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

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Abstract

Carbapenem-hydrolyzing 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 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 2014-2019 in comparison with genome sequences retrieved from 29 countries. By combining short-read and long-read sequencing, 47 and 132 complete *bla*_{OXA-48}-like plasmids were reconstructed for *E. coli* and *K. pneumoniae*, respectively. Distinct plasmid groups designated as pOXA-48, pOXA-181, and pOXA-232 were identified in the Netherlands and varied in size, % G+C, presence of antibiotic resistance genes, replicons and gene content. The pOXA-48 plasmids were variable, while pOXA-181 and pOXA-232 plasmids were conserved. A group of non-related pOXA-48 plasmids contained different resistance genes, non-IncL type replicons or carried no replicons. *K. pneumoniae* isolates carrying *bla*_{OXA-48} or *bla*_{OXA-232} were mostly resistant, while *E. coli* *bla*_{OXA-48}, *bla*_{OXA-181} and chromosomal *bla*_{OXA-48} or *bla*_{OXA-244} isolates were mostly sensitive for meropenem. Analysis of chromosomally localized *bla*_{OXA-48}-like alleles revealed that these were flanked by a direct repeat (DR) upstream of IS1R, which were found at multiple locations in the chromosome of distinct genetic backgrounds. In conclusion, the overall *bla*_{OXA-48}-like plasmid population in the Netherlands is conserved and similar to that reported for other countries, although a highly diverse *bla*_{OXA-48}-like plasmid subgroup was present. Chromosomally encoded *bla*_{OXA-48}-like alleles are from distinct genetic backgrounds and occurs at variable positions containing the DR, thereby indicating multiple independent transpositions.

Importance

OXA-48-type of carbapenem hydrolyzing 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 of *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) type 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*_{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*_{OXA-48}, *bla*_{OXA-162}, *bla*_{OXA-181}, *bla*_{OXA-204}, *bla*_{OXA-232}, and *bla*_{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 β 5- β 6-loop of the enzyme that can impact the efficiency of carbapenem hydrolysis (6–8). OXA-181 differs from OXA-48 by four amino acids substitutions (Thr104Ala, Asn110Asp, Glu168Gln and Ser171Ala) yet have both comparable carbapenem hydrolytic activity (9). OXA-232 differs from OXA-48 by five amino acid substitutions, four are identical to the differential OXA-181 mutations, however 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 hydrolyzing activity (11).

The most common plasmids that harbor *bla*_{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*_{OXA-181} gene is located on plasmids containing the *qnrS1* gene encoding for quinolone resistance and either ColE2, IncX3, IncN1 or IncT type of replicons (16, 17). Plasmids containing *bla*_{OXA-232} have the ColE-type of replicon and the backbone is identical to *bla*_{OXA-181} containing plasmids (10). The *bla*_{OXA-244} gene is located on an IncL plasmid and is suggested to be originated from *bla*_{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*_{OXA-48}-like gene has been found to be inserted at various chromosomal locations (18).

The global emergence of the carbapenem-hydrolyzing OXA-48 enzyme and OXA-48-like descendants on transmissible plasmids warrants a national surveillance. Currently, a paradigm shift occurs 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 health care institutions and countries, respectively. Therefore, the major goal of this study was to investigate the characteristics and contents

91 of *E. coli* and *K. pneumoniae* plasmids and chromosomes carrying *bla*_{OXA-48}-like genes obtained from
92 isolates submitted to the Dutch national CPE surveillance program in a global context using a
93 combination of NGS and TGS.

94

Results

Resistance to meropenem of CPE carrying *bla*_{OXA-48}-like plasmids. From 2014 till 2019, the National Institute for Public Health and the Environment (RIVM) received 1503 carbapenemase-producing *Enterobacterales*, 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, Suppl. file 1). 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 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) 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. *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 (R, 17/19; 89.5%, I, 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. 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 (Suppl. File 1). In general, there was a paucity of antibiotic resistance genes in most of the *bla*_{OXA-48}-like containing plasmids (Fig. 1b). Unweighted pair group method with arithmetic mean (UPGMA) clustering based on plasmid sequence comparison showed that the pOXA-232 plasmids containing the ColKP3 replicon were highly conserved (96-100% similarity). With 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. 2). 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 have the lowest %G+C content of 46.4% (Fig. 2). 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 ESBL gene *bla*_{CTXM-14b} (Fig. 1b, Fig. 2a). pOXA-48-3 plasmids resembled pOXA-48-5 plasmids, however 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. 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 differ 3.4 kB in size (Fig. 2a). 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. These plasmids had either non-IncL/M type of replicons *e.g.* IncR, IncY, IncF, or IncA or no known replicons (Fig. 1a + b). In addition, they had plasmid sizes that differed from the those in the different plasmid groups and %G+C content and predominantly originated from isolates from the Netherlands (Fig. 2).

