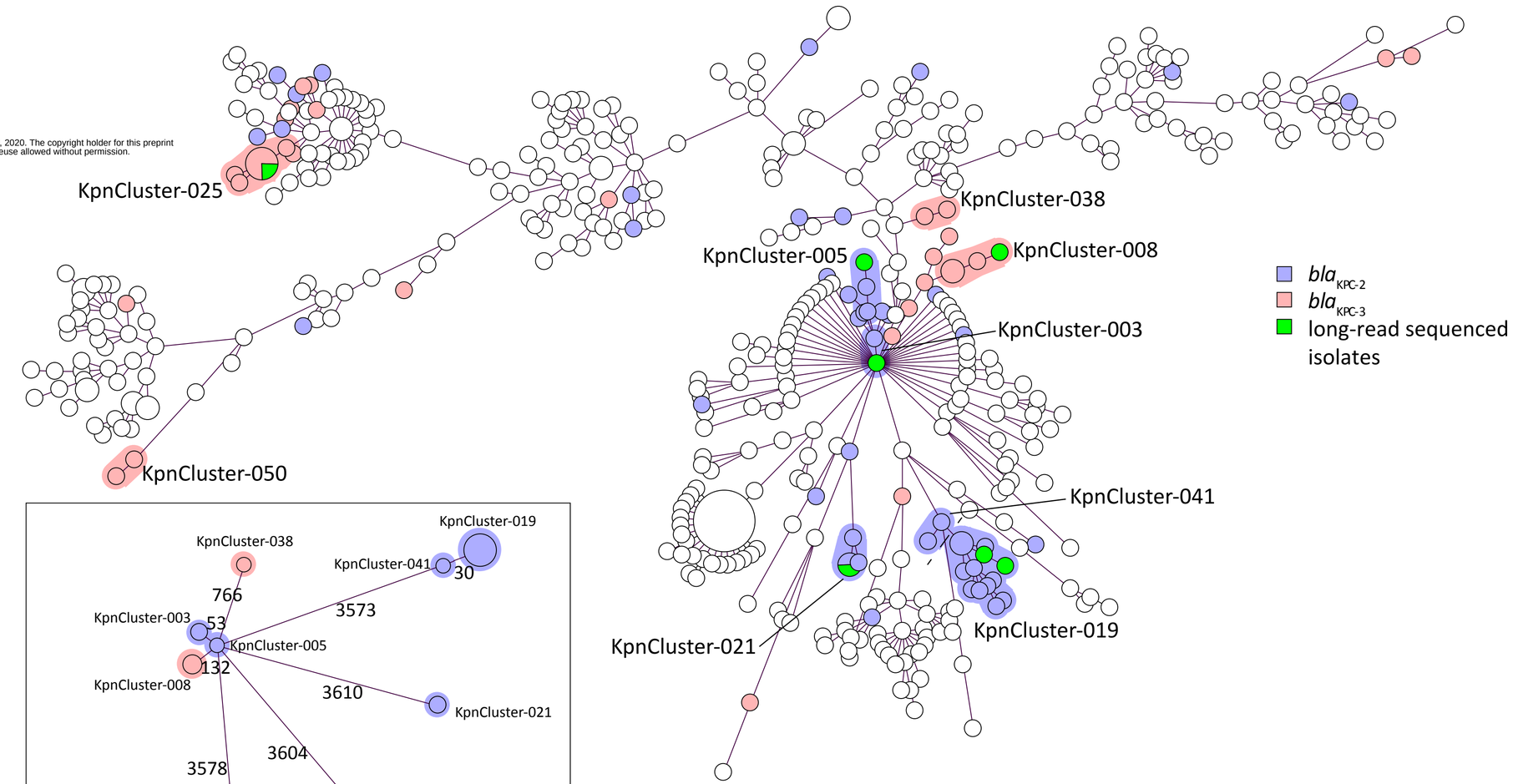

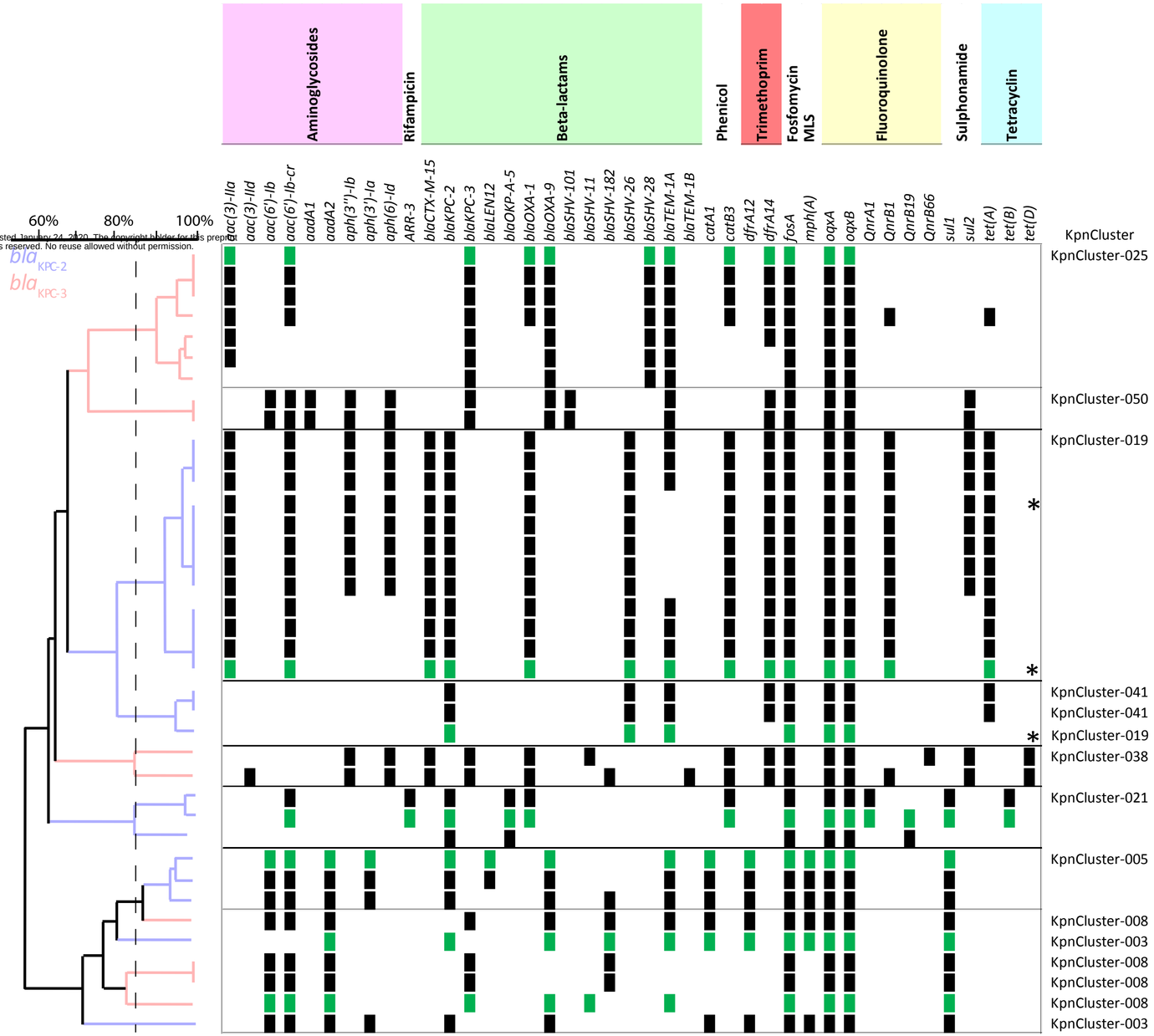

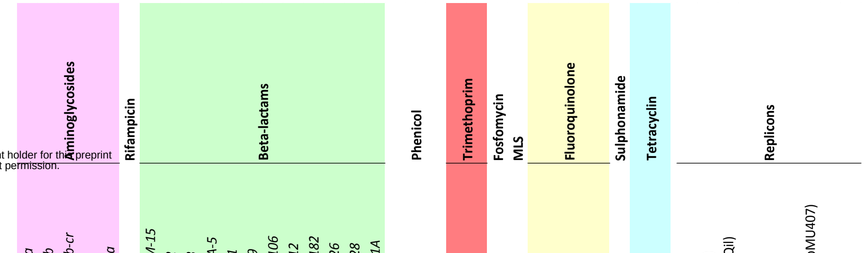

Genome(s)	Size (bp)	*bla* KPC transposon	KpnCluster	Cluster isolates with plasmid	Non-cluster isolates with plasmid
cRIVM_C008981			KpnCluster-003		
pRIVM_C008981_1	226,975	Tn*4401a*	(NL)	2/2	7/46
cRIVM_C014906			KpnCluster-019		
pRIVM_C014906_1	200,297	ΔTn*4401a* -like	(NL + Caribbean)	12/13	0/46
pRIVM_C014906_2	105,291			12/13	
pRIVM_C014906_3	15,947	ΔTn*4401a* -like		13/13	0/46
cRIVM_C018535					
pRIVM_C018535_1	200,152	ΔTn*4401a* -like		12/13	0/46
pRIVM_C018535_2	15,947	ΔTn*4401a* -like		13/13	0/46
cRIVM_C014947			KpnCluster-021		
pRIVM_C014947_1	250,606		(Caribbean)	2/3	
pRIVM_C014947_2	162,103			3/3	
pRIVM_C014947_3	61,919			3/3	
pRIVM_C014947_4	29,391	ΔTn*4401a* -like		3/3	0/46
pRIVM_C014947_5	4,938			3/3	
pRIVM_C014947_6	2,516			3/3	
cRIVM_C015139			KpnCluster-005		
pRIVM_C015139_1	209,317		(NL)	3/3	
pRIVM_C015139_2	98,806	Tn*4401a*		3/3	25/46
pRIVM_C015139_3	43,392			3/3	
cRIVM_C015274			KpnCluster-008		
pRIVM_C015274_1	115,525	Tn*4401a*	(NL)	4/4	6/46
