**bla\textsubscript{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 \(\text{bla}_{\text{OXA-48}}\)-like alleles and are abundant among *Enterobacteriales* in the Netherlands. Therefore, the objective here was to investigate the characteristics, gene content and diversity of the \(\text{bla}_{\text{OXA-48}}\)-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 \(\text{bla}_{\text{OXA-48}}\)-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 \(\text{virB2-virB3-virB9-virB10-virB11}\) 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-\(\text{IncL}\)-type replicons or no replicons. Whole genome multicore sequence typing revealed that the \(\text{bla}_{\text{OXA-48}}\)-like plasmids were found in a wide variety of genetic backgrounds in contrast to chromosomally encoded \(\text{bla}_{\text{OXA-48}}\)-like alleles. Chromosomally localized \(\text{bla}_{\text{OXA-48}}\) and \(\text{bla}_{\text{OXA-244}}\) alleles were located on genetic elements of variable sizes and comprised regions of pOXA-48 plasmids. The \(\text{bla}_{\text{OXA-48}}\)-like genetic element was flanked by a direct repeat upstream of \(\text{IS1R}\), and was found at multiple locations in the chromosomes of *E. coli*. Lastly, *K. pneumoniae* isolates carrying \(\text{bla}_{\text{OXA-48}}\) or \(\text{bla}_{\text{OXA-244}}\) were mostly resistant to meropenem, whereas *E. coli* \(\text{bla}_{\text{OXA-48}}\), \(\text{bla}_{\text{OXA-181}}\) and chromosomal \(\text{bla}_{\text{OXA-48}}\) or \(\text{bla}_{\text{OXA-244}}\) isolates were mostly sensitive. In conclusion, the overall \(\text{bla}_{\text{OXA-48}}\)-like plasmid population in the Netherlands is conserved and similar to that reported for other countries, confirming global dissemination of \(\text{bla}_{\text{OXA-48}}\)-like plasmids. Variations in size, presence of antibiotic resistance genes and gene content impacted pOXA-48, pOXA-181 and pOXA-232 plasmid architecture.
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.

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-lactamas) 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 worldwide among Enterobacteriales, including E. coli and K. pneumoniae [6]. The blaOXA-48-like genes make up the most prevalent carbapenemase-encoding genes found in Enterobacteriales in the Netherlands (44%), followed by blaNDM (27%) [4]. The OXA-48-like carbapenemases are encoded by the blaOXA-48, blaOXA-181, blaOXA-232, and blaOXA-244 genes. Other OXA-48-like, 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 β5–β6 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 (Thr104A, Asn110A, Glu168Gln and Ser171A), 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 blaOXA-48 belong to the IncI/M family, which are conjugative and have been described for E. coli and K. pneumoniae [12–15]. The blaOXA-181 gene is located on plasmids containing the qnrS1 gene coding for quinolone resistance and either the CoIE2, IncX3, IncN1 or IncT type of replicons [16, 17]. Plasmids containing blaOXA-232 have the CoIE-type replicon and the backbone is identical to blaOXA-181-containing plasmids [10]. The blaOXA-244 gene is located on an IncI plasmid and is suggested to originate from blaOXA-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 blaOXA-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 blaOXA-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 Enterobacteriales 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

Impact Statement

OXA-48-type carbapenem hydrolysing enzymes encoded by blaOXA-48-like genes from transmissible plasmids or chromosomes of Escherichia coli and Klebsiella pneumoniae have spread world-wide and are of concern. Dissecting the blaOXA-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.
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 (S: \(\leq 2\) mg l\(^{-1}\)), intermediate (I: \(>2\) to \(8\) mg l\(^{-1}\)) or resistant (R: \(>8\) mg l\(^{-1}\)) for meropenem.

