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

Antoni P. A. Hendrickx¹✉, Fabian Landman¹, Angela de Haan¹, Dyogo Borst¹, Sandra Witteveen¹, Marga G. van Santen-Verheувel¹, Han G. J. van der Heide¹, Leo M. Schouls¹ & The Dutch CPE surveillance Study Group*

Carbapenemase-producing *Klebsiella pneumoniae* emerged as a nosocomial pathogen causing morbidity and mortality in patients. For infection prevention it is important to track the spread of *K. pneumoniae* and its plasmids between patients. Therefore, the major aim was to recapitulate the 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 to 2019. Next-generation sequencing was combined with long-read third-generation sequencing to reconstruct 22 plasmids. wgMLST revealed five genetic clusters comprised of *K. pneumoniae* bla_{KPC-2} isolates and four clusters consisted of bla_{KPC-3} isolates. KpnCluster-019 bla_{KPC-2} isolates were found both in the Netherlands and the Caribbean islands, while bla_{KPC-3} cluster isolates only in the Netherlands. Each *K. pneumoniae* bla_{KPC-2} or bla_{KPC-3} cluster was characterized by a distinct resistome and plasmidome. However, the large and medium plasmids contained a variety of antibiotic resistance genes, conjugation machinery, cation transport systems, transposons, toxin/antitoxins, insertion sequences and prophage-related 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 transmission of a KpnCluster-019 bla_{KPC-2} strain between the Netherlands and the Caribbean.

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 bacteria⁵. 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 Tn4401, or variants hereof^{9–12}. Tn4401 consists of flanking imperfect repeat sequences, a Tn3 transposase gene, a Tn3 resolvase gene and the ISKpn6 and ISKpn7 insertion sequences¹⁰. The bla_{KPC-2} and bla_{KPC-3} carbapenemases are the most commonly identified variants that have spread globally and provide resistance to penicillins, carbapenems,

¹Center for Infectious Disease Control (CIb), National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. *A comprehensive list of consortium members appears at the end of the paper. ✉email: antoni.hendrickx@rivm.nl

<i>bla</i> _{KPC} allele	KpnCluster	MLST Sequence type	The Netherlands			Caribbean			Total
			S	I	R	S	I	R	
<i>bla</i> _{KPC-2}	KpnCluster-003	ST258			2				2
	KpnCluster-005	ST258			3				3
	KpnCluster-019	ST560	1	2		6	4		13
	KpnCluster-021	ST144					1	2	3
	KpnCluster-041	ST560				2			2
	Non-KpnCluster	variant	3	4	14	1	2	4	28
	Subtotal								51
<i>bla</i> _{KPC-3}	KpnCluster-008	ST512			4				4
	KpnCluster-025	ST307		1	6				7
	KpnCluster-038	ST11			2				2
	KpnCluster-050	ST13	1		1				2
	Non-KpnCluster	variant	3	5	8	1		1	18
	Subtotal								33
Total			8	12	40	10	7	7	84

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 mg/L), intermediate (I; ≥ 2 to 8 mg/L) and resistant (R; > 8 mg/L).

cephalosporins, cephamycins and monobactams^{13,14}. The KPC-2 and KPC-3 carbapenemases differ in only one amino acid as a histidine at position 272 is mutated to tyrosine (H272Y) in the KPC-3 variant¹⁵.

CPE isolates including *K. pneumoniae* are routinely sent to the National Institute for Public Health and the Environment (RIVM) and are typed by Illumina next-generation sequencing (NGS) in the Dutch National CPE Surveillance program to identify AMR genes and to determine possible transmission of strains¹⁶. NGS typically yields short sequence reads of 150 bases, thereby hampering the assembly of complete chromosomes and plasmids¹⁷. This is often due to large mobile genetic elements, such as insertion sequence elements, transposons, and other repetitive sequences e.g. tandem repeat regions of > 1500 bp in size. However, combining Illumina NGS sequencing with long-read third generation sequencing (TGS), which produces 1000 to 500,000 bases or longer sequence reads, can overcome this problem and enables the reconstruction of chromosomes and complete plasmids^{18,19}. Currently, the transmission of *K. pneumoniae* between persons in the Netherlands and the Caribbean and the impact hereof is not thoroughly understood. Five percent of the *K. pneumoniae* isolates collected in the Dutch National CPE Surveillance Program are retrieved from the Caribbean. It is also not clear whether plasmids of *K. pneumoniae* circulate endemically in the Netherlands or are introduced from the Caribbean. *bla*_{KPC}-type *K. pneumoniae* represent the third largest group (17.5%) of the *K. pneumoniae* isolates collected in the Dutch National CPE Surveillance Program after the *bla*_{OXA-48}-type (48.5%) and *bla*_{NDM-1}-type (24.3%) *K. pneumoniae*. While the prevalence of carbapenemase-producing *K. pneumoniae* and associated infections in the Netherlands is relatively low, the establishment of genomic surveillance of *K. pneumoniae* using TGS is of high importance^{20,21}. It provides for insights in the transmission of specific strains containing plasmids with AMR genes and/or virulence determinants. We therefore investigated the distribution of *K. pneumoniae* cluster isolates harboring *bla*_{KPC-2} or *bla*_{KPC-3} alleles obtained from the Dutch National CPE Surveillance Program and analyzed the contents of its plasmids using long-read third-generation sequencing.

Results

Distribution and genetic relationship of *bla*_{KPC-2} and *bla*_{KPC-3} carrying *K. pneumoniae*. A collection of 478 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 478 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 Kpn-Clusters) had unique classical MLST sequence types, of which ST144 (KpnCluster-021) and ST560 (KpnCluster-019) 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.

