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

Antoni P. A. Hendrickx1,2*, Fabian Landman1, Angela de Haan1, Dyogo Borst1, Sandra Witteveen1, Marga G. van Santen-Verheuvel1, 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*. Currently, carbapenemase-producing *Enterobacterales* (CPE) rank among the most problematic nosocomial pathogens with limited outlook on novel effective therapeutics2. 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 bacteria3. Most newly acquired AMR genes of *K. pneumoniae* are the result of horizontal gene transfer through conjugative plasmids4-8. 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 hereof9-12. 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 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
plasmids18,19. Currently, the transmission of longer sequence reads, can overcome this problem and enables the reconstruction of chromosomes and complete NGS sequencing with long-read third generation sequencing (TGS), which produces 1000 to 500,000 bases or carried the *bla* five distinct genetic clusters. Fifteen *K. pneumoniae* *bla* in the Dutch National CPE Surveillance Program are retrieved from the Caribbean. It is also not clear whether *bla* bean and the impact hereof is not thoroughly understood. Five percent of the 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 plasmids16,19. 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*OXA-23-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 importance20,21. 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.

### 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).

| *bla*KPC allele | KpnCluster | MLST | The Netherlands | Caribbean | Total |
|-----------------|------------|------|-----------------|-----------|-------|
|                 |            |      | S I R           | S I R     |       |
| *bla*KPC-2      | KpnCluster-003 | ST258 | 2               | 0         | 2     |
|                 | KpnCluster-005 | ST258 | 3               | 0         | 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    |
|                 | KpnCluster-008 | ST512 | 4               | 0         | 4     |
|                 | KpnCluster-025 | ST307 | 1 6             | 0         | 7     |
|                 | KpnCluster-038 | ST11  | 2               | 0         | 2     |
|                 | KpnCluster-050 | ST13  | 1 1             | 2         |       |
| Non-KpnCluster  | variant     | 3 5   | 8 1           | 1 18      | 33    |
| Subtotal        |            |       |                 |           | 33    |
| Total           |            | 8     | 12 40          | 10 7      | 84    |

Cephalosporins, cephamycins and monobactams13,14. 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.
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 Kpn-Cluster-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\textsubscript{KPC-2} or the bla\textsubscript{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\textsubscript{KPC-2} and KpnCluster-008 bla\textsubscript{KPC-3} cluster isolates with 53 to 132 alleles difference were also unrelated. KpnCluster-019 isolates are unique when compared to the bla\textsubscript{KPC-2} clusters KpnCluster-003, KpnCluster-005, and KpnCluster-021, in that they carried aminoglycoside (aac(3)-IIa), extended spectrum beta-lactams (bla\textsubscript{CTX-M-15}, bla\textsubscript{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\textsubscript{KPC-3} isolates contained the aminoglycoside (aac(3)-Iia) and beta-lactam AMR genes (bla\textsubscript{SHV-28}), while the other Kpn bla\textsubscript{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\textsubscript{KPC-2} and bla\textsubscript{KPC-3} isolates were resistant to meropenem (47/84; 56%). More specifically, seven of the 23 K. pneumoniae bla\textsubscript{KPC-2} cluster iso-

![Minimum spanning tree based on wgMLST of 478 sequenced K. pneumoniae isolates.](https://images.nature.com/nature/478/468/nn51778_F1a.jpg)
Figure 2. Resistome of *K. pneumoniae* bla\textsubscript{KPC-3} and bla\textsubscript{KPC-1} cluster isolates. *K. pneumoniae* bla\textsubscript{KPC-2} and bla\textsubscript{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\textsubscript{KPC-2} or bla\textsubscript{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\textsubscript{KPC-3} isolates were marked with blue branches, and *K. pneumoniae* bla\textsubscript{KPC-3} were marked magenta. Dutch KpnCluster-019 isolates were marked with an *. A dashed line marks the 85% cut off.