### 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). 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

| bla \(_{OXA-48}\)-like allele | E. coli | K. pneumoniae | Total |
|-----------------------------|---------|---------------|-------|
|                             | S       | I             | R     | All   | S     | I   | R     | All   |
| bla \(_{OXA-48}\)           | 145     | 10            | 2     | 157   | 102   | 63  | 21    | 374   |
| bla \(_{OXA-181}\)          | 33      | 2             | 1     | 36    | 14    | 3   | 4     | 57    |
| bla \(_{OXA-232}\)          | 2       | 2             | 1     | 4     | 21    | 19  | 19    |       |
| bla \(_{NDM-1}\)            | 28      | 2             | 30    | 1     | 1     | 2   | 32    |       |
| bla \(_{OXA-48}\)-like      | 1       | 3             | 21    | 25    | 25    | 25  | 25    |       |
| bla \(_{OXA-181}\)-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: \(\leq 2\) mg l\(^{-1}\)), intermediate (I: \(>2\) to \(8\) mg l\(^{-1}\)) or resistant (R: \(>8\) mg l\(^{-1}\)) for meropenem.
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 transposases 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 \( \text{bla}_{\text{OXA-48}} \)-like plasmids and chromosomes from NCBI (Table S1).

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.

These plasmids and chromosomes were stripped from their annotations and re-annotated again using Prokka v1.14.6. All chromosomes have the \( \text{dnaA} \) gene as a starting point in order to determine relative locations of \( \text{bla}_{\text{OXA-48}} \)-like alleles. For analysis of the plasmid gene content, the \( \text{bla}_{\text{OXA-48}} \) or \( \text{bla}_{\text{OXA-46}} \)-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.

| Plasmid/chromosome | Species | \( \text{bla}_{\text{OXA-48}} \) | \( \text{bla}_{\text{OXA-181}} \) | \( \text{bla}_{\text{OXA-232}} \) | \( \text{bla}_{\text{OXA-244}} \) | Total |
|--------------------|---------|----------------|----------------|----------------|----------------|-------|
| Plasmids           | \( E. \) coli | 30          | 16           | 1             |                | 47    |
|                    | \( K. \) pneumoniae | 108       | 10          | 14           |                | 132   |
| Plasmids NCBI      | \( E. \) coli | 14         | 35           | 49           |                | 49    |
|                    | \( K. \) pneumoniae | 81         | 10          | 4             |                | 114   |
| Chromosomes        | \( E. \) coli | 30         | 10          | 22           | 1              | 40    |
|                    | \( K. \) pneumoniae | 4         | 4            |               |                | 4     |
| Chromosomes NCBI   | \( E. \) coli | 6         | 1          | 7             |                | 7     |
|                    | \( K. \) pneumoniae | 1         | 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.
RESULTS
Resistance to meropenem of CPE carrying \textit{bla}_{\text{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 \textit{E. coli} (\(n=461\)) and \textit{K. pneumoniae} (\(n=645\)). PCR revealed that 272 \textit{E. coli} and 338 \textit{K. pneumoniae} isolates carried \textit{bla}_{\text{OXA-48}}-like alleles. Only the first submitted \textit{E. coli} or \textit{K. pneumoniae} isolate with a \textit{bla}_{\text{OXA-48}}-like allele per person in this study period was included. Therefore, 537 carbapenemase-producing \textit{E. coli} (\(n=230\)) and \textit{K. pneumoniae} (\(n=307\)) isolates were sequenced by NGS (Table S1). The majority of the \textit{E. coli} isolates were carrying \textit{bla}_{\text{OXA-48}}\textit{ bla}_{\text{OXA-181}}\textit{ bla}_{\text{OXA-244}}\textit{ bla}_{\text{OXA-181}}\textit{ bla}_{\text{OXA-244}}\textit{ 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 \textit{E. coli} isolates with \textit{bla}_{\text{OXA-48}} reached the clinical breakpoint for resistance (>8 mg l\(^{-1}\)) to meropenem. The \textit{bla}_{\text{OXA-244}} allele was found predominantly in \textit{E. coli} (30/32; 93.8\%) and was associated with a low MIC for meropenem.
**Fig. 2.** bla\textsubscript{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\textsubscript{OXA-48} and bla\textsubscript{OXA-232} alleles, of which the bla\textsubscript{OXA-48} allele was associated with resistance to meropenem (63/307; 20.5%). The bla\textsubscript{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\textsubscript{OXA-232} allele was exclusively found in *K. pneumoniae* and none of these isolates were meropenem-sensitive (resistant, 123/307; 40.1%). The bla\textsubscript{OXA-232} 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\textsubscript{OXA-232} allele was exclusively found in *K. pneumoniae* and none of these isolates were meropenem-sensitive (resistant, 123/307; 40.1%). The bla\textsubscript{OXA-232} 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.

