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bla$_{OXA-48}$-like genome architecture among carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* in the Netherlands

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Abstract

Carbapenem-hydrolysing enzymes belonging to the OXA-48-like group are encoded by *bla*$_{OXA-48}$-like alleles and are abundant among *Enterobacterales* in the Netherlands. Therefore, the objective here was to investigate the characteristics, gene content and diversity of the *bla*$_{OXA-48}$-like carrying plasmids and chromosomes of *Escherichia coli* and *Klebsiella pneumoniae* collected in the Dutch national surveillance from 2014 to 2019 in comparison with genome sequences from 29 countries. A combination of short-read genome sequencing with long-read sequencing enabled the reconstruction of 47 and 132 complete *bla*$_{OXA-48}$-like plasmids for *E. coli* and *K. pneumoniae*, respectively. Seven distinct plasmid groups designated as pOXA-48-1 to pOXA-48-5, pOXA-181 and pOXA-232 were identified in the Netherlands which were similar to internationally reported plasmids obtained from countries from North and South America, Europe, Asia and Oceania. The seven plasmid groups varied in size, G+C content, presence of antibiotic resistance genes, replicon family and gene content. The pOXA-48-1 to pOXA-48-5 plasmids were variable, and the pOXA-181 and pOXA-232 plasmids were conserved. The pOXA-48-1, pOXA-48-2, pOXA-48-3 and pOXA-48-5 groups contained a putative conjugation system, but this was absent in the pOXA-48-4, pOXA-181 and pOXA-232 plasmid groups. pOXA-48 plasmids contained the PemI antitoxin, while the pOXA-181 and pOXA-232 plasmids did not. Furthermore, the pOXA-181 plasmids carried a *virB2-virB3-virB9-virB10-virB11* type IV secretion system, while the pOXA-48 plasmids and pOXA-232 lacked this system. A group of non-related pOXA-48 plasmids from the Netherlands contained different resistance genes, non-IncL-type replicons or no replicons. Whole genome multilocus sequence typing revealed that the *bla*$_{OXA-48}$-like plasmids were found in a wide variety of genetic backgrounds in contrast to chromosomally encoded *bla*$_{OXA-48}$-like alleles. Chromosomally localized *bla*$_{OXA-48}$ and *bla*$_{OXA-244}$ alleles were located on genetic elements of variable sizes and comprised regions of pOXA-48 plasmids. The *bla*$_{OXA-48}$-like genetic element was flanked by a direct repeat upstream of IS1R, and was found at multiple locations in the chromosomes of *E. coli*. Lastly, *K. pneumoniae* isolates carrying *bla*$_{OXA-48}$ or *bla*$_{OXA-232}$ were mostly resistant for meropenem, whereas *E. coli bla*$_{OXA-48}$, *bla*$_{OXA-181}$ and chromosomal *bla*$_{OXA-48}$ or *bla*$_{OXA-244}$ isolates were mostly sensitive. In conclusion, the overall *bla*$_{OXA-48}$-like plasmid population in the Netherlands is conserved and similar to that reported for other countries, confirming global dissemination of *bla*$_{OXA-48}$-like plasmids. Variations in size, presence of antibiotic resistance genes and gene content impacted pOXA-48, pOXA-181 and pOXA-232 plasmid architecture.

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Abbreviations: AMR, antimicrobial resistance; CPE, carbapenemase-producing *Enterobacterales*; CRE, carbapenemase-resistant *Enterobacterales*; DR, direct repeat; ESBL, extended spectrum beta-lactamase; MIC, minimum inhibitory concentration; MST, minimum spanning tree; NGS, next-generation sequencing; ST, sequence type; TGS, third-generation sequencing; UPGMA, unweighted pair group method with arithmetic mean; VR, variable region; wgMLST, whole genome multilocus sequence typing.

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Two supplementary tables and supplementary files are available with the online version of this article.

DATA SUMMARY

The Illumina (NGS) sequence data set generated and analysed in this study is available in the European Nucleotide Archive (ENA) with study accession numbers PRJEB42331 (https://www.ebi.ac.uk/ena/browser/view/PRJEB42331) and PRJEB35685 (http://www.ebi.ac.uk/ena/data/view/PRJEB35685), and the Sequence Read Archive (SRA) with the study accession number PRJNA634885 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA634885/). The plasmid and chromosome sequences are deposited in GenBank of the National Center for Biotechnology Information (NCBI) and are available through the accession number PRJNA691727 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA691727). Relevant code was made available through https://github.com/BSR-AMR-RIVM/blaOXA-48-plasmids-Microbial-Genomics. The authors confirm that all supporting data, code, protocols and accession numbers have been provided within the article and through supplementary data files.

Impact Statement

OXA-48-type carbapenem hydrolysing enzymes encoded by *bla*OXA-48-like genes from transmissible plasmids or chromosomes of *Escherichia coli* and *Klebsiella pneumoniae* have spread world-wide and are of concern. Dissecting the *bla*OXA-48-like genome architecture at the molecular level by combining short-read and long-read sequencing will lead to understanding trends in the plasmid reservoir of *E. coli* and *K. pneumoniae* in the Netherlands and may enhance future international pathogen surveillance.

INTRODUCTION

Antimicrobial resistance (AMR) has dispersed among the family *Enterobacterales* and is a major concern for both hospitalized and non-hospitalized patients [1]. In carbapenemase-producing *Enterobacterales* (CPE), genes encoding carbapenemases are often located on transmissible plasmids that shuttle between bacterial strains of the same species, but also between distinct bacterial species and often confer resistance to carbapenem antibiotics [2, 3]. The predominant CPE species in the Netherlands from 2014 to 2019 were *Klebsiella pneumoniae* (43%), *Escherichia coli* (30%) and *Enterobacter cloacae* complex (13%) [4]. Carbapenemases are classified in Ambler classes A (i.e. KPC-types), B (i.e. IMP-, NDM- and VIM-types) and D (OXA β-lactamases) of carbapenem antibiotic-degrading enzymes [5]. The KPC, NDM, IMP, VIM and certain OXA-like enzymes are the most commonly identified variant carbapenemases that have spread world-wide among *Enterobacterales*, including *E. coli* and *K. pneumoniae* [6]. The $bla_{\text{OXA-48}}$-like genes make up the most prevalent carbapenemase-encoding genes found in *Enterobacterales* in the Netherlands (44%), followed by bla_{NDM} (27%) [4]. The OXA-48-like carbapenemases are encoded by the $bla_{\text{OXA-48}}$, $bla_{\text{OXA-162}}$, $bla_{\text{OXA-181}}$, $bla_{\text{OXA-204}}$, $bla_{\text{OXA-232}}$ and $bla_{\text{OXA-244}}$ genes. Other OXA-48-likes, such as OXA-245, OXA-484 and OXA-519, are less often reported groups of carbapenemases [6]. The distinction between the OXA-48-like carbapenemases is based on one to five specific amino acid substitutions in the $\beta5$–$\beta6$ loop of the enzyme that can impact the efficiency of carbapenem hydrolysis [6–8]. OXA-181 differs from OXA-48 by four amino acid substitutions (Thr104Ala, Asn110Asp, Glu168Gln and Ser171Ala), yet both have comparable carbapenem hydrolytic activity [9]. OXA-232 differs from OXA-48 by five amino acid substitutions, four of which are identical to the differential OXA-181 mutations, but OXA-232 contains an additional Arg214Ser substitution [10]. OXA-244 differs only by a single Arg214Gly mutation from OXA-48, and the OXA-244 together with OXA-181 enzymes have reduced carbapenem hydrolysing activity [11].