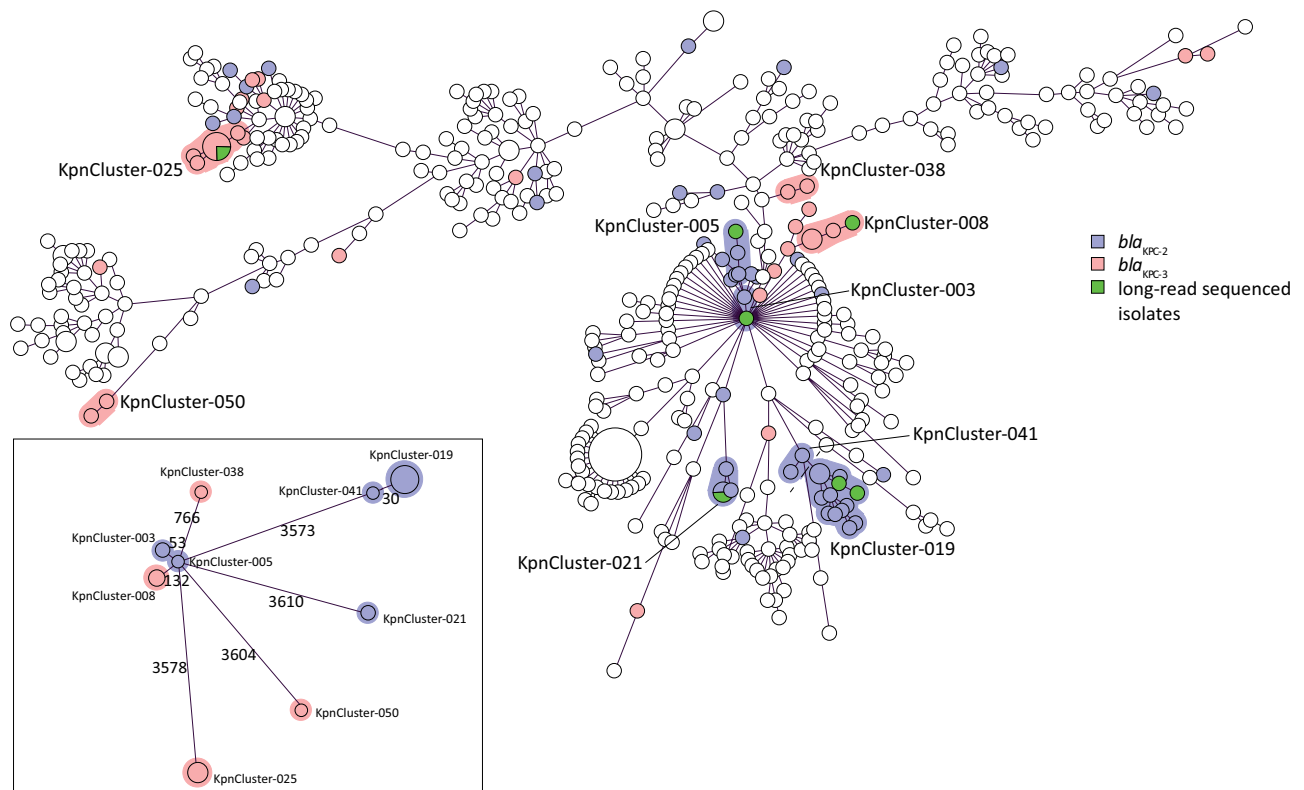


Figure 1. Minimum spanning tree based on wgMLST of 478 sequenced *K. pneumoniae* isolates. Circles represent *K. pneumoniae* isolates, and the sizes of the circles indicate the numbers of isolates. Lines connecting the circles represent the genetic distance in numbers of alleles; the longer the connecting line, the larger the genetic distance. *K. pneumoniae* bla_{KPC-2} isolates were marked blue and *K. pneumoniae* bla_{KPC-3} were marked magenta. *K. pneumoniae* bla_{KPC-2} or bla_{KPC-3} cluster isolates that were sequenced with TGS were marked green. Genetic clusters were indicated with either a blue or a magenta halo around the circles, if two or more isolates differ ≤ 20 alleles. A categorical coefficient was used for the clustering. Cluster names are indicated. Inset: genetic distance between the KpnClusters in which the allelic difference is indicated by numbers.

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). Based on wgMLST, the genetic distance between KpnCluster-019 and KpnCluster-041 was 30 alleles (0.6%) and for KpnCluster-003 and KpnCluster-005 53 alleles (1.1%). KpnCluster-008 differed 132 alleles (2.65%) from KpnCluster-005. While the allelic difference between these clusters was low, the other genetic clusters differed 3573 to 3610 alleles (71.8–72.5%) 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. Figure 1). All of the *K. pneumoniae* isolates contained the *fosA*, *oqxA* and *oqxB* genes. 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. Figure 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 (*blaCTX-M-15*, *blaSHV-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 (*blaSHV-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 iso-