**bla\textsubscript{OXA-48}-like plasmids cluster in distinct genogroups**

Comparison of the bla\textsubscript{OXA-48}-like plasmid sequences retrieved from the Netherlands with internationally reported bla\textsubscript{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\textsubscript{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\textsubscript{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 qnr\textsubscript{S1} 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\textsubscript{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′)-Vlb, aph(6′)-Id* and the extended spectrum beta-lactamase (ESBL) gene *bla\textsubscript{CTX-M-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\textsubscript{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\textsubscript{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-48-8 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
pOXA-48 plasmid gene content such as the presence or absence of AMR genes shaped the primary \( \text{bla}_{\text{OXA-48}} \)-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 \( \text{tra} \) 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
virB2-virB3-virB9-virB10-virB11 type IV secretion system, while the other pOXA-48 plasmids and pOXA-232 lacked this system. IS1 family transposes IS1R and IS1D, and IS4 family transposes IS10A were predominantly found in the pOXA-48 plasmids, and pOXA-181 plasmids were characterized by a variety of Tn3 family transposes. pOXA-232 plasmids did not contain IS or Tn3 elements.

**Distribution of isolates harbouring plasmid or chromosomally localized bla\textsubscript{OXA-48} and bla\textsubscript{OXA-244} alleles**

A fraction of the bla\textsubscript{OXA-48} (30/230; 13%) and bla\textsubscript{OXA-244} (10/230; 4.3%) alleles were located in the chromosomes of *E. coli* isolates, respectively (Table 2). Chromosomal bla\textsubscript{OXA-48} or bla\textsubscript{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\textsubscript{OXA-48} and bla\textsubscript{OXA-244} were non-randomly distributed in the MST and restricted to specific STs (Fig. 4a, Table S2). In contrast, plasmid-localized bla\textsubscript{OXA-48} and bla\textsubscript{OXA-244} 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\textsubscript{OXA-48} was found to be integrated in the chromosome (4/307; 1.3%), while none of the bla\textsubscript{OXA-181} or bla\textsubscript{OXA-232} alleles were located chromosomally. *K. pneumoniae* with either chromosome- or plasmid-localized bla\textsubscript{OXA-48}-like were randomly distributed in the MST (Fig. 4b). The presence of the bla\textsubscript{OXA-48} allele in the *E. coli* ST38 chromosomes was associated with the presence of the macrolide, trimethoprim and sulphonamide AMR genes mph\textsubscript{A}, dfr\textsubscript{A} and sul, while ST69 and ST127 were lacking the dfr\textsubscript{A} and sul genes (Fig. 4c). In contrast to *E. coli* ST38, the bla\textsubscript{OXA-48}-containing *K. pneumoniae* chromosomes were mostly devoid of AMR genes, with the exception of the fosfomycin and quinolone resistance genes \textit{fosA}, \textit{oqxA} and \textit{oqxB}.