The most common plasmids that harbour $bla_{\text{OXA-48}}$ belong to the IncL/M family, which are conjugative and have been described for *E. coli* and *K. pneumoniae* [12–15]. The $bla_{\text{OXA-181}}$ gene is located on plasmids containing the *qnrS1* gene coding for quinolone resistance and either the ColE2, IncX3, IncN1 or IncT type of replicons [16, 17]. Plasmids containing $bla_{\text{OXA-232}}$ have the ColE-type replicon and the backbone is identical to $bla_{\text{OXA-181}}$-containing plasmids [10]. The $bla_{\text{OXA-244}}$ gene is located on an IncL plasmid and is suggested to originate from $bla_{\text{OXA-48}}$ by a point mutation, which possibly occurred during integration in the *E. coli* ST38 chromosome [6, 11, 15]. Chromosome encoded OXA-48-like carbapenemases have been described previously in globally disseminated *E. coli* and *K. pneumoniae* [15, 18, 19]. In these chromosomes, the $bla_{\text{OXA-48}}$-like gene has been found to be inserted at various chromosomal locations [18].

The global emergence of the carbapenem-hydrolysing OXA-48 enzyme and OXA-48-like descendants on transmissible plasmids warrants national surveillance. Currently, a paradigm shift is occurring in national reference laboratories from next-generation sequencing (NGS) towards third generation long-read sequencing (TGS). This allows an in-depth study of CPE antibiotic resistance-plasmid biology and plasmid transmission within and between healthcare institutions and countries, respectively. Therefore, the major goal of this study was to investigate the characteristics and contents of *E. coli* and *K. pneumoniae* plasmids and chromosomes carrying $bla_{\text{OXA-48}}$-like genes obtained from isolates submitted to the Dutch national CPE surveillance programme in a global context using a combination of NGS and TGS.

METHODS

Bacterial isolates

For the Dutch National CPE Surveillance programme, medical microbiology laboratories from the Netherlands routinely send *Enterobacterales* isolates with a meropenem minimum inhibitory concentration (MIC) of >0.25 mg l^{-1} and/or an imipenem MIC of >1 mg l^{-1} or genotypic or

Table 1. Resistance to meropenem per *E. coli* or *K. pneumoniae* isolate carrying bla_{OXA-48}-like alleles in 2014–2019

	E. coli				*K. pneumoniae*				Total
***bla*$_{OXA-48}$-like allele**	**S**	**I**	**R**	**All**	**S**	**I**	**R**	**All**	
bla_{OXA-48}	145	10	2	157	102	52	63	217	374
$bla_{OXA-181}$	33	2	1	36	14	3	4	21	57
$bla_{OXA-232}$						2	17	19	19
$bla_{OXA-244}$	28	2		30	1	1		2	32
bla_{NDM-1}, bla_{OXA-48}-like					1	3	21	25	25
bla_{NDM-5}, bla_{OXA-48}-like			4	4			14	14	18
Other		1	2	3	2	3	4	9	12
Total	206	15	9	230	120	64	123	307	537

Based on the clinical breakpoints according to EUCAST, the isolates were classified as sensitive (S: ≤2 mgl^{-1}), intermediate (I: >2 to 8 mg l^{-1}) or resistant (R: >8 mg l^{-1}) for meropenem.

phenotypic evidence of carbapenemase production to the National Institute of Public Health and the Environment through Type-Ned, an online platform [3]. The low MIC threshold for submission was chosen to monitor CPE instead of carbapenem-resistant *Enterobacterales* (CRE), because CPE represent a reservoir for the spread of antibiotic resistance genes. In this study, 537 carbapenemase-producing *E. coli* and *K. pneumoniae* isolates carrying bla_{OXA-48}-like alleles (bla_{OXA-48}, $bla_{OXA-181}$, $bla_{OXA-232}$) were included and were collected from 1 January 2014 until 31 December 2019 (Table S1, Suppl. File 1, available in the online version of this article). Only the first submitted *E. coli* or *K. pneumoniae* isolate with a bla_{OXA-48}-like allele per person in this study period was included.

Antimicrobial susceptibility testing

Resistance to carbapenem was confirmed by assessing the MIC for meropenem using an Etest (bioMérieux). Based on the clinical breakpoints according to EUCAST, the isolates were classified as sensitive (≤2 mgl^{-1}), intermediate (>2 mgl^{-1} and ≤8 mgl^{-1}) and resistant (>8 mgl^{-1}) to meropenem. Isolates were analysed for carbapenemase production using the carbapenem inactivation method (CIM) [20].

Next-generation sequencing

E. coli and *K. pneumoniae* isolates were subjected to NGS using the Illumina HiSeq 2500 (BaseClear). The antibiotic resistance gene profile and plasmid replicon compositions in all of the isolates were determined by interrogating the ResFinder (version 3.1.0) and PlasmidFinder (version 2.0.2) databases available from the Center for Genomic Epidemiology [21, 22]. 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. The resulting NGS-derived data, such as resistance genes, replicons and whole genome multilocus sequence typing (wgMLST) profiles, were imported into BioNumerics version 7.6.3 for subsequent comparative analyses (Applied Maths).

Long-read third-generation sequencing

High-molecular-weight DNA was isolated using an in-house developed protocol as described previously [3]. The Oxford Nanopore protocol SQK-LSK108 (https://community.nanoporetech.com) and the expansion kit for native barcoding EXP-NBD104 was used (Oxford Nanopore Technologies). A shearing step was performed using g-TUBEs (Covaris) to obtain an average DNA fragment size of 8 kb for isolates from 2014 to 2018. To obtain larger DNA fragments, this shearing step was omitted for isolates from 2019 and subsequently SQK-LSK109 was followed (Oxford Nanopore Technologies). The DNA was repaired using FFPE and end-repair kits (New England BioLabs) followed by ligation of barcodes with 1× bead clean up using AMPure XP (Beckman Coulter Nederland) after each step as described in SQK-LSK108 and SQK-LSK109. 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 were performed using Albacore 2.3.1 and a single FASTA file per isolate was extracted from the FAST5 files using Poretools 0.5.1 [23]. Fifty base pairs were trimmed at both sides and only reads larger than 5000 bp were used in further analyses. Illumina and Nanopore data were used in a hybrid assembly performed by Unicycler v0.4.4 [24]. Illumina data were not trimmed before running Unicycler, which was operated using default settings and verbosity 2. The resulting contig files were annotated using Prokka v1.14.6 and were subsequently loaded into BioNumerics for further analyses [25].