lates (30%) and 13 of the 15 bla\textsubscript{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\textsubscript{KPC} clusters with ≥3 isolates per cluster, revealed 22 plasmids with varying sizes (Fig. 3). Plasmids containing either the bla\textsubscript{KPC-2} or bla\textsubscript{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 IncFI1(K), IncHI2 and IncHI2a, or IncFIB(pQfi) (Fig. 3). The small plasmids (<50 kb) contained ColIa or IncX3/IncIa/IncPα 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
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 \(\text{bla}_{\text{KPC}}\)2 KpnCluster-019 isolates were obtained from both the Caribbean and the Netherlands, while \(\text{bla}_{\text{KPC}}\)3 KpnCluster-021 isolates originated only from the Caribbean (Table 1, Fig. 3). In the KpnCluster-019 isolate RIVM\_C014906, three copies of the \(\text{bla}_{\text{KPC}}\)2 gene were present, while other cluster isolates had only one \(\text{bla}_{\text{KPC}}\)2 copy. One copy was located in the chromosome, one copy in the 200 kb plasmid PIVM\_C014906\_1 and a third copy on the 16 kb plasmid PIVM\_C014906\_3. All these three \(\text{bla}_{\text{KPC}}\)2 copies were located on a highly similar \(\text{Tn}4401\_a\)-derived \(\Delta\text{Tn}4401\_a\)-like transposon of 5.6 kb in this strain. The chromosomes contained this \(\Delta\text{Tn}4401\_a\)-like transposon in the exact same region. KpnCluster-003, KpnCluster-005, KpnCluster-008 and KpnCluster-021 contained \(\text{bla}_{\text{KPC}}\)2-like transposon \(\text{Tn}4401\_a\) 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 PIVM\_C014947\_1 from KpnCluster-021 carried the largest number of genes and this number decreased by the decreasing size of the plasmids. Most of the plasmids contained genes with unknown function. The large and medium sized plasmids contained the \(\text{kclA}\) gene, encoding an antirestriction protein implicated in the facilitation of \(\text{bla}_{\text{KPC}}\)2 allele transfer.23 None of the plasmids contained known virulence determinants such as \(\text{rmpA2, rmpA2, iroBC}\), or \(\text{icuABC}\) implicated in hypervirulence.24,25. Comparison of the large plasmids revealed that PIVM\_C008981\_1 and PIVM\_C015139\_1 from KpnCluster-003 and KpnCluster-005 displayed 90% similarity (Fig. 4). Plasmid PIVM\_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 \(\text{silE}\) and \(\text{silF}\) genes encoding a silver-binding protein and a silver exporting ATPase, \(\text{cufSRCFB}\) genes implicated in cation efflux, the \(\text{copABCDC-panE}\) genes involved in copper resistance and the \(\text{arsHACBAD}\) arsenic resistance gene cluster. These large plasmids also contained \(\text{fcdRABCDE}\) implicated in Fe(3+)-dicitrate transport, the \(\text{traiDSQCAVM-ylpA}\) plasmid conjugation gene cluster, and the \(\text{hihA-higA1}\) anti-toxins, except PIVM\_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 thereof. 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-putH Type IV secretion system. pRIVM_C014947_3 contained a merPT mercuric transport system, while pRIVM_C014906_3 and pRIVM_C015274_3 carried a cecC colicin-E3. The plasmid pRIVM_C014947_5 contained the bldA 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 blaKPC 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 blaKPC the carbapenemase allele was located on a ∆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 283 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_C014906_2, pRIVM_C015139_1, pRIVM_C015274_2 and pRIVM_C015274_3, 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 blaKPC 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 ygbMLKJ1-blaKPC-C015274_region 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 Enterobacteriace phage P4.

**Discussion**
We showed that a K. pneumoniae ST560 strain carrying blaKPC-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, blaKPC 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.
circulating extensively drug-resistant (XDR) K. pneumoniae ST258 and ST307 strains. 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\textit{KPC}-2 allele, two on two different plasmids and one in the chromosome. The localization of bla\textit{KPC}-2 on the chromosome and additional bla\textit{KPC}-2 copies have been reported previously and is further complicating the understanding of transmission of multidrug-resistant \textit{K. pneumoniae}.

Thirdly, KpnCluster-019 and also KpnCluster-021 isolates from the Caribbean harbored the bla\textit{KPC}-2 allele on a 5.6 kb \textit{\Delta}Tn4401a-like transposon, while the other isolates from the other genetic clusters from the Netherlands contained \textit{bla} \textit{KPC} on a 10 kb \textit{Tn}4401a transposon. Most global descriptions of \textit{K. pneumoniae} \textit{bla} \textit{KPC} the past decade have been associated with \textit{Tn}4401a or isoforms hereof. The traditional association of \textit{bla} \textit{KPC} with the \textit{Tn}4401a transposon has possibly been eroded in \textit{K. pneumoniae} isolates from the Caribbean.

**Figure 5.** \textit{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.
to a smaller variant. This is the first report of identification of a 5.6 kb $\Delta Tn4401\text{-}a$-like $bla$KPC-2 transposon of $K.\ pneumoniae$ in the Netherlands. Preliminary surveillance data analysis revealed that the $\Delta Tn4401\text{-}a$-like element carrying $bla$KPC-2 and smaller variants disseminated among $E.\ cloacae$, $S.\ marcesens$, $K.\ oxytoca$ and $E.\ coli$ in the Netherlands (unpublished data). Future work will seek to understand the dissemination of the $\Delta Tn4401\text{-}a$-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\cite{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\cite{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\cite{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\cite{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