pRIVM_C015274_2	43,380			4/4	
pRIVM_C015274_3	13,636			2/4	
cRIVM_C015451			KpnCluster-025		
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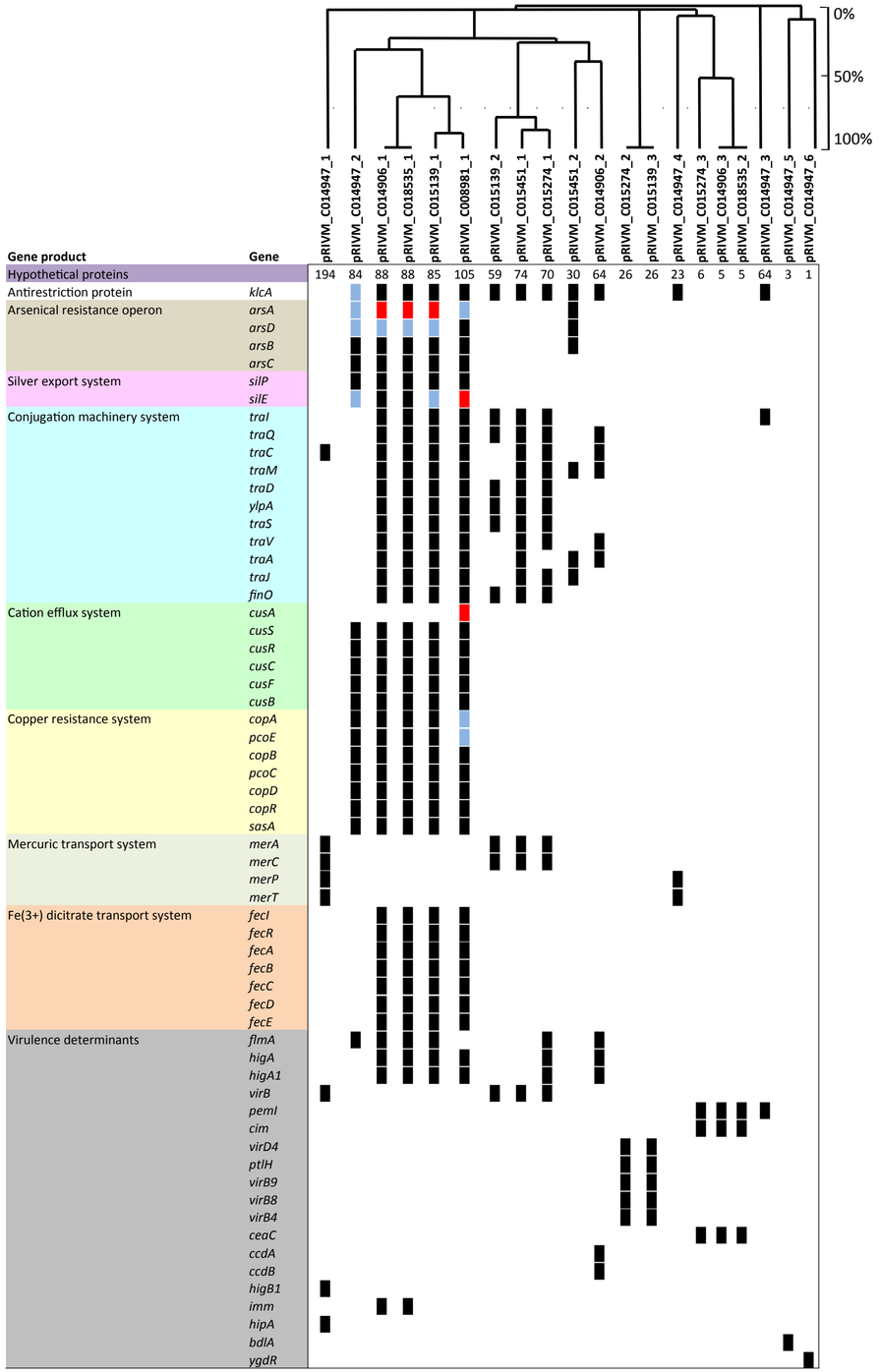
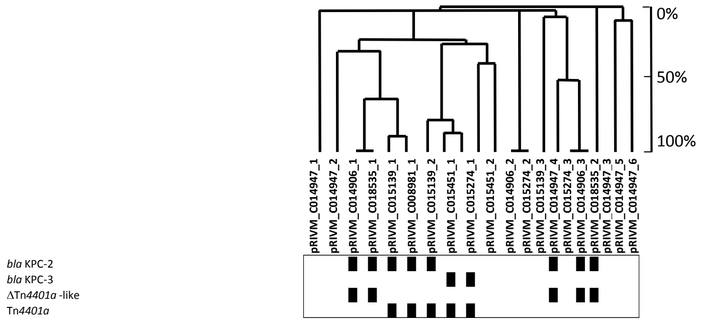
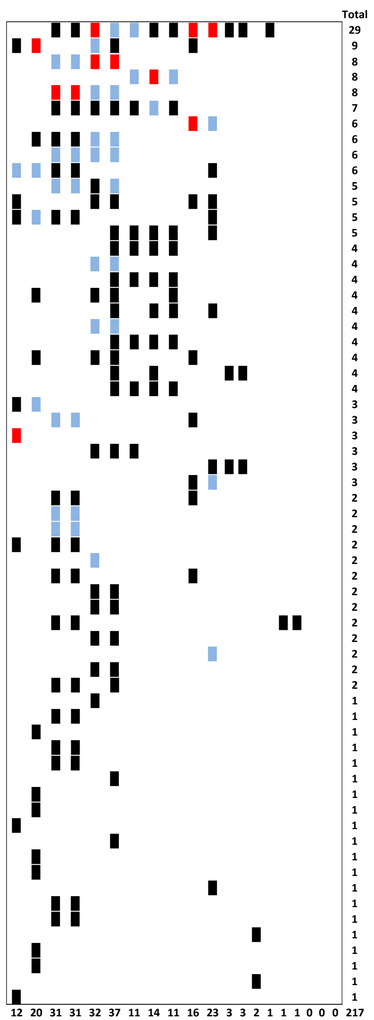
pRIVM_C015451_2	68,684			6/7	





Transposon	Type	Total
IS1 family transposase	IS1A	29
	IS1D	9
	IS1R	8
	IS1X2	8
	ISKpn14	8
	ISPmi3	7
IS110 family transposase	IS1663	6
	IS4321R	6
	IS5075	6
	ISEsa2	6
IS1182 family transposase	ISKpn6	5
IS1595 family transposase	ISSsu9	5
IS200/IS605 family transposase	IS609	5
IS21 family transposase	ISKpn7	5
IS3 family transposase	IS103	4
	IS1222	4
	IS1400	4
	ISEc52	4
	ISEcl1	4
	ISEhe3	4
	ISKpn11	4
	ISKpn18	4
	ISKpn38	4
	ISKpn8	4
	ISSen4	3
IS4 family transposase	IS421	3
	ISVsa5	3
IS481 family transposase	ISKpn28	3
IS5 family transposase	IS903	3
	ISKpn26	3
	ISThi1	2
IS6 family transposase	IS15	2
	IS15DII	2
	IS26	2
	IS6100	2
IS66 family transposase	ISCfr14	2
	ISCro1	2
	ISEc22	2
	ISKpn24	2
	ISSal1	2
	ISSgsp1	2
ISAs1 family transposase	ISKpn31	2
ISL3 family transposase	ISCfr12	2