**Architecture of chromosome-localized bla\textsubscript{OXA-48} or bla\textsubscript{OXA-244} allelic regions**

In *E. coli*, the bla\textsubscript{OXA-48} or bla\textsubscript{OXA-244} alleles were positioned in distinct regions in the chromosome relative to \textit{dnaA} (Fig. 5a, Table 3). Chromosomally residing bla\textsubscript{OXA-48} or bla\textsubscript{OXA-244} were located on different genetic elements with variable sizes of ~2.6, ~11 or ~20 kb. The bla\textsubscript{OXA-48}-like genetic element was flanked by IS1 family transposes IS1R and IS1D and had the IS1R-IS1D-bla\textsubscript{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/IS1D sequence. The chromosomal insertion sites of bla\textsubscript{OXA-48}-like genes and length of the insertion element varied per sequence type. In contrast to the bla\textsubscript{OXA-48} allele, the chromosomally residing bla\textsubscript{OXA-244} allele was not found in the ST127 genetic background. In *K. pneumoniae*, bla\textsubscript{OXA-48} was also found to be embedded between two IS4 family transposes IS10A genes. Comparison of pOXA-48 plasmids with the chromosomal bla\textsubscript{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\textsubscript{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 four *E. coli* chromosomes with bla\textsubscript{OXA-48}-like, compared to 4.6× in the five *K. pneumoniae* chromosomes containing bla\textsubscript{OXA-48} alleles. 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\textsubscript{OXA-48} region was flanked by one single DR

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**Fig. 4.** Distribution of chromosome- or plasmid-localized bla\textsubscript{OXA-48} or bla\textsubscript{OXA-244}. (a) MST of *E. coli* in which chromosome- or plasmid-localized bla\textsubscript{OXA-48} or bla\textsubscript{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.
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<sub>OXA-48</sub>-like chromosomal insertion site, direct repeat and insertion element

| Species | MLST ST | Carba allele | Location of bla<sub>OXA-48</sub>-like | Location of bla<sub>OXA-48</sub>-like fragment | Size | No. of DRs in chromosome | DRs flanking bla<sub>OXA-48</sub>-like |
|---------|---------|--------------|--------------------------------------|-----------------------------------------------|------|-------------------------|-------------------------------------|
| E. coli | 38      | bla<sub>OXA-48</sub> | 1226615  1227412                      | 1227631  1216632                              | −10999 | 6                       | 1                                   |
| cRIVM_C0112087 | 38      | bla<sub>OXA-48</sub> | 837858   838655                       | 838907   818476                               | −20431 | 10                      | 2                                   |
| cRIVM_C014187 | 38      | bla<sub>OXA-48</sub> | 3322083  3322880                      | 3323099  3321208                              | −1891  | 5                       | 2                                   |
| cRIVM_C017997 | 38      | bla<sub>OXA-48</sub> | 1271878  1272675                      | 1272927  1261895                              | −11032 | 7                       | 2                                   |
| cRIVM_C018220 | 38      | bla<sub>OXA-48</sub> | 4497614  4498411                      | 4497362  4517799                              | 20437  | 11                      | 2                                   |
| cRIVM_C018563 | 38      | bla<sub>OXA-48</sub> | 1225857  1226654                      | 1226873  1215874                              | −10999 | 5                       | 2                                   |
| cRIVM_C018567 | 38      | bla<sub>OXA-48</sub> | 4429086  4429883                      | 4428834  4449271                              | 20437  | 11                      | 2                                   |