Plasmid content analysis

For annotation a Conda environment was set up with packages to facilitate a Snakemake pipeline which could process samples in bulk, and perform initial annotation with Prokka and enhancement with BLAST+ [26, 27]. Prokka annotation

Table 2. *bla*$_{OXA-48}$-like plasmids and chromosomes analysed in this study

		Carbapenemase allele				
Plasmid/chromosome	**Species**	***bla*$_{OXA-48}$**	***bla*$_{OXA-181}$**	***bla*$_{OXA-232}$**	***bla*$_{OXA-244}$**	**Total**
Plasmids	*E. coli*	30	16	1		47
	K. pneumoniae	108	10	14		132
Plasmids NCBI	*E. coli*	14	35			49
	K. pneumoniae	81	10	22	1	114
Chromosomes	*E. coli*	30			10	40
	K. pneumoniae	4				4
Chromosomes NCBI	*E. coli*	6			1	7
	K. pneumoniae	1				1
Total		274	71	37	12	394

Plasmids included in this study are complete and circular only, while the chromosomes were either circular or linear DNA.
NCBI indicates plasmids or chromosomes retrieved from the National Center for Biotechnology Information.

was executed in two stages: in the first stage it identified the coordinates of candidate genes with Prodigal, and in the second step it predicted these genes by utilizing user-set databases and its default the SWISS-PROT database [28, 29]. SWISS-PROT was used as it is a curated protein sequence database striving to provide a high level of annotation. To preserve the speed of the initial annotation we prepared a small database by combining sequence data from the ResFinder (version 3.1.0) database and the PlasmidFinder (version 2.0.2) database [21, 22]. If Prokka was unable to predict a gene it labelled the coordinate as a hypothetical protein. In order to reduce the hypothetical proteins in our annotation we used a set of custom Python scripts to extract and prepare them for BLAST+. After alignment with BLAST+, the supplemented Python code was used to replace the hypothetical proteins in the initial annotation file with their best alignment match (https://github.com/BSR-AMR-RIVM/blaOXA-48-plasmids-Microbial-Genomics). BioNumerics was used to extract and analyse the presence of annotated genes and tranposases in the different plasmids. The data were plotted, analysed and visualized in Excel. The presence of the direct repeat (DR) was analysed by searching for GGTAATGACTCCAAC using the BioNumerics sequence search feature in the sequence viewer.

Plasmid and chromosome comparisons

BioNumerics was used to compare complete plasmid DNA sequence and circular and linear chromosome datasets. Linear assembly contigs were omitted. Plasmid groups were identified based on 'all-to-all' primary DNA sequence comparison in BioNumerics in combination with unweighted pair group method with arithmetic mean (UPGMA) clustering. Plasmids with ≥80% sequence identity were considered to belong to the same plasmid group. The CLC Genomics Workbench version 12.0 software (www.qiagenbioinformatics.com) was used to retrieve *bla*$_{OXA-48}$-like plasmids and chromosomes from NCBI (Table S1). These plasmids and chromosomes were stripped from their annotations and re-annotated again using Prokka v1.14.6. All chromosomes have the *dnaA* gene as a starting point in order to determine relative locations of *bla*$_{OXA-48}$-like alleles. For analysis of the plasmid gene content, the *bla*$_{OXA-48}$ or *bla*$_{OXA-48}$-like allele was set as the starting point.

Minimum spanning tree, UPGMA, MLST and wgMLST analyses

The BioNumerics software was used to generate a minimum spanning tree (MST) or a UPGMA hierarchical clustering as described previously [3]. The categorical coefficient was used to calculate the MST and the MST was based on in-house *E. coli* and *K. pneumoniae* wgMLST schemes. The NGS data of the *K. pneumoniae* and *E. coli* isolates were used for classical MLST and wgMLST analyses using in-house wgMLST schemes made in the SeqSphere software version 6.0.2 (Ridom). The in-house *K. pneumoniae* wgMLST scheme comprised 4978 genes (3471 core-genome and 1507 accessory-genome targets) using *K. pneumoniae* MGH 78,578 (NC_009648.1) as a reference genome. The in-house *E. coli* wgMLST scheme comprised 4503 genes (3199 core-genome and 1304 accessory-genome targets) using *E. coli* 536 (CP000247.1) as a reference genome.

Ethics statement

The bacterial isolates belong to the medical microbiological laboratories participating in the Dutch National CPE Surveillance programme and were obtained as part of routine clinical care in recent years. Since no identifiable personal data were collected and data were analysed and processed anonymously, written or verbal patient consent was not required. According to the Dutch Medical Research Involving Human Subjects Act (WMO) this study was exempt from review by an Institutional Review Board.

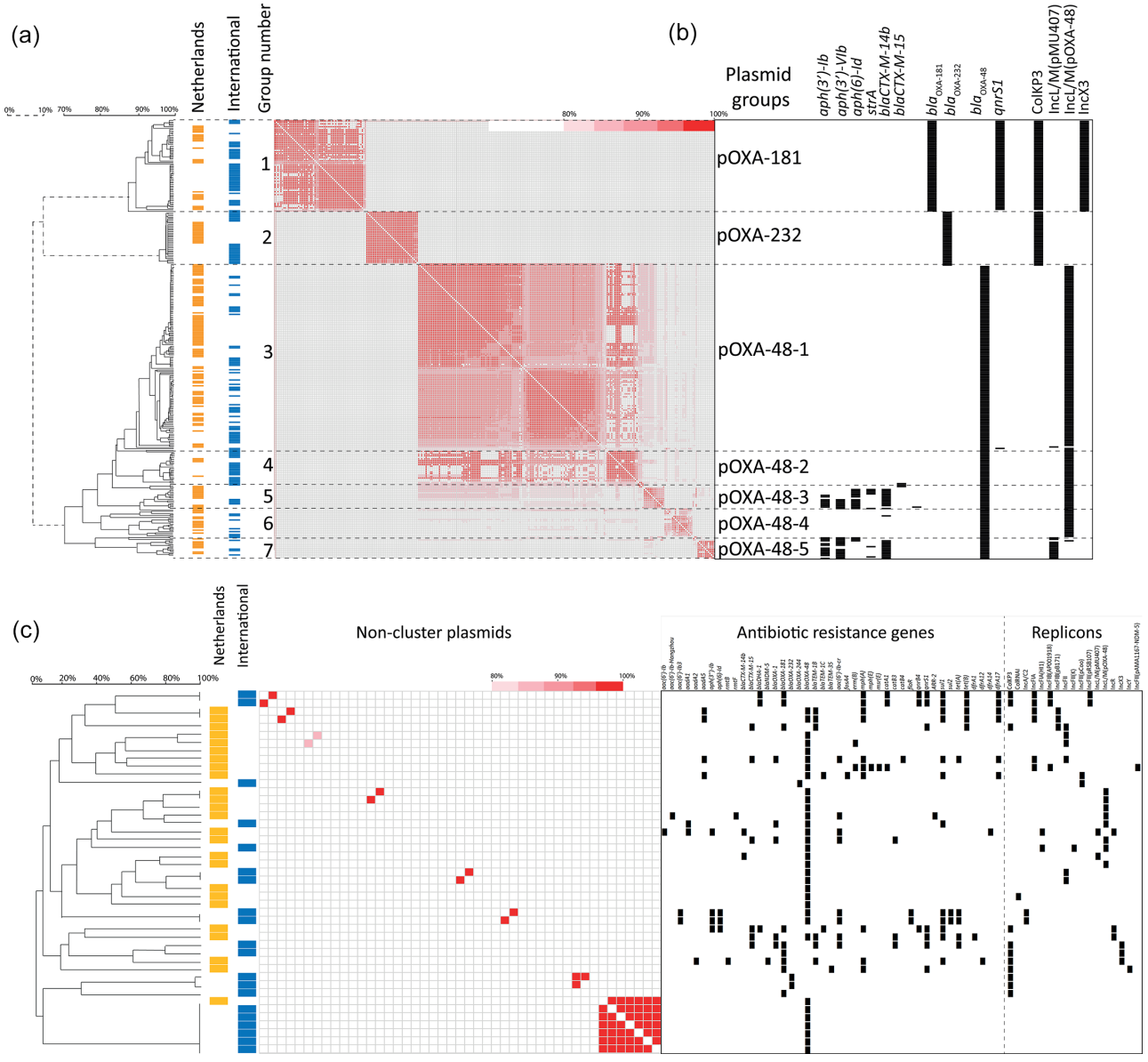

Fig. 1. Genetic clustering of bla_{OXA-48}-like plasmids in seven groups. (a) UPGMA clustering based on plasmid DNA sequences revealed seven distinct groups of bla_{OXA-48}-like plasmids. These groups were designated as pOXA-xxx (e.g. pOXA-48-1). Plasmids retrieved from the Netherlands are indicated in orange and international plasmids in blue. Group numbers are indicated. A heatmap shows the percentage of sequence identity, where red is 100% identical and white 0% identical. (b) The presence of AMR genes and replicons among the plasmids is indicated with black squares. Plasmids are depicted in rows and the AMR genes and replicons in columns. (c) As in (a) and (b) for the non-cluster plasmids.