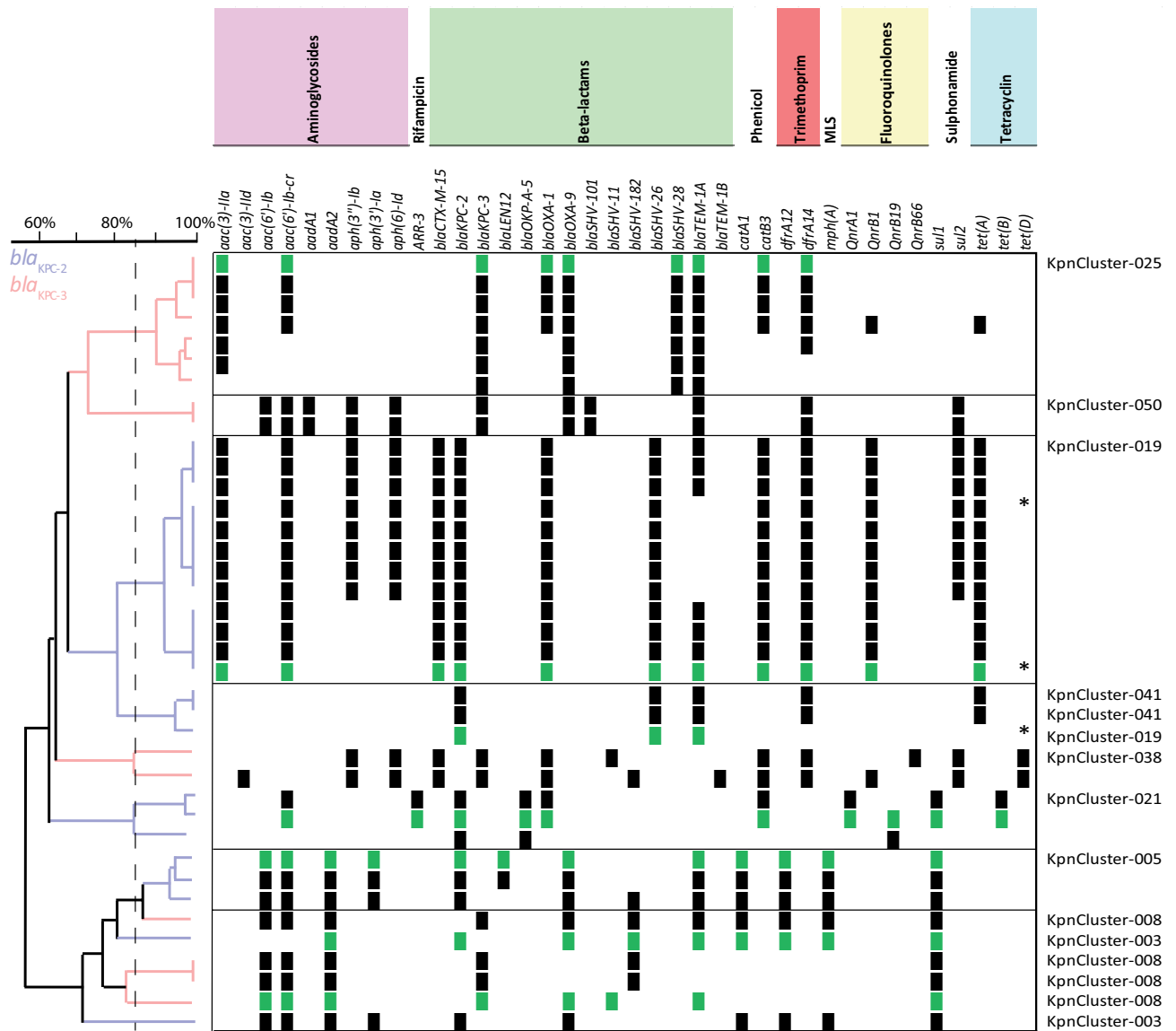


Figure 2. Resistome of *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3} cluster isolates. *K. pneumoniae* bla_{KPC-2} and bla_{KPC-3} cluster isolates were indicated on the y-axis and AMR genes on the x-axis. All isolates analysed contained the *fosA*, *oqxA* and *oqxB* AMR genes and were not included in this figure. The clustering was based on the presence (squares) and absence of AMR genes. Antibiotic classes are indicated above the AMR genes in different colors. Resistance genes in *K. pneumoniae* bla_{KPC-2} or bla_{KPC-3} cluster isolates that were sequenced with TGS were marked with green squares. Genetic relatedness was depicted in an UPGMA tree in which *K. pneumoniae* bla_{KPC-2} isolates were marked with blue branches, and *K. pneumoniae* bla_{KPC-3} were marked magenta. Dutch KpnCluster-019 isolates were marked with an *. A dotted line marks the 85% cut off.

lates (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 with ≥ 3 isolates per cluster, revealed 22 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. Sixteen of the 22 plasmids contained AMR genes from various classes and five 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

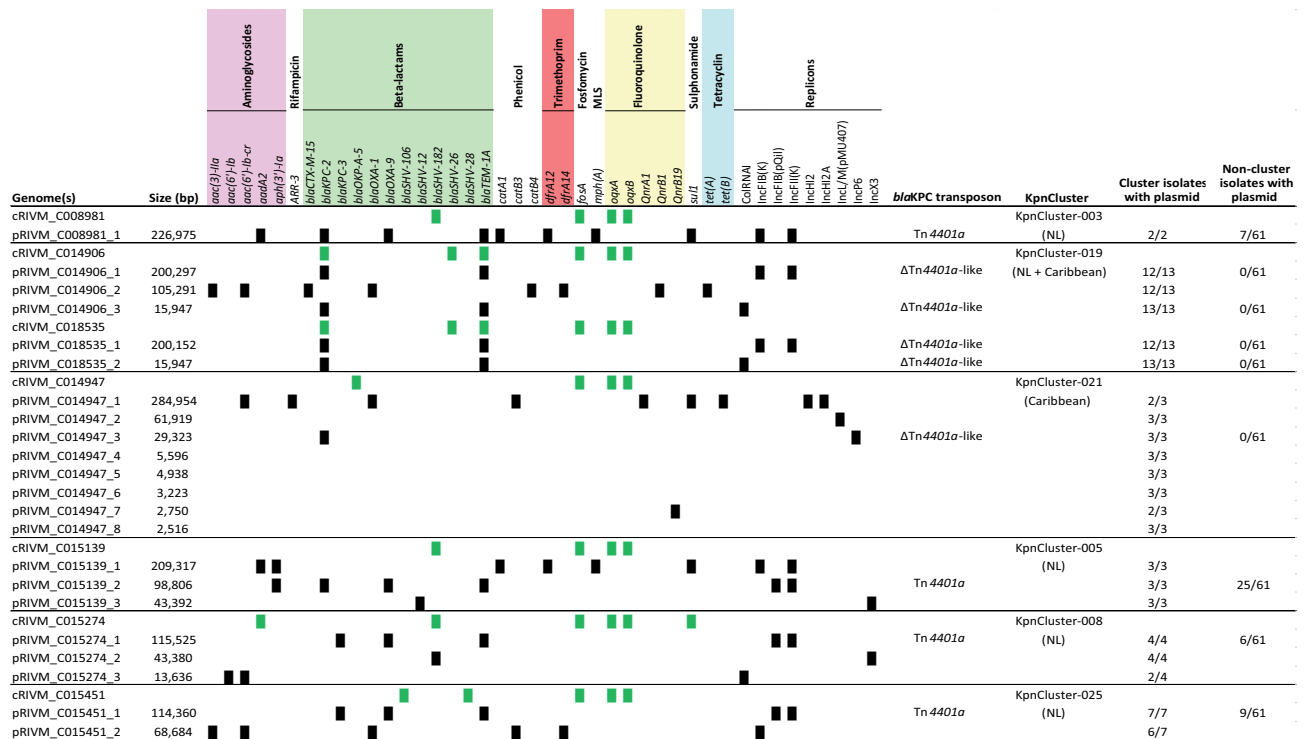


Figure 3. Antimicrobial resistance genes on chromosomes and plasmids. The presence of AMR genes among the 22 plasmids of seven TGS sequenced isolates is indicated with black squares and for the chromosomes using green squares. Chromosomes (cRIVM_C0xxxx) and plasmids (pRIVM_C0xxxx) are depicted on the Y-axis, and AMR genes on the x-axis. Antibiotic classes are indicated above the AMR genes in different colors.

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

The *bla*_{KPC-2} KpnCluster-019 isolates were obtained from both the Caribbean and the Netherlands, while *bla*_{KPC-2} KpnCluster-021 isolates originated only from the Caribbean (Table 1, Fig. 3). In the KpnCluster-019 isolate RIVM_C014906, three copies of the *bla*_{KPC-2} gene were present, while other cluster isolates had only one *bla*_{KPC} copy. One copy was located in the chromosome, one copy in the 200 kb plasmid pRIVM_C014906_1 and a third copy on the 16 kb plasmid pRIVM_C014906_3. All these three *bla*_{KPC-2} copies were located on a highly similar *Tn4401a*-derived Δ*Tn4401a*-like transposon of 5.6 kb in this strain. The chromosomes contained this Δ*Tn4401a*-like transposon in the exact same region. KpnCluster-003, KpnCluster-005, KpnCluster-008 and KpnCluster-025 consist of isolates that were obtained in the Netherlands and in these isolates the *bla*_{KPC} allele was located on a *Tn4401a* transposon of 10 kb.