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 region 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 *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, indicating these plasmids are non-conjugative (Fig. 3b). In pOXA-48-4 plasmids, the *tra* conjugation system is incomplete, while pOXA-48-5 plasmids contain 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 lacked this system. IS1 family transposases IS1R, 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 harboring 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 type 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 minimum spanning tree (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} and *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 kb, ~11 kb or ~20 kb. The *bla*_{OXA-48}-like genetic element was flanked by IS1 family transposase 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 was 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. The *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-nucleotide direct repeat (DR) GGTAATGACTCCAAC was typically located directly upstream IS1R thereby flanking the *bla*_{OXA-48}-like insertion element. This DR sequence occurs on average 1 to 2x in pOXA-48-1 to pOXA-48-5 plasmids, except in pOXA-181 and pOXA-232 plasmids (Fig. 5c). The DR was found on average 9x in the 47 *E. coli* chromosomes with *bla*_{OXA-48}-like, compared to 4.6x the 5 *K. pneumoniae* chromosomes containing *bla*_{OXA-48}. The DR occurred on average 9x, 8x, and 4x in *E. coli* ST38, ST69 and ST127, respectively. In only four of the 52 chromosomes analyzed, the *bla*_{OXA-48} region was flanked by one single DR sequence if the orientation of the carbapenemase allele was in reverse orientation (Table 3). In one chromosome, no DR sequence or truncates hereof were found. In *K. pneumoniae*, in two ST45 of the four isolates with *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 alleles and 44 *bla*_{OXA-48}-like containing chromosomes of *E. coli* and *K. pneumoniae* isolates obtained from the Dutch national CPE surveillance program 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 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 IS sequences, in addition to conjugation machinery may attributed 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 been 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 (20–22). *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 antimicrobial resistance plasmids to new genetic

backgrounds. To date, no double combinations of *bla*_{OXA-48}-like of alleles 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 found occasionally (6, 23). 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, suggests that multiple transposition and recombination events have occurred. The chromosomal *bla*_{OXA-48} segment likely 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, but not in pOXA-181 and 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 harbor 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 distinct *bla*_{OXA-48}-like alleles had different meropenem susceptibilities in *K. pneumoniae* and *E. coli* isolates, indicating that not each allele results in the same resistance phenotype. Especially *K. pneumoniae* containing *bla*_{OXA-232} were highly resistant, which can possibly be attributed to a high copy number of pOXA-232 plasmids (24). Alternatively, OXA-48 enzyme production, an altered affinity for meropenem, or other determinants such as porins, efflux pumps or the presence of additional extended-spectrum β -lactamases can be responsible for this phenomenon as well (25, 26).

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.

Materials and methods

Bacterial isolates. For the Dutch National CPE Surveillance program, medical microbiology laboratories from the Netherlands routinely send *Enterobacterales* isolates with a meropenem minimum inhibitory concentration (MIC) of >0.25 mg/L and/or an imipenem MIC of >1 mg/L or genotypical or phenotypical 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 January 1st 2014 until December 31st 2019 (Table S1, Suppl. File 1). 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 Inc., Marcy l'Etoile, France). Based on the clinical breakpoints according to EUCAST, the isolates were classified as sensitive (≤2 mg/L), intermediate (>2 mg/L and ≤8 mg/L) and resistant (>8 mg/L) to meropenem. Isolates were analyzed for carbapenemase production using the carbapenem inactivation method (CIM) (27).

Next-generation sequencing. *E. coli* and *K. pneumoniae* isolates were subjected to next-generation sequencing (NGS) using the Illumina HiSeq 2500 (BaseClear, Leiden, the Netherlands). 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 (28, 29). 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, wgMLST profiles were imported into BioNumerics version 7.6.3 for subsequent comparative analyses (Applied Maths, Sint-Martens-Latem, Belgium).