	ISEc38	1
	ISKpn25	1
	ISStma11	1
ISNCY family transposase	ISBcen27	1
	ISKpn21	1
	ISLad2	1
	ISSen7	1
Transposon Tn552 DNA-invertase	Tn552	1
Tn3 family transposase		1
	IS3000	1
	ISEc63	1
	ISPa38	1
	ISPsy42	1
	ISSba14	1
	ISXc4	1
	Tn2	1
	TnAs1	1
	TnAs2	1
	TnAs3	1
Transposon Tn3 resolvase		1
Total		**217**



12	**20**	**31**	**31**	**32**	**37**	**11**	**14**	**11**	**16**	**23**	**3**	**3**	**2**	**1**	**1**	**0**	**0**	**0**

***bla*$_{KPC}$ allele**	**KpnCluster**	**wgMLST**	**The Netherlands**			**Caribbean**			
		Cluster type	S	I	R	S	I	R	Total
bla$_{KPC-2}$	KpnCluster-003	2521			2				2
	KpnCluster-005	123			3				3
	KpnCluster-019	2494	1	2		6	4		13
	KpnCluster-021	2588					1	2	3
	KpnCluster-041	2494				2			2
	Non-KpnCluster	variant	3	4	14	1	2	4	28
	Subtotal								51
bla$_{KPC-3}$	KpnCluster-008	53			4				4
	KpnCluster-025	1257		1	6				7
	KpnCluster-038	1752			2				2
	KpnCluster-050	1969	1		1				2
	Non-KpnCluster	variant	3	5	8	1		1	18
	Subtotal								33
	Total		8	12	40	10	7	7	84

Plasmid	KpnCluster	*bla*$_{KPC}$ allele	Bacterial species	Plasmid	Query Coverage (%)	Identity (%)	Accession number	Country	Year	Reference