Comparison of the *K. pneumoniae* plasmid content among clusters. An UPGMA clustering based on the DNA sequence of the 22 plasmids from distinct genetic clusters revealed that the majority of the plasmids were unrelated (Fig. 4). The largest two plasmids pRIVM_C008981_1 from KpnCluster-003 and pRIVM_C014947_1 from Kpncluster-021 carried the largest number of genes and this number decreased by the decreasing size of the plasmids. Most these plasmid located genes had unknown function. The large and medium sized plasmids contained the *klcA* gene, encoding an antirestriction protein implicated in the facilitation of *bla*_{KPC} allele transfer²². None of the plasmids contained known virulence determinants such as *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 from KpnCluster-021 was not related to any other of the large plasmids. Despite the low similarity, these large plasmids from KpnClusters-003, -005, and -019, 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* anti-toxins, except pRIVM_C014947_1 from KpnCluster-021. In addition, the large plasmids also contained a proportion of plasmid-specific and thus *K. pneumoniae* cluster specific gene content (Suppl. Figure 2).

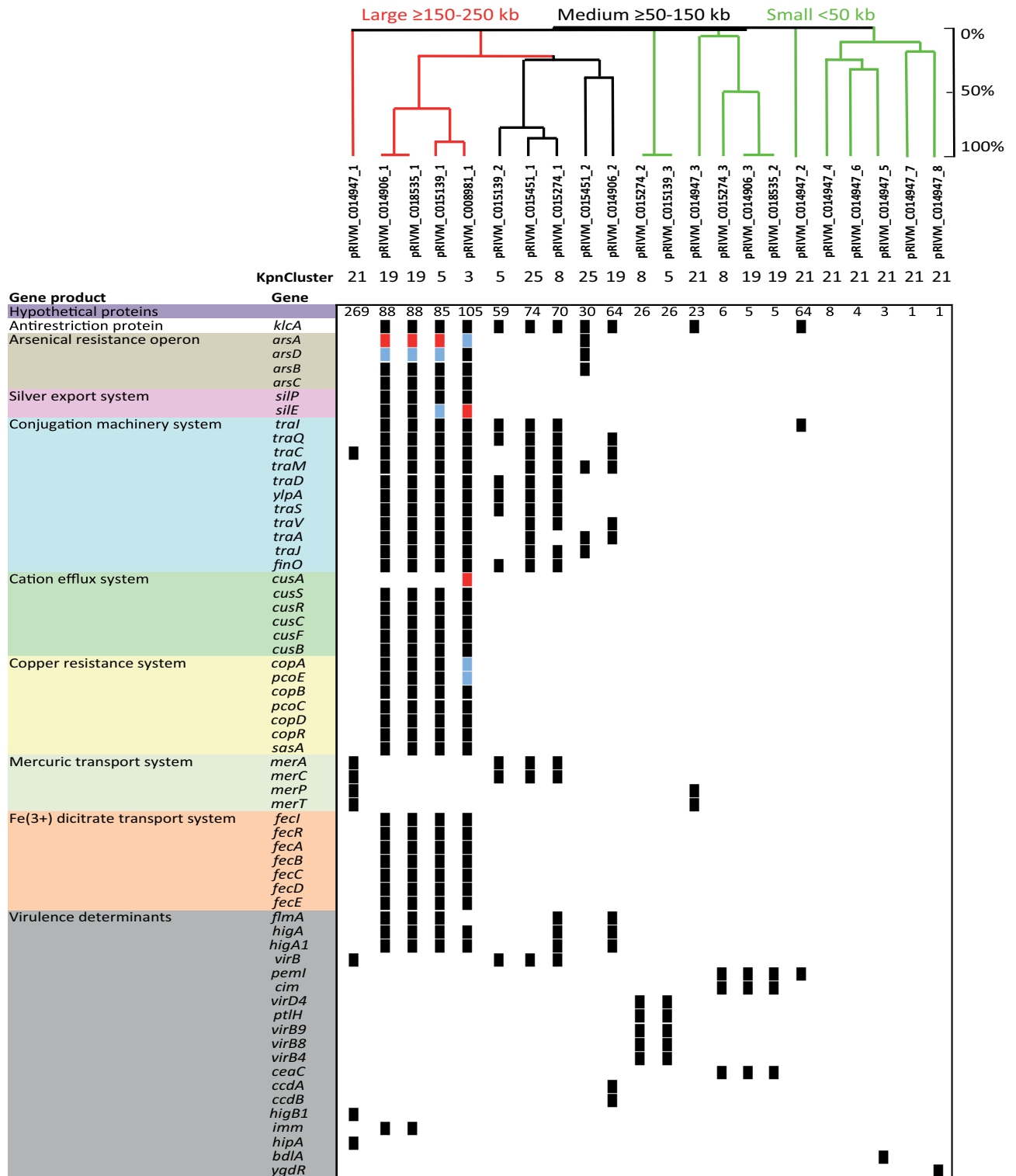


Figure 4. *K. pneumoniae* plasmid gene content. An UPGMA clustering was performed based on the plasmid DNA sequence for the determination of the genetic relation among the 22 plasmids. Similarity is indicated on the y-axis using a scale from 0 (not similar) to 100% (identical). A similarity of ≥ 85 to 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 22 plasmids of seven TGS sequenced isolates. If a gene was present twice, blue squares were used and more than 2, red squares were used. Colors indicated different groups of genes with a specific function. In the UPGMA tree, large plasmids are indicated in red, medium plasmids in black and small plasmids in green color.

Three medium-sized plasmids contained the *virB* virulence regulon transcriptional activator and the *merAC* mercuric reductase and transport protein. While pRIVM_C015274_1 from KpnCluster-008 and pRIVM_C015451_1 from KpnCluster-025 contained a plasmid conjugation gene cluster, plasmids pRIVM_C014906_2, pRIVM_C015139_2, and pRIVM_C015451_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_3 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 from distinct clusters. The large and medium sized plasmids contained the most transposase sequences, and each plasmid had its unique transposon signature (Fig. 5). The IS1, IS110 and IS3 transposase families dominated in the *K. pneumoniae* plasmids among genetic clusters. 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 *Tn4401a* transposon, except in pRIVM_C014906_1 and pRIVM_C01835_1 from KpnCluster-019. In the small plasmids carrying a *bla*_{KPC}, the carbapenemase allele was located on a Δ *Tn4401a*-like transposon. The large plasmids pRIVM_C008981_1, pRIVM_C015139_1 and pRIVM_C014906_1 from KpnClusters-003, -005, and -019 harbored 37, 32 and 31 annotated transposases, respectively. In contrast, the largest plasmid pRIVM_C014947_1 of 285 kb from KpnCluster-021 contained only 16 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) from KpnCluster-005 and KpnCluster-008 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 15 of the 22 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 of *K. pneumoniae* 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 the eight plasmids 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. The fact that 15 of the 22 plasmids analyzed in this study were found previously in distinct hosts, suggest international spread of these plasmids.