| cRIVM_C018583 | 38      | bla<sub>OXA-48</sub> | 5316678  5317475                      | 5317694  5297296                              | −20398 | 9                       | 2                                   |
| cRIVM_C018699 | 38      | bla<sub>OXA-48</sub> | 4450722  4451519                      | 4450470  4470907                              | 20437  | 13                      | 2                                   |
| cRIVM_C018707 | 38      | bla<sub>OXA-48</sub> | 4437017  4437814                      | 4436798  4457202                              | 20404  | 11                      | 2                                   |
| cRIVM_C028536 | 38      | bla<sub>OXA-48</sub> | 1293474  1294271                      | 1294490  1283491                              | −10999 | 7                       | 2                                   |
| cRIVM_C028568 | 38      | bla<sub>OXA-48</sub> | 4379381  4381078                      | 4378876  4390834                              | 11947  | 12                      | 2                                   |
| cRIVM_C028613 | 38      | bla<sub>OXA-48</sub> | 102972   103769                        | 102478   104598                                | 2120   | 8                       | 2                                   |
| cRIVM_C028803 | 38      | bla<sub>OXA-48</sub> | 4458895  4459692                      | 4458676  4447908                              | 20404  | 9                       | 2                                   |
| cRIVM_C029020 | 38      | bla<sub>OXA-48</sub> | 5148725  5149522                      | 5149741  5129343                              | −20398 | 5                       | 2                                   |
| cRIVM_C029033 | 38      | bla<sub>OXA-48</sub> | 102972   103769                        | 102433   104975                                | 2542   | 12                      | 2                                   |
| cRIVM_C029042 | 38      | bla<sub>OXA-48</sub> | 4342207  4343004                      | 4341988  4362392                              | 20404  | 13                      | 2                                   |
| cRIVM_C029951 | 38      | bla<sub>OXA-48</sub> | 106793   107590                        | 106574   126972                                | 20398  | 6                       | 2                                   |
| cRIVM_C029952 | 38      | bla<sub>OXA-48</sub> | 445075   4455872                      | 4454823  4475254                               | 20431  | 8                       | 2                                   |
| cRIVM_C030197 | 38      | bla<sub>OXA-48</sub> | 3998627  3999424                      | 3998408  4001066                              | 2658   | 7                       | 2                                   |
| cRIVM_C030300 | 38      | bla<sub>OXA-48</sub> | 3998582  3999379                      | 3998088  4001021                              | 2658   | 7                       | 2                                   |
| cRIVM_C030371 | 38      | bla<sub>OXA-48</sub> | 5246611  5247408                      | 5247627  5227229                              | −20398 | 5                       | 2                                   |
| cRIVM_C030453 | 38      | bla<sub>OXA-48</sub> | 1219470  1220267                      | 1209487  1220734                               | −11247 | 10                      | 2                                   |
| CP032145_1    | 38      | bla<sub>OXA-48</sub> | 844693   845490                       | 845709   827237                                | −18472 | 9                       | 2                                   |
| CP040390_1    | 38      | bla<sub>OXA-48</sub> | 4461613  4462410                      | 4461394  4479866                               | 18472  | 11                      | 2                                   |
| cRIVM_C010151 | 69      | bla<sub>OXA-48</sub> | 3898487  3899284                      | 3900200  3879422                               | −20778 | 4                       | 2                                   |