RESULTS

Resistance to meropemen of CPE carrying bla_{OXA-48}-like plasmids

From 2014 to 2019, the National Institute for Public Health and the Environment (RIVM) received 1503 CPE, of which the majority (*n*=1106) were *E. coli* (*n*=461) and *K. pneumoniae* (*n*=645). PCR revealed that 272 *E. coli* and 338 *K. pneumoniae* isolates carried bla_{OXA-48}-like alleles. Only the first submitted *E. coli* or *K. pneumoniae* isolate with a bla_{OXA-48}-like allele per person in this study period was included. Therefore, 537 carbapenemase-producing *E. coli* (*n*=230) and *K. pneumoniae* (*n*=307) isolates were sequenced by NGS (Table S1). The majority of the *E. coli* isolates were carrying bla_{OXA-48}, $bla_{OXA-181}$ and $bla_{OXA-244}$ alleles and had MICs for meropenem that were below the clinical breakpoint of 2 mg l^{-1} for sensitivity according to EUCAST (206/230; 89.6%) (Table 1). Only 2/157 (1.3%) of the *E. coli* isolates with bla_{OXA-48} reached the clinical breakpoint for resistance (>8 mg l^{-1}) to meropenem. The $bla_{OXA-244}$ allele was found predominantly in *E. coli* (30/32; 93.8%) and was associated with a low MIC for meropenem.

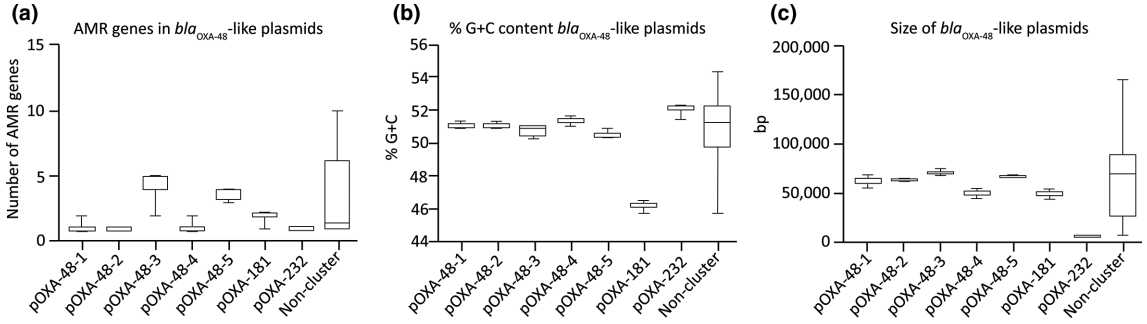

Fig. 2. *bla*$_{OXA-48}$-like plasmids have distinct molecular characteristics. (a) The number of AMR genes among the pOXA-48-like plasmid groups, (b) the G+C content (%) of the distinct pOXA-48-like plasmid groups and (c) the size (kb) of the pOXA-48-like plasmid groups. Bars, the variation per group.

K. pneumoniae carried mostly *bla*$_{OXA-48}$, *bla*$_{OXA-181}$ and *bla*$_{OXA-232}$ alleles, of which the *bla*$_{OXA-48}$ allele was associated with resistance to meropenem (63/307; 20.5%). The *bla*$_{OXA-181}$ allele was found in both *E. coli* and *K. pneumoniae*, and conferred resistance to meropenem in 4/21 (19%) of the *K. pneumoniae* isolates and 1/36 (2,8%) of the *E. coli* isolates. The *bla*$_{OXA-232}$ allele was exclusively found in *K. pneumoniae* and none of these isolates were meropenem-sensitive (resistant, 17/19; 89.5%, intermediate, 2/19; 10.5%). Combinations of *bla*$_{OXA-48}$-like alleles with either *bla*$_{NDM-1}$ or *bla*$_{NDM-5}$ resulted in high MICs for meropenem. For all *bla*$_{OXA-48}$-like alleles and double allele combinations, *K. pneumoniae* was more resistant (123/307; 40.1%) than *E. coli* (9/230; 3.9%). Due to initial limited resources, a subset (220/537; 41%) of the isolates submitted in 2018 and 2019 were sequenced with Nanopore long-read sequencing enabling the reconstruction of 47 and 132 complete *bla*$_{OXA-48}$-like plasmids for *E. coli* and *K. pneumoniae*, respectively (Table 2).

bla$_{OXA-48}$-like plasmids cluster in distinct genogroups

Comparison of the *bla*$_{OXA-48}$-like plasmid sequences retrieved from the Netherlands with internationally reported *bla*$_{OXA-48}$-like plasmids revealed clustering of the plasmids in a pOXA-232 group, a pOXA-181 group and five distinct pOXA-48 groups (Fig. 1a). A number of plasmids did not cluster with any of the other plasmids and were designated as the 'non-cluster' group (Fig. 1c). Plasmids identified in the Netherlands were similar to internationally reported plasmids that were obtained from 29 different countries from North and South America, Europe, Asia and Oceania (File. S1). In general, there was a paucity of antibiotic resistance genes in most of the *bla*$_{OXA-48}$-like-containing plasmids (Fig. 1a, b). UPGMA clustering based on plasmid sequence comparison showed that the pOXA-232 plasmids containing the ColKP3 replicon were highly conserved (96–100% similarity). At 6.2 kb in size, the pOXA-232 plasmids were the smallest *bla*$_{OXA-48}$-like plasmids and carried a single replicon, but had the highest average G+C content of 52.2% (Fig. 2a–c). In contrast, pOXA-181 plasmids carried the *qnrS1* allele and ColKP3 and IncX3 replicons and were also conserved (90–100%) (Fig. 1a, b). The pOXA-181 plasmids were on average 51.3 kb in size and had the lowest G+C content of 46.4% (Fig. 2b, c). Despite the high sequence conservation of pOXA-181 and pOXA-232 plasmids, they were found in CPE with distinct chromosomal backgrounds (Table S2). The largest and most variable group comprised *bla*$_{OXA-48}$-containing plasmids with an IncL/M(pOXA-48) type of replicon and could be divided into five subgroups, pOXA-48-1 to pOXA-48-5. The sequence conservation among pOXA-48-1 plasmids ranged from 80 to 100% (Fig. 1a). pOXA-48-1 plasmids were on average 64 kB with a G+C content of 51.2% and differed only from pOXA-48-2 plasmids by 0.1 kb. pOXA-48–3 was characterized by the presence of the aminoglycoside resistance genes *aph(3')-Ib*, *aph(3')-VIb, aph(6')-Id* and the extended spectrum beta-lactamase (ESBL) gene *bla*$_{CTXM-14b}$ (Fig. 1b). pOXA-48-3 plasmids resembled pOXA-48-5 plasmids, but most of the pOXA-48-5 plasmids lacked the *aph(6')-Id* gene and contained a distinct IncL/M(pMU407) replicon (Fig. 1b). pOXA-48-4 plasmids lacked these aminoglycoside resistance genes and these plasmids were smaller in size (Fig. 2). pOXA-48-3 and pOXA-48-5 had on average four AMR genes, one replicon per plasmid, a highly similar G+C content of 50.9 and 50.7%, respectively, but differed by 3.4 kB in size (Fig. 2). Non-cluster *bla*$_{OXA-48}$-like plasmids were distinct from those in the other groups and carried a wide variety of AMR genes resulting in distinct resistomes (Fig. 2a). These plasmids had either non-IncL/M-type replicons (e.g. IncR, IncY, IncF or IncA) or no known replicons (Fig. 1c). In addition, they had plasmid sizes that differed from those in the different plasmid groups and in G+C content and predominantly originated from isolates from the Netherlands (Fig. 2b, c).