pRIVM_C008981_1	KpnCluster-003	*bla*$_{KPC-2}$	*K. pneumoniae*	pGMI16-005_01	35	99.96	CP028181.1	Denmark	2013	
pRIVM_C014906_1	KpnCluster-019	*bla*$_{KPC-2}$	*K. pneumoniae*	pKPN1482-1	63	99.91	CP020842.1	USA	2014	Long *et al*., 2017
pRIVM_C014906_2			*K. quasipneumoniae*	plasmid pG747	97	99.84	CP034137.1	Nigeria	2013	
pRIVM_C014906_3		*bla*$_{KPC-2}$	*K. pneumoniae*	plasmid unnamed5	58	99.97	CP033630.1	Italy	2013	Roe *et al*., 2019
pRIVM_C018535_1	KpnCluster-019	*bla*$_{KPC-2}$	*K. pneumoniae*	pKPN1482-1	63	99.91	CP020842.1	USA	2014	Long *et al*., 2017
pRIVM_C018535_2		*bla*$_{KPC-2}$	*K. pneumoniae*	plasmid unnamed5	58	99.97	CP033630.1	Italy	2013	Roe *et al*., 2019
pRIVM_C014947_1	KpnCluster-021		*S. enterica*	pSJO-60984	93	99.99	CP025277.1	USA	2007	
pRIVM_C014947_2			*K. quasipneumoniae*	pDA33145-152	42	99.48	CP029598.1	USA	1994	Nicoloff *et al*., 2019
pRIVM_C014947_3			*K. pneumoniae*	plasmid unnamed3	90	99.98	CP032170.1	USA	2015	
pRIVM_C014947_4		*bla*$_{KPC-2}$	*K. pneumoniae*	pA1705-KPC	93	99.97	MH909348.1	China	2013	