### Table 2. BLAST similarity analysis of $K.\ pneumoniae$ plasmids.

| Plasmid       | KpnCluster | $bla$KPC allele | Bacterial species | Plasmid          | Query coverage (%) | Identity (%) | Accession number  | Country | Year | Reference          |
|---------------|------------|-----------------|-------------------|------------------|--------------------|--------------|-------------------|---------|------|--------------------|
| pRIVM_C008981_1 | KpnCluster-003 | $bla$KPC-2 | $K.\ pneumoniae$ | pGM16-005_01    | 35                 | 99.96        | CP028181.1       | Denmark | 2013 |                    |
| pRIVM_C014906_1 | KpnCluster-019 | $bla$KPC-2 | $K.\ pneumoniae$ | pKP11482-1       | 63                 | 99.91        | CP020842.1       | USA     | 2014 | Long et al. (2017) |
| pRIVM_C014906_2 | KpnCluster-019 | $K.\ quasi\text{-}pneumoniae$ | plasmid pG747 | 97               | 99.84              | CP034137.1   | Nigeria           | 2013    |      |                    |
| pRIVM_C014906_3 | $bla$KPC-2 | $K.\ pneumoniae$ | unnamed5 | 58               | 99.97              | CP033630.1   | Italy             | 2013    |      | Roe et al. (2019)  |
| pRIVM_C018535_1 | $bla$KPC-2 | $K.\ pneumoniae$ | pKP11482-1       | 63               | 99.91              | CP020842.1   | USA               | 2014    |      | Long et al. (2017) |
| pRIVM_C018535_2 | $bla$KPC-2 | $K.\ pneumoniae$ | 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 | $K.\ pneumoniae$ | unnamed3 | 90               | 99.98              | CP032170.1   | USA               | 2015    |      |                    |
| pRIVM_C014947_3 | $bla$KPC-2 | $K.\ pneumoniae$ | pA1705-KPC | 93               | 99.97              | MH090348.1   | China             | 2013    |      |                    |
| pRIVM_C014947_4 | $K.\ pneumoniae$ | pKP118-2079_5kb | 100              | 100               | MT090963.1   | China             | 2018    |      |                    |
| pRIVM_C014947_5 | $E.\ hormaechei$ | pC4_003 | 100              | 99.97              | CP042543.1   | Australia         | 2007    |      |                    |
| pRIVM_C014947_6 | $K.\ pneumoniae$ | pD17KP0032-3 | 100              | 100               | CP052331.1   | S. Korea          | 2017    |      |                    |
| pRIVM_C014947_7 | $E.\ hormaechei$ | unnamed3 | 97               | 100               | CP035388.1   | UK                | 2016    |      |                    |
| pRIVM_C014947_8 | $E.\ hormaechei$ | pC45-004 | 100              | 99.69              | CP042555.1   | Australia         | 2013    |      |                    |
| 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    |      |                    |
sequencing. Nevertheless, we identified plasmids in K. pneumoniae blaKPC-2 and blaKPC-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 community13,29,30. 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 blaSHV-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 blaKPC-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 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 blaKPC-2 allele or the blaKPC-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/periurethra (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 genome32. 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 sequencing18,37. 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 × g for 2 min. The pellet was washed and resuspended in 500 µl of 150 mM NaCl. The suspension was spun down at 5000 × g for 5 min and the pellet was resuspended in 100 µl of Quick-Extract DNA Extraction Solution (Lucigen) and 0.1 µl Ready-Lyse Lysozyme solution (Epicentre) and incubated for 1 h at 37 °C. Subsequently, 85 µl 10 mM Tris 1 mM EDTA pH = 8 (1 × TE), 10 µl protease K (> 600 mAU/mL, Qiangen) and 5 µl 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 × volume 3 M sodium acetate pH = 5.2 and 2.5 × volume ice cold 100% ethanol. Precipitated DNA was spun down at 13,000 × g for 15 min and pellets were washed with 1 ml 70% ethanol followed by another centrifugation at 13,000 × g for 5 min. After drying, the pellet was dissolved in 200 µl 1 × TE and diluted to 1 µg 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 previously18. 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 ≤ 0.4% different). An UPGMA clustering of *K. pneumoniae* 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 TG5 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 ≥ 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 Blasting website (https://blast.ncbi.nlm.nih.gov) website and date from October 2019.

**Plasmid content analysis.** Bioinformatics 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 laborato-ries 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. FL, 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. Halaby1, R. Steingrover2, J. W. T. Cohen Stuart3, D. C. Melles3, K. van Dijk6, I. J. B. Spijkenier5, D. W. Notermans9, 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 Jong8, A. Majier-Reuwer18, M. van Trijp19, A. J. van Griethuysen20, A. Ott21, E. Bathoorn22, J. C. Sinnige23, E. Heikens24, E. I. G. B. de Brauwer25, F. S. Stals25, W. Silvis26, J. W. Dorigo-Zetsma27, K. Waar28, S. P. van Mens29, N. Roescher29, A. Voss31, H. Wertheim32, B. C. G. C. Slingerland33, H. M. E. Frenay33, T. Schulin34, B. M. W. Diederen35,36, 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. Overdeves50, R. W. Bosboom51, T. A. M. Trieneke52, M. J. H. M. Wolfhagen53 & S. Paltansing54

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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 AMC, 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, Ampia 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. 28Centre 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, Izaia 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, Diakonessenhuis, 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, Isala Hospital, Zwolle, 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.