Gene content determines distinct *bla*$_{OXA-48}$-like plasmid architecture

Analysis of the gene content of representative plasmids from the seven distinct plasmid groups revealed a group-associated gene content (Fig. 3). pOXA-48 plasmids had conserved plasmid regions, designated as regions 1, 2 and 3 and a central variable region (VR) which displayed variability in gene content and length (Fig. 3a). Plasmid region 2 was absent in pOXA-48-4 plasmids. The variations in

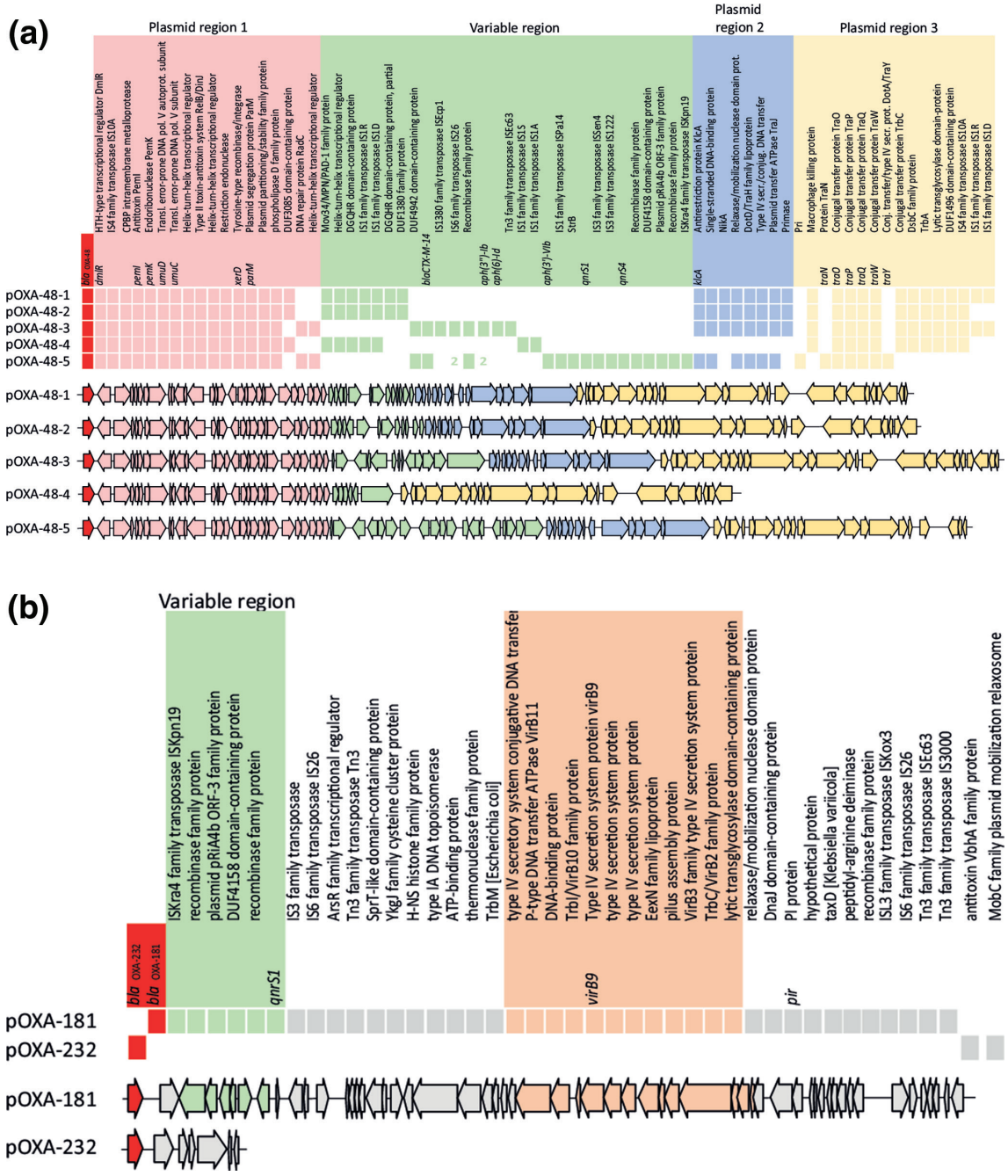

Fig. 3. Differences in bla_{OXA-48}-like plasmid architecture. (a) Diversity in pOXA-48-1 to pOXA-48-5 plasmid gene content. Complete plasmids were visualized in a linear way with the bla_{OXA-48}-like allele at starting position 1. The presence and absence of genes is indicated among representative plasmids from the plasmid groups. Colours indicate different groups of genes corresponding to different regions in the plasmid, or the variable region. Plasmid regions are labelled above the plasmid sequence. (b) Similar to (a) but displaying diversity in pOXA-181 and pOXA-232 plasmid gene content.

pOXA-48 plasmid gene content such as the presence or absence of AMR genes shaped the primary bla_{OXA-48}-like plasmid architecture, and varied among the different plasmid groups. While the pOXA-48-1, pOXA-48-2, pOXA-48-3 and pOXA-48-5 groups contained the *klcA* anti-restriction gene and a putative conjugation system, these features were absent in the pOXA-48-4, pOXA-181 and pOXA-232 plasmid groups (Fig. 3b). In pOXA-48-4 plasmids, the *tra* conjugation system was incomplete, while pOXA-48-5 plasmids contained a full conjugation system. pOXA-48 plasmids contained the PemI antitoxin, while the pOXA-181 and pOXA-232 plasmids did not. pOXA-181 plasmids carried a