pRIVM_C014947_5			*E. hormaechei*	p34998-4.921kb	100	98.72	CP010381.1	USA	2011	Chavda *et al.*, 2016
pRIVM_C014947_6			*E. hormaechei*	unnamed4	100	92.18	CP035389.1	UK	2016	
pRIVM_C015139_1	KpnCluster-005		*K. pneumoniae*	plasmid 2	100	99.99	LR130549.1	Australia	2018	
pRIVM_C015139_2		*bla*$_{KPC-2}$	*K. pneumoniae*	pUJ-83KPC	99	98.55	MG700549.1	Germany	2017	
pRIVM_C015139_3			*K. pneumoniae*	pBK13043-2	100	99.89	CP020839.1	USA	2004	Long *et al.*, 2017
pRIVM_C015274_1	KpnCluster-008	*bla*$_{KPC-3}$	*K. pneumoniae*	plasmid p2	87	99.91	CP019774.1	Switzerland	2015	Ruppe *et al*., 2017
pRIVM_C015274_2			*K. pneumoniae*	pBK13043-2	100	99.95	CP020839.1	USA	2004	Long *et al*., 2017
pRIVM_C015274_3			*K. pneumoniae*	ColEST258	100	100	JN247853.1	Italy	2012	Garcia-Fernandez *et al*., 2012
pRIVM_C015451_1	KpnCluster-025	*bla*$_{KPC-3}$	*K. pneumoniae*	pKPC	99	99.99	CP043971.1	France	2019	
pRIVM_C015451_2			*K. pneumoniae*	p911021-tetA	66	99.55	MG288679.1	China	2019	

Plasmid(s)	*bla*$_{KPC}$ allele	Phage region(s) length (kb)	Most Common Phage	Accession number
pRIVM_C008981_1	*bla*$_{KPC-2}$	11.3	Escherichia phage RCS47	NC_042128