Continued
Table 3. Continued

| Species   | MLST ST | Carba allele | Location of \(\text{bla}_{\text{OXA-48}}\)-like | Location of \(\text{bla}_{\text{OXA-48}}\)-like fragment | Size | No. of DRs in chromosome | DRs flanking \(\text{bla}_{\text{OXA-48}}\)-like |
|-----------|---------|--------------|---------------------------------|---------------------------------|------|--------------------------|--------------------------|
| E. coli   |         | \(\text{bla}_{\text{OXA-48}}\) | Start                   | End                   | Start                   | End                   |                          |                          |
| cRIVM_C018576 | 69      | \(\text{bla}_{\text{OXA-48}}\) | 749018                  | 749815               | 750731                  | 747656               | −3075                   | 8                         |
| cRIVM_C030256 | 69      | \(\text{bla}_{\text{OXA-48}}\) | 749016                  | 749813               | 750729                  | 747654               | −3075                   | 8                         |
| cRIVM_C030443 | 69      | \(\text{bla}_{\text{OXA-48}}\) | 102728                  | 103525               | 102609                  | 116091              | 13582                    | 12                        |
| cRIVM_C036689 | 99      | \(\text{bla}_{\text{OXA-48}}\) | 79721                   | 80518                | 79502                   | 99900               | 20398                    | 5                         |
| cRIVM_C014046 | 127     | \(\text{bla}_{\text{OXA-48}}\) | ND                      | ND                   | ND                      | ND                  | 20673                    | 3                         |
| cRIVM_C017887 | 127     | \(\text{bla}_{\text{OXA-48}}\) | 4003811                 | 4004608              | 4003592                 | 4023990            | 20398                    | 4                         |
| cRIVM_C018150 | 127     | \(\text{bla}_{\text{OXA-48}}\) | ND                      | ND                   | ND                      | ND                  | ND                      | 8                         |
| cRIVM_C028497 | 127     | \(\text{bla}_{\text{OXA-48}}\) | 3939883                 | 3940680              | 3939664                 | 3960068            | 20404                    | 4                         |
| cRIVM_C028620 | 127     | \(\text{bla}_{\text{OXA-48}}\) | 3968348                 | 3969145              | 3968129                 | 3988333            | 20404                    | 3                         |
| cRIVM_C028724 | 127     | \(\text{bla}_{\text{OXA-48}}\) | 3884417                 | 3885214              | 3884198                 | 3904602            | 20404                    | 2                         |
| cRIVM_C028786 | 127     | \(\text{bla}_{\text{OXA-48}}\) | 4000159                 | 4000956              | 3999940                 | 4020344            | 20404                    | 6                         |
| cRIVM_C029324 | 127     | \(\text{bla}_{\text{OXA-48}}\) | 3910758                 | 3911555              | 3910539                 | 3930943            | 20404                    | 2                         |
| cRIVM_C018249 | 349     | \(\text{bla}_{\text{OXA-48}}\) | 89993                   | 90790                | 89454                   | 91619              | 2165                     | 15                        |
| cRIVM_C011352 | 361     | \(\text{bla}_{\text{OXA-48}}\) | 80789                   | 81568                | 80376                   | 809696             | 20592                    | 36                        |
| cRIVM_C018404 | 940     | \(\text{bla}_{\text{OXA-48}}\) | 3841159                 | 3841956              | 3840907                 | 3859484            | 18577                    | 25                        |
| cRIVM_C029894 | 1722    | \(\text{bla}_{\text{OXA-48}}\) | 102501                  | 103298               | 101585                  | 115864             | 14279                    | 2                         |
| CP38505_1     | nd      | \(\text{bla}_{\text{OXA-48}}\) | 102956                  | 103753               | 102737                  | 123135             | 20398                    | 10                        |
| CP090302_1     | nd      | \(\text{bla}_{\text{OXA-48}}\) | 80907                   | 81704                | 80688                   | 82533              | 1845                     | 25                        |
| K. pneumoniae |         | \(\text{bla}_{\text{OXA-48}}\) |                     |                     |                     |                     |                          |                          |
| cRIVM_C014073 | 45      | \(\text{bla}_{\text{OXA-48}}\) | 4405354                 | 4406151              | 4404860                 | 4425910            | 21050                    | 6                         |
| cRIVM_C018300 | 45      | \(\text{bla}_{\text{OXA-48}}\) | 4348865                 | 4349642              | 4348626                 | 4369024            | 20398                    | 7                         |
| cRIVM_C015657 | 101     | \(\text{bla}_{\text{OXA-48}}\) | 2622228                 | 263025               | 262095                  | 264541             | 2446                     | 0                         |
| cRIVM_C015043 | 377     | \(\text{bla}_{\text{OXA-48}}\) | 2534722                 | 2535669              | 2535788                 | 2515390            | −20398                   | 9                         |
| NZ-CP090023_1 | nd      | \(\text{bla}_{\text{OXA-48}}\) | 1406533                 | 1407330              | 1405017                 | 1407743            | 2726                     | 1                         |

Size of the \(\text{bla}_{\text{OXA-48}}\)-like insertion element excludes the IS1R-IS1D elements. If a number denotes a ‘−’, the \(\text{bla}_{\text{OXA-48}}\)-like element is in reverse orientation. The location of \(\text{bla}_{\text{OXA-48}}\)-like and the \(\text{bla}_{\text{OXA-48}}\)-like fragment in the chromosome is indicated by locations relative to \(\text{dnaA}\).
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-232} alleles, but not chromosomal bla_{OXA-181} and bla_{OXA-244} 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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