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 1). The most commonly found prophage sequence in *Klebsiella* chromosomes was the *Enterobacteria* phage P4.

Discussion

We showed that a *K. pneumoniae* ST560 strain carrying *bla*_{KPC-2} from KpnCluster-019 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 22 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. Some of the cluster isolates had unique MLST sequence types (ST144 and ST560) which were not published previously and differ from globally

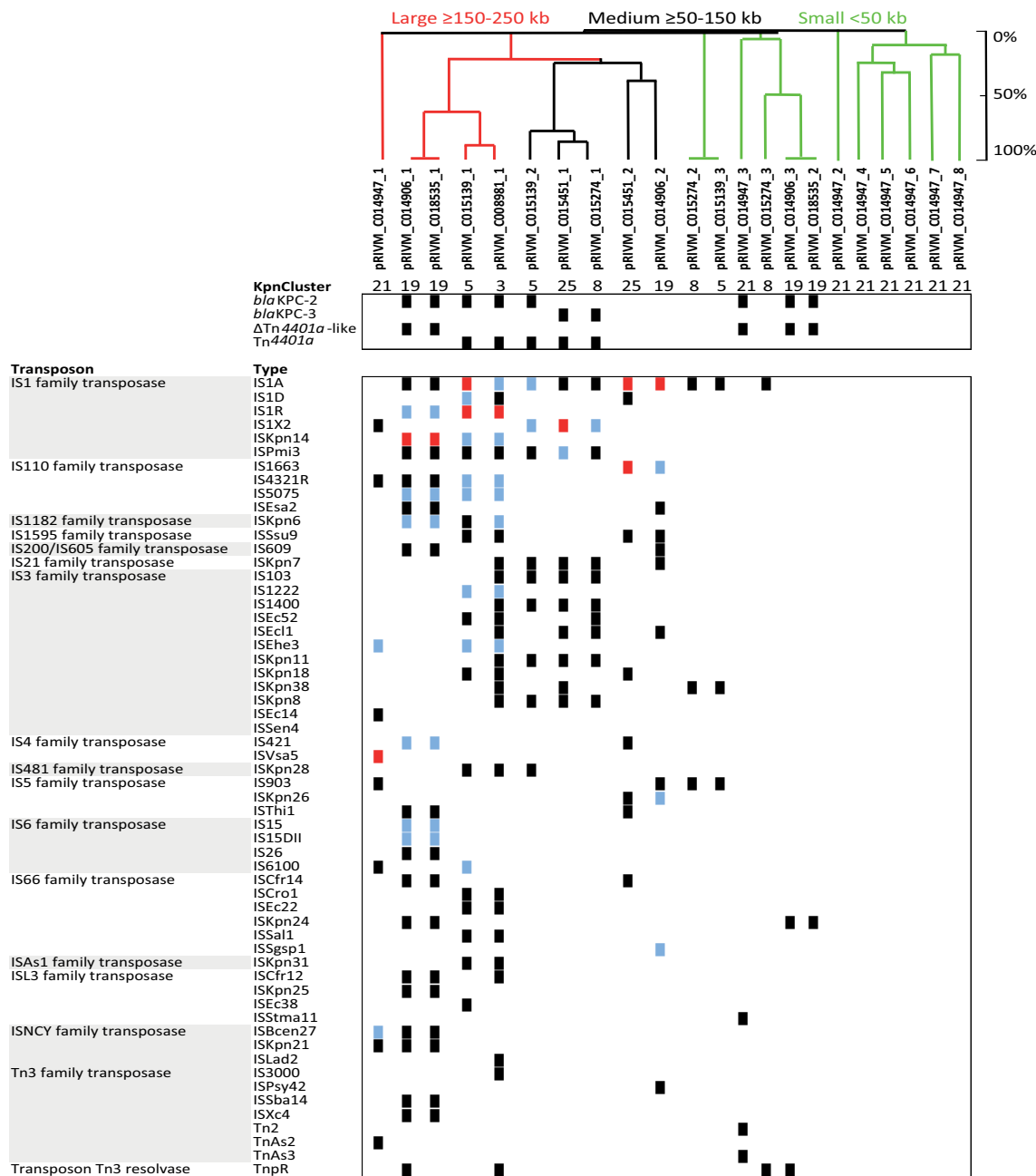


Figure 5. *K. pneumoniae* plasmid-localized transposases. The presence (black squares) and absence is indicated of annotated transposases among the 22 plasmids of six TGS sequenced isolates. The plasmids are indicated on the x-axis. If a transposon was present twice, blue squares were used and more than 2, red squares were used. The light grey area indicates specific transposons found in only one plasmid. In the UPGMA tree, large plasmids are indicated in red, medium plasmids in black and small plasmids in green color.

circulating extensively drug-resistant (XDR) *K. pneumoniae* ST258 and ST307 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 Tn4401a-like transposon, while the other isolates from the other genetic clusters from the Netherlands contained *bla*_{KPC} on a 10 kb Tn4401a transposon. Most global descriptions of *K. pneumoniae* *bla*_{KPC} the past decade have been associated with Tn4401a or isoforms hereof⁹. The traditional association of *bla*_{KPC} with the Tn4401a transposon has possibly been eroded in *K. pneumoniae* isolates from the Caribbean