pRIVM_C014906_1	*bla*$_{KPC-2}$	7, 3.2, 23.3, 5.1, 5.7	Stx2-converting phage 1717	NC_011357
pRIVM_C014906_2		43.6	Escherichia phage RCS47	NC_042128
pRIVM_C014906_3	*bla*$_{KPC-2}$			
pRIVM_C018535_1	*bla*$_{KPC-2}$	7, 3.2, 23.3, 5.1, 5.7	Stx2-converting phage 1717	NC_011357
pRIVM_C018535_2	*bla*$_{KPC-2}$			
pRIVM_C014947_1		10.9	Bacillus phage Shanette	NC_028983
pRIVM_C014947_2		30.6, 15.1	Enterobacteria phage P1, Escherichia phage RCS47	NC_005856, NC_042128
pRIVM_C014947_3				
pRIVM_C014947_4	*bla*$_{KPC-2}$			
pRIVM_C014947_5				
pRIVM_C014947_6				
pRIVM_C015139_1		23.5, 11.3	Stx2-converting phage 1717, Escherichia phage RCS47	NC_011357, NC_042128
pRIVM_C015139_2	*bla*$_{KPC-2}$	30.8	Escherichia phage RCS47	NC_042128
pRIVM_C015139_3		24.8	Escherichia phage RCS47	NC_042128
pRIVM_C015274_1	*bla*$_{KPC-3}$	38.7	Escherichia phage RCS47	NC_042128
pRIVM_C015274_2		24.8	Escherichia phage RCS47	NC_042128
pRIVM_C015274_3				
pRIVM_C015451_1	*bla*$_{KPC-3}$	39.5	Escherichia phage RCS47	NC_042128
pRIVM_C015451_2		20.6	Staphylococcus phage SPbeta-like	NC_029119