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. A shearing step was performed using g-TUBE's (Covaris) to obtain an average DNA fragment size of 8 kb for isolates from 2014-2018. This shearing step was omitted for isolates from 2019. The DNA was repaired using FFPE and end-repair kits (New England BioLabs) followed by ligation of barcodes with bead clean up using AMPure XP (Beckman Coulter) after each step. Barcoded isolates were pooled and sequencing adapters were added by ligation. The final library was loaded onto a MinION flow cell (MIN-106 R9.4.1). The 48-hour sequence run was started without live base calling enabled on a MinION device connected to a desktop computer. After the sequence run, base calling and de-multiplexing was performed using Albacore 2.3.1 and a single FASTA file per isolate was extracted from the FAST5 files using Poretools 0.5.1 (30). Illumina and Nanopore data were used in a hybrid assembly performed by Unicycler v0.4.4 (31). The resulting contig files were annotated using Prokka and were subsequently loaded into BioNumerics for further analyses (32).

Plasmid content analysis. For annotation a Conda environment was set up with packages to facilitate a Snakemake pipeline which could process samples in bulk, perform initial annotation with Prokka and enhancement with BLAST+ (33, 34). Prokka annotation was executed in two stages, in the first stage it identified the coordinates of candidate genes with Prodigal, in the second step it predicted these genes by utilizing user set databases and its default the Swissprot database (35, 36). To preserve the speed of the initial annotation we prepared a small database by combining sequence data from the ResFinder database and the PlasmidFinder database (28, 29). If Prokka was unable to predict a gene it will label 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 (Suppl. File 2). BioNumerics was used to extract and analyze the presence of annotated genes and transposases in the different plasmids. The data was plotted, analyzed and visualized in Excel.

Plasmid and chromosome comparisons. BioNumerics was used to compare complete plasmid DNA sequence and circular and linear chromosome datasets. Linear assembly contigs were omitted in this study. 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. All chromosomes have the *dnaA* gene as 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.

Ethics statement. The bacterial isolates belong to the medical microbiological laboratories participating in the Dutch National CPE Surveillance program and were obtained as part of routine clinical care in the past years. Since no identifiable personal data were collected and data were analyzed 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.

Minimum Spanning Tree and UPGMA analyses. The BioNumerics software was used to generate a minimum spanning tree (MST) or an UPGMA hierarchical clustering as described previously (3). The MST was based on in-house *E. coli* and *K. pneumoniae* wgMLST schemes. The categorical coefficient was used to calculate the MST.

NGS and plasmid data availability. The Illumina (NGS) sequence data set generated and analyzed in this study are available in NCBI in the European Nucleotide Archive (ENA) under project number xxx. The plasmid sequences are deposited in Genbank and available through the accession numbers xxx.

Figure legends

Figure 1. Genetic clustering of *bla*_{OXA-48}-like plasmids. (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 black. 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) Similar as (a) and (b) for the non-cluster plasmids.

Figure 2. *bla*_{OXA-48}-like plasmids have distinct characteristics. (a) Graphs show the distinct characteristics e.g. the number of AMR genes, the size in kilo base pairs and the %G+C content of the distinct pOXA-48-like groups of plasmids. Each dot represents a value per plasmid. Scale bars indicated the variation per group.

Figure 3. *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. Colors indicated 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 as (a) but displays diversity in pOXA-181 and pOXA-232 plasmid gene content.

Figure 4. Distribution of chromosome- or plasmid-localized *bla*_{OXA-48} or *bla*_{OXA-244}. (a) Minimum spanning tree of *E. coli* in which chromosome- or plasmid-localized *bla*_{OXA-48} or *bla*_{OXA-244} alleles are indicated by different colors. (b) Similar as in (a) but for *K. pneumoniae*. (c) The presence of AMR genes among the chromosomes analyzed 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 colors.

Figure 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 open reading frames of which *bla*_{OXA-48} is depicted in red and *bla*_{OXA-244} in light red. DR indicated the direct repeat sequence GGTAATGACTCCAAC located upstream IS1R. Sequence types are depicted by ST and sizes of the different insertion sequences are indicated in kilobase (kb). (c) Frequency of the DR sequence in *bla*_{OXA-48}-like plasmids and chromosomes of *E. coli* and *K. pneumoniae*.

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

<i>bla</i> _{OXA-48} -like allele	<i>E. coli</i>				<i>K. pneumoniae</i>				Total
	S	I	R	All	S	I	R	All	
<i>bla</i> _{OXA-48}	145	10	2	157	102	52	63	217	374
<i>bla</i> _{OXA-181}	33	2	1	36	14	3	4	21	57
<i>bla</i> _{OXA-232}						2	17	19	19
<i>bla</i> _{OXA-244}	28	2		30	1	1		2	32
<i>bla</i> _{NDM-1} , <i>bla</i> _{OXA-48} -like					1	3	21	25	25
<i>bla</i> _{NDM-5} , <i>bla</i> _{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 mg/L), intermediate (I; >2 to 8 mg/L) and resistant (R; >8 mg/L) for meropenem.