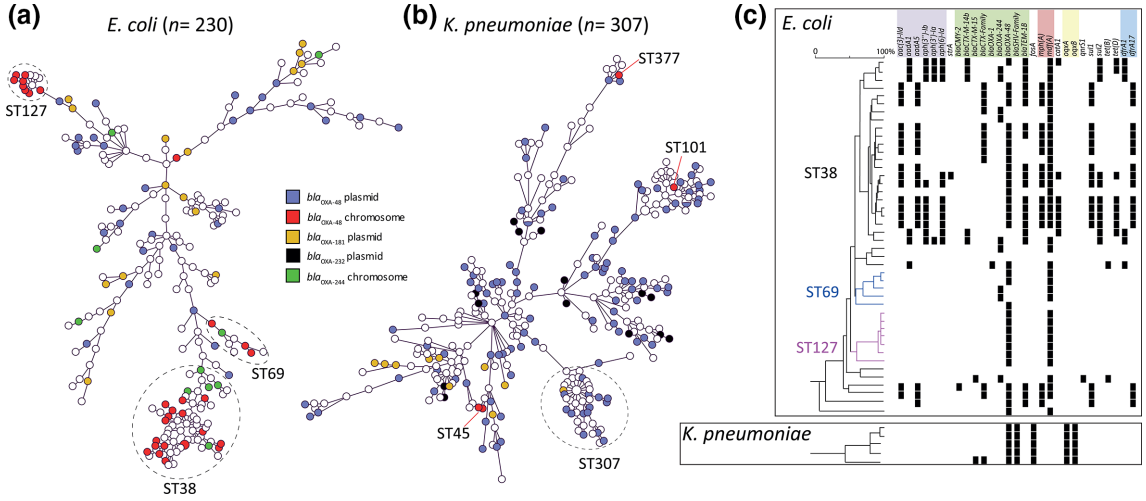

Fig. 4. Distribution of chromosome- or plasmid-localized bla_{OXA-48} or $bla_{OXA-244}$. (a) MST of *E. coli* in which chromosome- or plasmid-localized bla_{OXA-48} or $bla_{OXA-244}$ alleles are indicated by different colours. (b) Similar to (a) but for *K. pneumoniae*. (c) The presence of AMR genes among the chromosomes analysed in this study is indicated with black squares. Chromosomes are depicted in rows and the AMR genes in columns. Antibiotic classes are indicated above the AMR genes in different colours.

virB2-virB3-virB9-virB10-virB11 type IV secretion system, while the other pOXA-48 plasmids and pOXA-232 lacked this system. IS1 family transposases IS1R and IS1D, and IS4 family transposase IS10A were predominantly found in the pOXA-48 plasmids, and pOXA-181 plasmids were characterized by a variety of Tn3 family transposases. pOXA-232 plasmids did not contain IS or Tn3 elements.

Distribution of isolates harbouring plasmid or chromosomally localized bla_{OXA-48} and $bla_{OXA-244}$ alleles

A fraction of the bla_{OXA-48} (30/230; 13%) and $bla_{OXA-244}$ (10/230; 4.3%) alleles were located in the chromosomes of *E. coli* isolates, respectively (Table 2). Chromosomal bla_{OXA-48} or $bla_{OXA-244}$ occurred in *E. coli* isolates with the MLST sequence types ST38, ST69 and ST127 among other STs (Fig. 4a, Table S2). The STs were all unrelated and were multiple locus variants from ST38. The chromosome-localized bla_{OXA-48} and $bla_{OXA-244}$ were non-randomly distributed in the MST and restricted to specific STs (Fig. 4a, Table S2). In contrast, plasmid-localized bla_{OXA-48} occurred in *E. coli* isolates from a variety of non-related STs and were found randomly dispersed among the MST, except in ST38, ST69 and ST127. In four *K. pneumoniae* isolates, bla_{OXA-48} was found to be integrated in the chromosome (4/307; 1.3%), while none of the $bla_{OXA-181}$ or $bla_{OXA-232}$ alleles were located chromosomally. *K. pneumoniae* with either chromosome- or plasmid-localized bla_{OXA-48}-like were randomly distributed in the MST (Fig. 4b). The presence of the bla_{OXA-48} allele in the *E. coli* ST38 chromosomes was associated with the presence of the macrolide, trimethoprim and sulphonamide AMR genes *mph(A)*, *dfrA* and *sul*, while ST69 and ST127 were lacking the *dfrA* and *sul* genes (Fig. 4c). In contrast to *E. coli* ST38, the bla_{OXA-48}-containing *K. pneumoniae* chromosomes were mostly devoid of AMR genes, with the exception of the fosfomycin and quinolone resistance genes *fosA*, *oqxA* and *oqxB*.

Architecture of chromosome-localized bla_{OXA-48} or $bla_{OXA-244}$ allelic regions

In *E. coli*, the bla_{OXA-48} or $bla_{OXA-244}$ alleles were positioned in distinct regions in the chromosome relative to *dnaA* (Fig. 5a, Table 3). Chromosomally residing bla_{OXA-48} or $bla_{OXA-244}$ were located on different genetic elements with variable sizes of ~2.6,~11 or ~20 kb. The bla_{OXA-48}-like genetic element was flanked by IS1 family transposases IS1R and IS1D and had the IS1R-IS1D-bla_{OXA-48}-insert-IS1R-IS1D structure or variants thereof (Fig. 5b). The sizes of the genetic elements were determined as the sequence in between the flanking IS1R and IS1D, thereby excluding the size of the IS1R/1D sequence. The chromosomal insertion sites of bla_{OXA-48}-like genes and length of the insertion element varied per sequence type. In contrast to the bla_{OXA-48} allele, the chromosomally residing $bla_{OXA-244}$ allele was not found in the ST127 genetic background. In *K. pneumoniae*, bla_{OXA-48} was also found to be embedded between two IS4 family transposase IS10A genes. Comparison of pOXA-48 plasmids with the chromosomal bla_{OXA-48} insertions revealed that these chromosomal insertions resembled variable regions of plasmid region 1 (Fig. 5b). A 15-nt DR ggtaatgactccaac was typically located directly upstream IS1R, thereby flanking the bla_{OXA-48}-like insertion element. This DR sequence occurs on average once or twice in pOXA-48-1 to pOXA-48-5 plasmids and non-cluster plasmids, except in pOXA-181 and pOXA-232 plasmids (Fig. 5c). The DR was found on average 9× in the 47 *E. coli* chromosomes with bla_{OXA-48}-like, compared to 4.6× in the five *K. pneumoniae* chromosomes containing bla_{OXA-48}. The DR occurred on average 9, 8 and 4× in *E. coli* ST38, ST69 and ST127, respectively. In only four of the 52 chromosomes analysed, the bla_{OXA-48} region was flanked by one single DR