Plasmid	KpnCluster	<i>bla</i> _{KPC} allele	Bacterial species	Plasmid	Query coverage (%)	Identity (%)	Accession number	Country	Year	Reference
pRIVM_C008981_1	KpnCluster-003	<i>bla</i> _{KPC-2}	<i>K. pneumoniae</i>	pGMI16-005_01	35	99.96	CP028181.1	Denmark	2013	
pRIVM_C014906_1	KpnCluster-019	<i>bla</i> _{KPC-2}	<i>K. pneumoniae</i>	pKPN1482-1	63	99.91	CP020842.1	USA	2014	Long et al. (2017) ⁴²
pRIVM_C014906_2			<i>K. quasipneumoniae</i>	plasmid pG747	97	99.84	CP034137.1	Nigeria	2013	
pRIVM_C014906_3		<i>bla</i> _{KPC-2}	<i>K. pneumoniae</i>	unnamed5	58	99.97	CP033630.1	Italy	2013	Roe et al. (2019) ⁴³
pRIVM_C018535_1		<i>bla</i> _{KPC-2}	<i>K. pneumoniae</i>	pKPN1482-1	63	99.91	CP020842.1	USA	2014	Long et al. (2017) ⁴²
pRIVM_C018535_2		<i>bla</i> _{KPC-2}	<i>K. pneumoniae</i>	unnamed5	58	99.97	CP033630.1	Italy	2013	Roe et al. (2019) ⁴³
pRIVM_C014947_1	KpnCluster-021		<i>S. enterica</i>	pSJO-60984	93	99.99	CP025277.1	USA	2007	
pRIVM_C014947_2			<i>K. pneumoniae</i>	unnamed3	90	99.98	CP032170.1	USA	2015	
pRIVM_C014947_3		<i>bla</i> _{KPC-2}	<i>K. pneumoniae</i>	pA1705-KPC	93	99.97	MH909348.1	China	2013	
pRIVM_C014947_4			<i>K. pneumoniae</i>	pKP18-2079_5kb	100	100	MT090963.1	China	2018	
pRIVM_C014947_5			<i>E. hormaechei</i>	pC4_003	100	99.97	CP042543.1	Australia	2007	
pRIVM_C014947_6			<i>K. pneumoniae</i>	pD17KP0032-3	100	100	CP052331.1	S. Korea	2017	
pRIVM_C014947_7			<i>E. hormaechei</i>	unnamed3	97	100	CP035388.1	UK	2016	
pRIVM_C014947_8			<i>E. hormaechei</i>	pC45-004	100	99.69	CP042555.1	Australia	2013	
pRIVM_C015139_1	KpnCluster-005		<i>K. pneumoniae</i>	plasmid 2	100	99.99	LR130549.1	Australia	2018	
pRIVM_C015139_2		<i>bla</i> _{KPC-2}	<i>K. pneumoniae</i>	pUJ-83KPC	99	98.55	MG700549.1	Germany	2017	
pRIVM_C015139_3			<i>K. pneumoniae</i>	pBK13043-2	100	99.89	CP020839.1	USA	2004	Long et al. (2017) ⁴²
pRIVM_C015274_1	KpnCluster-008	<i>bla</i> _{KPC-3}	<i>K. pneumoniae</i>	plasmid p2	87	99.91	CP019774.1	Switzerland	2015	Ruppe et al. (2017) ⁴⁴
pRIVM_C015274_2			<i>K. pneumoniae</i>	pBK13043-2	100	99.95	CP020839.1	USA	2004	Long et al. (2017) ⁴²
pRIVM_C015274_3			<i>K. pneumoniae</i>	ColEST258	100	100	JN247853.1	Italy	2012	Garcia-Fernandez et al. (2012) ⁴⁵
pRIVM_C015451_1	KpnCluster-025	<i>bla</i> _{KPC-3}	<i>K. pneumoniae</i>	pKPC	99	99.99	CP043971.1	France	2019	
pRIVM_C015451_2			<i>K. pneumoniae</i>	p911021-tetA	66	99.55	MG288679.1	China	2019	

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

to a smaller variant. This is the first report of identification of a 5.6 kb $\Delta Tn4401a$ -like *bla*_{KPC-2} transposon of *K. pneumoniae* in the Netherlands. Preliminary surveillance data analysis revealed that the $\Delta Tn4401a$ -like element carrying *bla*_{KPC-2} and smaller variants disseminated among *E. cloacae*, *Serratia marcescens*, *K. oxytoca* and *E. coli* in the Netherlands (unpublished data). Future work will seek to understand the dissemination of the $\Delta Tn4401a$ -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 study²¹. 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 cephamycin²⁷. The increase in meropenem resistance observed in our study is possibly correlated with improved ability of KPC-3 enzymes to hydrolyze the meropenem antibiotic¹⁵. Alternatively, additional beta-lactamase genes such as *bla*_{OXA-1}, *bla*_{OXA-9} or *bla*_{TEM-1A} may contribute to increased resistance for meropenem²⁸.

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

Plasmid(s)	<i>bla</i> _{KPC} allele	Phage region(s) length (kb)	Most common phage	Accession number
pRIVM_C008981_1	<i>bla</i> _{KPC-2}	11.3	Escherichia phage RCS47	NC_042128
pRIVM_C014906_1	<i>bla</i> _{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	<i>bla</i> _{KPC-2}			
pRIVM_C018535_1	<i>bla</i> _{KPC-2}	7, 3.2, 23.3, 5.1, 5.7	Stx2-converting phage 1717	NC_011357
pRIVM_C018535_2	<i>bla</i> _{KPC-2}			
pRIVM_C014947_1		10.9	Bacillus phage Shanette	NC_028983
pRIVM_C014947_2				
pRIVM_C014947_3	<i>bla</i> _{KPC-2}			
pRIVM_C014947_4				
pRIVM_C014947_5				
pRIVM_C014947_6				
pRIVM_C014947_7				
pRIVM_C014947_8				
pRIVM_C015139_1		23.5, 11.3	Stx2-converting phage 1717, Escherichia phage RCS47	NC_011357, NC_042128
pRIVM_C015139_2	<i>bla</i> _{KPC-2}	30.8	Escherichia phage RCS47	NC_042128
pRIVM_C015139_3		24.8	Escherichia phage RCS47	NC_042128
pRIVM_C015274_1	<i>bla</i> _{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	<i>bla</i> _{KPC-3}	39.5	Escherichia phage RCS47	NC_042128
pRIVM_C015451_2		20.6	Staphylococcus phage SPbeta-like	NC_029119

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

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 systems, cation efflux systems including Fe(3+)-dicitrate transport, and genes encoding for silver, copper and arsenic resistance. The small plasmids contained putative virulence determinants. The presence of these systems may contribute to the success of transmission of specific *K. pneumoniae* strains in the hospital setting or the community^{13,29,30}. Escherichia RCS47 prophage sequences were found on medium and large plasmids in the cluster isolates analyzed. In contrast, the chromosomes contained a variety of prophage-related sequences. RCS47 is a P1-like bacteriophage carrying the ESBL-encoding *bla*_{SHV-2} gene was isolated from a clinical *E. coli* strain³¹. The prevalence of P1-like prophages in animal and human *E. coli* strain collections was 12.6%³¹. The presence of P1-like phage sequences in plasmids of a snapshot of the *K. pneumoniae* population in the Netherlands and the Caribbean suggest that the role of P1-like phages in disseminating antibiotic resistance may be underestimated³².

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

Methods

Bacterial isolates. For the Dutch National carbapenemase-producing Enterobacterales (CPE) Surveillance program, medical microbiology laboratories from the Netherlands and the Caribbean routinely send CPE isolates with a meropenem minimum inhibitory concentration (MIC) of ≥ 0.25 $\mu\text{g/ml}$ and/or an imipenem MIC of ≥ 1 $\mu\text{g/ml}$ 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 person 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 mg/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 previously³³.