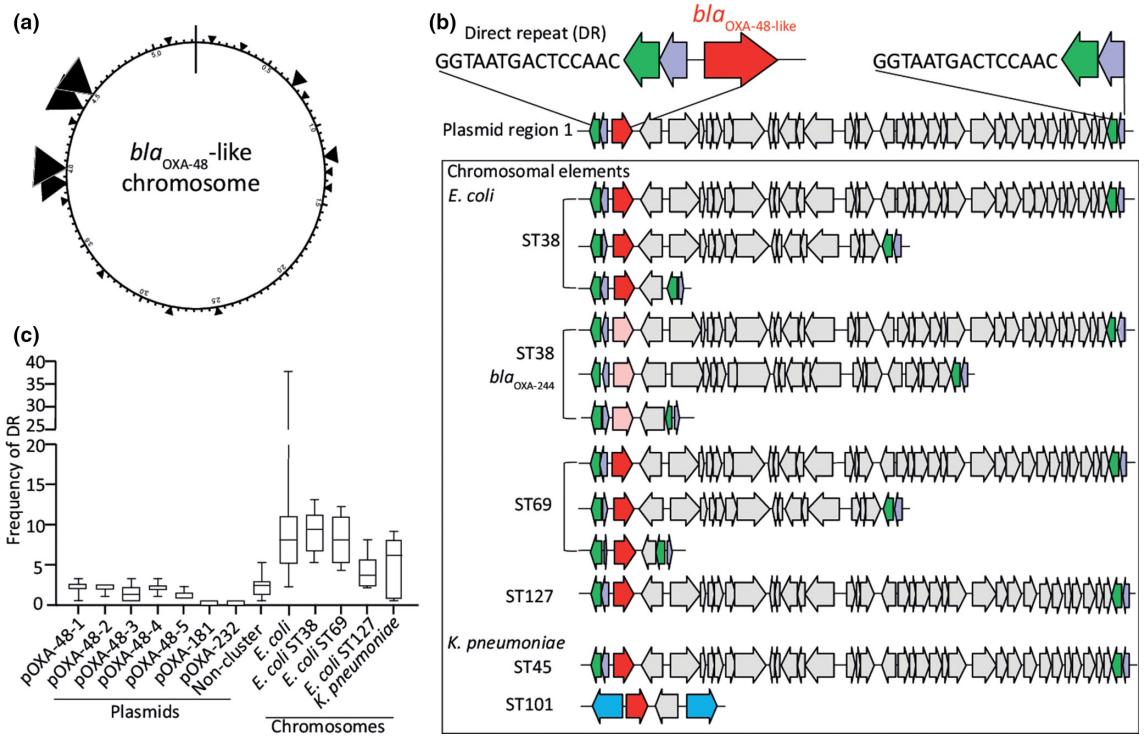

Fig. 5. Distinct integration sites of variable bla_{OXA-48} and $bla_{OXA-244}$ elements in the chromosome. (a) Artificial chromosome in which the different bla_{OXA-48}-like insertion positions are indicated by triangles. (b) Comparison of plasmid region 1 with chromosomal insertion sites of bla_{OXA-48}-like. Arrows indicate ORFs of which bla_{OXA-48} is depicted in red and $bla_{OXA-244}$ in light red. DR indicates the direct repeat sequence ggtaatgactccaac located upstream of IS1R. Sequence types are depicted by ST and sizes of the different insertion sequences are indicated in kilobases (kb). (c) Frequency of the DR sequence in bla_{OXA-48}-like plasmids and chromosomes of *E. coli* and *K. pneumoniae*.

sequence if the orientation of the carbapenemase allele was in reverse orientation (Table 3). In one chromosome, no DR sequence or truncates thereof were found. In *K. pneumoniae*, in two of the four ST45 isolates bla_{OXA-48} was inserted in the same location in the chromosome through a highly comparable genetic element (Fig. 5b). In a more distantly related *K. pneumoniae* ST101 isolate, a mobile genetic element of ~2.4 kb bla_{OXA-48} was localized in a distinct region, as also for the chromosomes retrieved from NCBI (Fig. 5b).

DISCUSSION

We dissected the architecture of 179 complete plasmids carrying bla_{OXA-48}-like and 44 bla_{OXA-48}-like alleles containing chromosomes of *E. coli* and *K. pneumoniae* isolates obtained from the Dutch national CPE surveillance programme in comparison with bla_{OXA-48}-like plasmids and chromosomes reported in the NCBI databank. The overall bla_{OXA-48}-like plasmid population in the Netherlands is conserved and compares to internationally reported plasmids. Most of the bla_{OXA-48}-like plasmids from both *E. coli* and *K. pneumoniae* could be clustered into seven distinct genotypic plasmid groups, which were characterized by a paucity in AMR genes, marked differences in gene content, replicon family, size and G+C content. This suggests the plasmids studied here have distinct origins and have transferred horizontally among CPE world-wide. In contrast to pOXA-181 and pOXA-232 plasmids, which were highly conserved, a group of pOXA-48 plasmids were diverse in genetic composition with sequence variation as high as 20%. The presence of a variety of transposases and insertion sequences, in addition to conjugation machinery, may be attributable to the genetic diversity of the pOXA-48 plasmids, in particular in the pOXA-48-3 and pOXA-48-5 plasmid subgroups.

There was an additional group of genetically highly diverse bla_{OXA-48}-like plasmids obtained in the Netherlands with a large range in G+C content, a variety of IncL and non-IncL-type replicons (IncR, IncFII or IncY), AMR genes and low inter-plasmid similarity. This suggests the presence of a potentially recently introduced set of plasmids that have not yet widely spread in the Netherlands. OXA-48 plasmids with either an IncR, IncFII or IncY replicon have only recently been described and are relatively rare [30–32]. The presence of these variable and rare bla_{OXA-48}-like plasmids suggest that the current OXA-48 plasmid reservoir may larger than currently reported. bla_{OXA-48}-like plasmids occurred in globally disseminated *E. coli* and *K. pneumoniae* isolates with known genetic backgrounds such as *E. coli* ST38 and *K. pneumoniae* ST307, but also multiple new STs, demonstrating continuous dissemination of AMR plasmids to new genetic backgrounds. To date, no double combinations of bla_{OXA-48}-like of alleles

Table 3. Characteristics of the *bla*$_{OXA-48}$-like chromosomal insertion site, direct repeat and insertion element

Species	MLST ST	Carba allele	Location of *bla*$_{OXA-48}$-like		Location of *bla*$_{OXA-48}$-like fragment		Size	No. of DRs in chromosome	DRs flanking *bla*$_{OXA-48}$-like
E. coli			**Start**	**End**	**Start**	**End**			
cRIVM_C012087	38	*bla* $_{OXA-48}$	1226615	1227412	1227631	1216632	−10999	6	1
cRIVM_C014115	38	*bla* $_{OXA-48}$	837858	838655	838907	818476	−20431	10	2
cRIVM_C014187	38	*bla*$_{OXA-48}$	3322083	3322880	3323099	3321208	−1891	5	2
cRIVM_C017997	38	*bla*$_{OXA-48}$	1271878	1272675	1272927	1261895	−11032	7	2
cRIVM_C018220	38	*bla*$_{OXA-48}$	4497614	4498411	4497362	4517799	20437	11	2
cRIVM_C018563	38	*bla*$_{OXA-48}$	1225857	1226654	1226873	1215874	−10999	5	2
cRIVM_C018567	38	*bla*$_{OXA-48}$	4429086	4429883	4428834	4449271	20437	11	2
cRIVM_C018583	38	*bla*$_{OXA-244}$	5316678	5317475	5317694	5297296	−20398	9	2
cRIVM_C018699	38	*bla*$_{OXA-48}$	4450722	4451519	4450470	4470907	20437	13	2
cRIVM_C018707	38	*bla*$_{OXA-48}$	4437017	4437814	4436798	4457202	20404	11	2
cRIVM_C028536	38	*bla*$_{OXA-48}$	1293474	1294271	1294490	1283491	−10999	7	2
cRIVM_C028568	38	*bla*$_{OXA-48}$	4379381	4380178	4378887	4390834	11947	12	2
cRIVM_C028613	38	*bla*$_{OXA-244}$	102972	103769	102478	104598	2120	8	2
cRIVM_C028803	38	*bla*$_{OXA-48}$	4458895	4459692	4458676	4479080	20404	9	2
cRIVM_C029020	38	*bla*$_{OXA-48}$	5148725	5149522	5149741	5129343	−20398	5	2
cRIVM_C029033	38	*bla*$_{OXA-244}$	102972	103769	102433	104975	2542	12	2
cRIVM_C029042	38	*bla*$_{OXA-48}$	4342207	4343004	4341988	4362392	20404	13	2
cRIVM_C029951	38	*bla*$_{OXA-244}$	106793	107590	106574	126972	20398	6	2
cRIVM_C029952	38	*bla*$_{OXA-48}$	4455075	4455872	4454823	4475254	20431	8	2
cRIVM_C030197	38	*bla*$_{OXA-48}$	3998627	3999424	3998408	4001066	2658	7	2
cRIVM_C030300	38	*bla*$_{OXA-48}$	3998582	3999379	3998088	4001021	2933	7	2
cRIVM_C030371	38	*bla*$_{OXA-244}$	5246611	5247408	5247627	5227229	−20398	5	2
cRIVM_C030453	38	*bla*$_{OXA-48}$	1219470	1220267	1209487	1220734	−11247	10	2
CP032145_1	38	*bla*$_{OXA-48}$	844693	845490	845709	827237	−18472	9	2
CP040390_1	38	*bla*$_{OXA-48}$	4461613	4462410	4461394	4479866	18472	11	2
cRIVM_C010151	69	*bla*$_{OXA-48}$	3898487	3899284	3900200	3879422	−20778	4	2

Continued

Table 3. Continued

Species	MLST ST	Carba allele	Location of bla_{OXA-48}-like		Location of bla_{OXA-48}-like fragment		Size	No. of DRs in chromosome	DRs flanking bla_{OXA-48}-like
E. coli			**Start**	**End**	**Start**	**End**			
cRIVM_C018576	69	bla_{OXA-48}	749018	749815	750731	747656	−3075	8	1
cRIVM_C030256	69	bla_{OXA-48}	749016	749813	750729	747654	−3075	8	1
cRIVM_C030443	69	$bla_{OXA-244}$	102728	103525	102509	116091	13582	12	2
cRIVM_C036689	99	bla_{OXA-48}	79721	80518	79502	99900	20398	5	2
cRIVM_C014046	127	bla_{OXA-48}	ND	ND	ND	ND	20673	3	2
cRIVM_C017887	127	bla_{OXA-48}	4003811	4004608	4003592	4023990	20398	4	2
cRIVM_C018150	127	bla_{OXA-48}	ND	ND	ND	ND	ND	8	2
cRIVM_C028497	127	bla_{OXA-48}	3939883	3940680	3939664	3960068	20404	4	2
cRIVM_C028620	127	bla_{OXA-48}	3968348	3969145	3968129	3988533	20404	3	2
cRIVM_C028724	127	bla_{OXA-48}	3884417	3885214	3884198	3904602	20404	2	2
cRIVM_C028786	127	bla_{OXA-48}	4000159	4000956	3999940	4020344	20404	6	2
cRIVM_C029324	127	bla_{OXA-48}	3910758	3911555	3910539	3930943	20404	2	2
cRIVM_C018249	349	$bla_{OXA-244}$	89993	90790	89454	91619	2165	15	2
cRIVM_C011532	361	$bla_{OXA-244}$	80789	81568	80376	100968	20592	36	2
cRIVM_C018404	940	bla_{OXA-48}	3841159	3841956	3840907	3859484	18577	25	2
cRIVM_C029494	1722	$bla_{OXA-244}$	102501	103298	101585	115864	14279	2	2
CP38505_1	nd	$bla_{OXA-244}$	102956	103753	102737	123135	20398	10	2
CP050382_1	nd	$bla_{OXA-244}$	80907	81704	80688	82533	1845	25	2
K. pneumoniae									
cRIVM_C014073	45	bla_{OXA-48}	4405354	4406151	4404860	4425910	21050	6	2
cRIVM_C018500	45	bla_{OXA-48}	4348845	4349642	4348626	4369024	20398	7	2
cRIVM_C015657	101	bla_{OXA-48}	262228	263025	262095	264541	2446	0	0
cRIVM_C015043	377	bla_{OXA-48}	2534772	2535569	2535788	2515390	-20398	9	2
NZ-CP040023_1	nd	bla_{OXA-48}	1406533	1407330	1405017	1407743	2726	1	1

Size of the bla_{OXA-48}-like insertion element excludes the IS1R-IS1D elements. If a number denotes a '−', the bla_{OXA-48}-like element is in reverse orientation. The location of bla_{OXA-48}-like and the bla_{OXA-48}-like fragment in the chromosome is indicated by locations relative to *dnaA*.

have been detected in one strain, although combinations with other carbapenemase alleles such as either bla_{NDM-1} or bla_{NDM-5} exist.

In this study, we also detected chromosomally localized bla_{OXA-48} and $bla_{OXA-244}$ alleles, but not chromosomal $bla_{OXA-181}$ and $bla_{OXA-232}$ alleles. This is in contrast to reports from other countries, where chromosomally localized $bla_{OXA-181}$ and $bla_{OXA-232}$ alleles have been described and found occasionally [6, 33]. Possibly, fragments containing $bla_{OXA-181}$ and $bla_{OXA-232}$ alleles failed to integrate by the lack of appropriate transposases, direct repeat target sequences in the plasmids or a suitable genetic background. Chromosomal insertion of bla_{OXA-48} or $bla_{OXA-244}$ may have occurred through IS1R-mediated transposition and recombination of OXA-48 plasmid sequences into *E. coli* and *K. pneumoniae* chromosomes with distinct genetic compositions [15]. The various lengths and compositions of bla_{OXA-48}-like segments and a variety of locations in the chromosome suggest that multiple transposition and recombination events have occurred. The chromosomal bla_{OXA-48} segment probably originated from plasmids belonging to the pOXA-48-1 to pOXA-48-5 groups. A potential insertion target site, a 15 bp direct repeat, was present in multiple copies in the chromosome and was found only in pOXA-48-1 to pOXA-48-5 plasmids and non-cluster plasmids, but not in pOXA-181 or pOXA-232 plasmids. This direct repeat was also found more frequently in *E. coli* than in *K. pneumoniae* chromosomes, which may explain why more *E. coli* than *K. pneumoniae* isolates harbour chromosomal bla_{OXA-48}/$bla_{OXA-244}$ and not $bla_{OXA-181}$/$bla_{OXA-232}$.

The majority of the bla_{OXA-48}-containing *K. pneumoniae* isolates in this study had MICs for meropenem above the clinical breakpoint, in contrast to *E. coli*, which were mostly sensitive. The bla_{OXA-48}-like alleles had different meropenem susceptibilities in *K. pneumoniae* and *E. coli* isolates, indicating that not all alleles result in the same resistance phenotype. In particular, *K. pneumoniae* containing $bla_{OXA-232}$ were highly resistant, which can possibly be attributed to a high copy number of pOXA-232 plasmids [34]. Alternatively, OXA-48 enzyme production, an altered affinity for meropenem, or other determinants such as outer membrane proteins, porins, efflux pumps or the presence of additional ESBLs can be responsible for this phenomenon as well [35, 36].

In conclusion, long-read sequencing of isolates from the Dutch National CPE surveillance contributed to the dissection of the architecture of bla_{OXA-48}-like plasmids and bla_{OXA-48}-like chromosome insertions of CPE in the Netherlands. Conjugation machinery, transposable elements and/or virulence determinants may contribute to plasmid diversification and dissemination, and represent important features that warrant future investigation. Additional long-read sequencing efforts of plasmids of CPE are required to monitor the changing plasmid reservoir involved in the spread of antibiotic resistance determinants in the Netherlands and beyond.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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