Next-generation sequencing, MLST and wgMLST. All 84 *K. pneumoniae* isolates were subjected to next-generation sequencing (NGS) using the Illumina HiSeq 2500 (BaseClear, Leiden, the Netherlands). The NGS data of the *K. pneumoniae* isolates were used for classical MLST and wgMLST analyses using the in-house wgMLST scheme in SeqSphere software version 6.0.2 (Ridom GmbH, Münster, Germany). The in-house *K. pneumoniae* wgMLST scheme was comprised of 4978 genes (3471 core-genome and 1507 accessory-genome targets) using *K. pneumoniae* MGH 78,578 (NC_009648.1) as a reference genome²¹. For classical MLST, the existing scheme was used and cluster nomenclature were depicted in Table 1³⁴. The resulting data was imported into Bionumerics version 7.6.3 for subsequent comparative analyses (Applied Maths, Sint-Martens-Latem, Belgium). The antibiotic resistance gene profile and plasmid replicon compositions in all of the isolates were determined by interrogating the online ResFinder (version 3.1.0) and PlasmidFinder (version 2.0.2) databases available at the Center for Genomic Epidemiology website (<https://cge.cbs.dtu.dk/services/>)^{35,36}. For ResFinder, a 90% identity threshold and a minimum length of 60% were used as criteria, whereas for PlasmidFinder, an identity of 95% was utilized.

Long-read third-generation sequencing. One *K. pneumoniae* isolate per genetic KpnCluster was sequenced using long-read third-generation Nanopore sequencing^{18,37}. High molecular weight DNA was isolated using an in-house developed protocol. Bacteria were grown overnight in 1.5 ml Brain heart infusion broth and culture was spun down at $13,000 \times g$ for 2 min. The pellet was washed and resuspended in 500 μ l of 150 mM NaCl. The suspension was spun down at $5000 \times g$ for 5 min and the pellet was resuspended in 100 μ l of Quick-Extract DNA Extraction Solution (Lucigen) and 0.1 μ l Ready-Lyse Lysozyme solution (Epicentre) and incubated for 1 h at 37 °C. Subsequently, 85 μ l 10 mM Tris 1 mM EDTA pH=8 (1 \times TE), 10 μ l proteinase K (>600 mAU/mL, Qiagen) and 5 μ l 20% sodium dodecyl sulfate solution were added, and the mixture was incubated at 56 °C for 30 min. DNA was precipitated overnight at -20 °C by adding 0.1 \times volume 3 M sodium acetate pH=5.2 and 2.5 \times volume ice cold 100% ethanol. Precipitated DNA was spun down at $13,000 \times g$ for 15 min and pellets were washed with 1 ml 70% ethanol followed by another centrifugation at $13,000 \times g$ for 5 min. After drying, the pellet was dissolved in 200 μ l 1 \times TE and diluted to 1 μ g with Nuclease-free water.

The Oxford Nanopore protocol SQK-LSK108 (<https://community.nanoporetech.com>) and the expansion kit for native barcoding EXP-NBD104 was used. Briefly, a shearing step was performed using g-TUBE's (Covaris) to obtain an average DNA fragment size of 8 kb. The DNA was repaired using FFPE and end-repair kits (New England Biolabs) followed by ligation of barcodes with bead clean up using AMPure XP (Beckman Coulter) after each step. Barcoded isolates were pooled and sequencing adapters were added by ligation. The final library was loaded onto a MinION flow cell (MIN-106 R9.4.1). The 48-h sequence run was started without live base calling enabled on a MinION device connected to a desktop computer. After the sequence run, base calling and de-multiplexing was performed using Albacore 2.3.1 and a single FASTA file per isolate was extracted from the FAST5 files using Poretools 0.5.1³⁸. Illumina and Nanopore data were used in a hybrid assembly performed by Unicycler v0.4.4³⁹. The resulting contig files were annotated using Prokka and were subsequently loaded into BioNumerics for further analyses⁴⁰.

Minimum spanning tree and UPGMA analyses. The BioNumerics software was used to generate a minimum spanning tree (MST) or an UPGMA hierarchical clustering as described previously¹⁶. The MST was based on an in-house *K. pneumoniae* wgMLST scheme. The categorical coefficient was used to calculate the MST. wgMLST clusters were defined as a minimum of two isolates of which the genetic distance between the two isolates was ≤ 20 genes ($20/4978 \leq 0.4\%$ different). An UPGMA clustering of *K. pneumoniae* *bla*_{KPC-2} and *bla*_{KPC-3} isolates was performed based on the presence and/or absence of antibiotic resistance genes per isolate.

Plasmid reconstruction by read mapping. The CLC Genomics Workbench version 12.0 software (www.qiagenbioinformatics.com) was used to reconstruct plasmids. For this, complete plasmids obtained by TGS were used as a scaffold to map the trimmed NGS reads of isolates that were from the same genetic wgMLST cluster. A plasmid was scored "present" in an isolate if reads mapped to a reference plasmid of interest and $\geq 85\%$ of the consensus sequence size in kilo bases was reconstructed. Linear DNA fragments < 5 kb were omitted in this study. Nucleotide BLAST analyses on plasmid sequences were performed using the <https://blast.ncbi.nlm.nih.gov> website and date from October 2019.

Plasmid content analysis. Bionumerics was used to extract and analyze annotated genes and transposases in the 22 different plasmids. The data was plotted in Excel. Phaster, the PHAge Search Tool Enhanced Release website (<https://phaster.ca/>) was used to determine the presence of phage sequences in the plasmids and searches date from October 2019⁴¹.

Ethics statement. The bacterial isolates used in this study belong to the medical microbiological laboratories participating in the Dutch National CPE Surveillance program and was obtained as part of routine clinical care in the past. Since no identifiable personal data were collected and data were analyzed anonymously, written or verbal patient consent was not required for this study and was therefore not obtained. According to the Dutch

Medical Research Involving Human Subjects Act (WMO) this study was considered exempt from review by an Institutional Review Board.

Data availability

The Illumina (NGS) and plasmid sequence data sets generated and analyzed in this study are freely available in the Sequence Read Archive (SRA) under BioProject ID PRJNA634885 and in Genbank under accession numbers as depicted in Supplementary Table 2.

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Author contributions

A.P.A.H. and L.M.S. were involved in the experimental design, coordinated the whole work, summarized the data, prepared the figures and wrote the manuscript. FL, ADH and MGSV performed laboratory experiments. F.L., D.B., S.W., M.G.S.V., and H.V.D.H. processed sequencing data and curated databases. D.B. and H.V.D.H. were involved in bioinformatic analyses. Members of the Dutch CPE surveillance Study Group provided bacterial isolates used in this study. All authors and contributors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to A.P.A.H.

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The Dutch CPE surveillance Study Group

T. Halaby², R. Steingrover³, J. W. T. Cohen Stuart⁴, D. C. Melles⁵, K. van Dijk⁶, I. J. B. Spijkerman⁷, D. W. Notermans⁸, J. H. Oudbier⁹, M. L. van Ogtrop¹⁰, A. van Dam¹¹, M. den Reijer¹², J. A. J. W. Kluytmans¹³, M. P. M. van der Linden¹⁴, E. E. Mattsson¹⁵, M. van der Vusse¹⁶, E. de Jong¹⁷, A. Maijer-Reuwer¹⁸, M. van Trijp¹⁹, A. J. van Griethuysen²⁰, A. Ott²¹, E. Bathoorn²², J. C. Sinnige²³, E. Heikens²⁴, E. I. G. B. de Brauwert²⁵, F. S. Stals²⁵, W. Silvis²⁶, J. W. Dorigo-Zetsma²⁷, K. Waar²⁸, S. P. van Mens²⁹, N. Roescher³⁰, A. Voss³¹, H. Wertheim³², B. C. G. C. Slingerland³³, H. M. E. Frenay³³, T. Schulin³⁴, B. M. W. Diederren^{35,36}, L. Bode³⁷, M. van Rijn³⁸, S. Dinant³⁹, M. Damen³⁹, P. de Man⁴⁰, M. A. Leversteijn-van Hall⁴¹, E. P. M. van Elzakker⁴², A. E. Muller⁴³, P. Schneeberger⁴⁴, D. W. van Dam⁴⁵, A. G. M. Buiting⁴⁶, A. L. M. Vlek⁴⁷, A. Stam⁴⁸, A. Troelstra⁴⁹, I. T. M. A. Overdeest⁵⁰, R. W. Bosboom⁵¹, T. A. M. Trienekens⁵², M. J. H. M. Wolfhagen⁵³ & S. Paltansing⁵⁴

²Analytical Diagnostic Center N.V. Curaçao, Willemstad, The Netherlands. ³St. Maarten Laboratory Services, Cay Hill, The Netherlands. ⁴Department of Medical Microbiology, Noordwest Ziekenhuisgroep, Alkmaar, The Netherlands. ⁵Department of Medical Microbiology, Meander Medical Center, Amersfoort, The Netherlands. ⁶Department of Medical Microbiology and Infection Control, Amsterdam UMC, Amsterdam, The Netherlands. ⁷Department of Medical Microbiology, Academic Medical Center, Amsterdam UMC, Amsterdam, The Netherlands. ⁸Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, The Netherlands. ⁹Comicro, Hoorn, The Netherlands. ¹⁰Department of Medical Microbiology, Onze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands. ¹¹Public Health Service, Public Health Laboratory, Amsterdam, The Netherlands. ¹²Department of Medical Microbiology and Infection Prevention, Gelre Hospitals, Apeldoorn, The Netherlands. ¹³Microvida Laboratory for Microbiology, Department of Infection Control, Amphia Hospital, Breda, The Netherlands. ¹⁴Department of Medical Microbiology, IJsselland Hospital, Capelle a/d IJssel, The Netherlands. ¹⁵Department of Medical Microbiology, Reinier de Graaf Groep, Delft, The Netherlands. ¹⁶Department of Medical Microbiology, Deventer Hospital, Deventer, The Netherlands. ¹⁷Department of Medical Microbiology, Slingeland Hospital, Doetinchem, The Netherlands. ¹⁸Department of Medical Microbiology, ADRZ Medisch Centrum, Goes, The Netherlands. ¹⁹Department of Medical Microbiology and Infection Prevention, Groene Hart Hospital, Gouda, The Netherlands. ²⁰Department of Medical Microbiology, Gelderse Vallei Hospital, Ede, The Netherlands. ²¹Department of Medical Microbiology, Certe, Groningen, The Netherlands. ²²Department of Medical Microbiology, University of Groningen, University Medical Center, Groningen, The Netherlands. ²³Regional Laboratory of Public Health, Haarlem, The Netherlands. ²⁴Department of Medical Microbiology, St Jansdal Hospital, Harderwijk, The Netherlands. ²⁵Department of Medical Microbiology and Infection Control, Zuyderland Medical Centre, Heerlen, The Netherlands. ²⁶LabMicTA, Regional Laboratory of Microbiology Twente Achterhoek, Hengelo, The Netherlands. ²⁷Department of Medical Microbiology, CBSL, Tergooi Hospital, Hilversum, The Netherlands. ²⁸Izore Centre for Infectious Diseases Friesland, Leeuwarden, The Netherlands. ²⁹Department of Medical Microbiology, Maastricht University Medical Centre, Maastricht, The Netherlands. ³⁰Department of Medical Microbiology and Immunology, St Antonius Hospital, Nieuwegein, The Netherlands. ³¹Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands. ³²Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands. ³³Regional Laboratory Medical Microbiology (RLM), Dordrecht, The Netherlands. ³⁴Department of Medical Microbiology, Laurentius Hospital, Roermond, The Netherlands. ³⁵Department of Medical Microbiology, Bravis Hospital, Roosendaal, The Netherlands. ³⁶Department of Medical Microbiology, ZorgSaam Hospital Zeeuws-Vlaanderen, Terneuzen, The Netherlands. ³⁷Department of Medical Microbiology, Erasmus University Medical Center, Rotterdam, The Netherlands. ³⁸Department of Medical Microbiology, Ikazia Hospital, Rotterdam, The Netherlands. ³⁹Department of Medical Microbiology, Maasstad Hospital, Rotterdam, The Netherlands. ⁴⁰Department of Medical Microbiology, Sint Franciscus Gasthuis, Rotterdam, The Netherlands. ⁴¹Department of Medical Microbiology, Alrijne Hospital, Leiden, The Netherlands. ⁴²Department of Medical Microbiology, Haga Hospital, 's-Gravenhage, The Netherlands. ⁴³Department of Medical Microbiology, HMC Westeinde Hospital, 's-Gravenhage, The Netherlands. ⁴⁴Department of Medical Microbiology and Infection Control, Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands. ⁴⁵Department of Medical Microbiology and Infection Control, Zuyderland Medical Centre, Sittard-Geleen, The Netherlands. ⁴⁶Department of Medical Microbiology and Immunology, Elisabeth-TweeSteden (ETZ) Hospital, Tilburg, The Netherlands. ⁴⁷Department of Medical Microbiology and Immunology, Diaconessenhuis, Utrecht, The Netherlands. ⁴⁸Department of Medical Microbiology, Saltro Diagnostic Centre, Utrecht, The Netherlands. ⁴⁹Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands. ⁵⁰Department of Medical Microbiology, PAMM, Veldhoven, The Netherlands. ⁵¹Laboratory for Medical Microbiology and Immunology, Rijnstate Hospital, Velp, The Netherlands. ⁵²Department of Medical Microbiology, VieCuri Medical Center, Venlo, The Netherlands. ⁵³Laboratory of Medical Microbiology and Infectious Diseases, Isala Hospital, Zwolle, The Netherlands. ⁵⁴Franciscus Gasthuis & Vlietland, Microbiology and Infection Prevention, Schiedam, The Netherlands.