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

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

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

Species	MLST ST	Carba allele	Location of <i>bla</i> _{OXA-48} -like	Location of <i>bla</i> _{OXA-48} -like fragment	Size	Nr. of DR's in chromosome	DR's flanking <i>bla</i> _{OXA-48} -like		
<i>E. coli</i>									
cRIVM_C012086	38	<i>bla</i> _{OXA-48}	1226615	1227412	1227631	1216632	-10999	6	1
cRIVM_C014114	38	<i>bla</i> _{OXA-48}	837858	838655	838907	818476	-20431	10	2
cRIVM_C014186	38	<i>bla</i> _{OXA-48}	3322083	3322880	3323099	3321208	-1891	5	2
cRIVM_C017996	38	<i>bla</i> _{OXA-48}	1271878	1272675	1272927	1261895	-11032	7	2
cRIVM_C018219	38	<i>bla</i> _{OXA-48}	4497614	4498411	4497362	4517799	20437	11	2
cRIVM_C018562	38	<i>bla</i> _{OXA-48}	1225857	1226654	1226873	1215874	-10999	5	2
cRIVM_C018566	38	<i>bla</i> _{OXA-48}	4429086	4429883	4428834	4449271	20437	11	2
cRIVM_C018582	38	<i>bla</i> _{OXA-244}	5316678	5317475	5317694	5297296	-20398	9	2
cRIVM_C018698	38	<i>bla</i> _{OXA-48}	4450722	4451519	4450470	4470907	20437	13	2
cRIVM_C018706	38	<i>bla</i> _{OXA-48}	4437017	4437814	4436798	4457202	20404	11	2
cRIVM_C019174	38	<i>bla</i> _{OXA-48}	1293474	1294271	1294490	1283491	-10999	7	2
cRIVM_C019187	38	<i>bla</i> _{OXA-48}	4379381	4380178	4378887	4390834	11947	12	2
cRIVM_C019211	38	<i>bla</i> _{OXA-48}	5148725	5149522	5149741	5129343	-20398	5	2
cRIVM_C019217	38	<i>bla</i> _{OXA-244}	102972	103769	102433	104975	2542	12	2
cRIVM_C019381	38	<i>bla</i> _{OXA-244}	102972	103769	102478	104598	2120	8	2
cRIVM_C019486	38	<i>bla</i> _{OXA-48}	4458895	4459692	4458676	4479080	20404	9	2
cRIVM_C019528	38	<i>bla</i> _{OXA-48}	4342207	4343004	4341988	4362392	20404	13	2
cRIVM_C019616	38	<i>bla</i> _{OXA-244}	106793	107590	106574	126972	20398	6	2
cRIVM_C019622	38	<i>bla</i> _{OXA-48}	4455075	4455872	4454823	4475254	20431	8	2
cRIVM_C000059	38	<i>bla</i> _{OXA-48}	3998627	3999424	3998408	4001066	2658	7	2
cRIVM_C028470	38	<i>bla</i> _{OXA-48}	3998582	3999379	3998088	4001021	2933	7	2
cRIVM_C028944	38	<i>bla</i> _{OXA-244}	5246611	5247408	5247627	5227229	-20398	5	2
cRIVM_C029551	38	<i>bla</i> _{OXA-48}	1219470	1220267	1209487	1220734	-11247	10	2
CP032145_1	38	<i>bla</i> _{OXA-48}	844693	845490	845709	827237	-18472	9	2
CP040390_1	38	<i>bla</i> _{OXA-48}	4461613	4462410	4461394	4479866	18472	11	2
cRIVM_C010150	69	<i>bla</i> _{OXA-48}	3898487	3899284	3900200	3879422	-20778	4	2
cRIVM_C018575	69	<i>bla</i> _{OXA-48}	749018	749815	750731	747656	-3075	8	1
cRIVM_C000053	69	<i>bla</i> _{OXA-48}	749016	749813	750729	747654	-3075	8	1
cRIVM_C029547	69	<i>bla</i> _{OXA-244}	102728	103525	102509	116091	13582	12	2
cRIVM_C018100	99	<i>bla</i> _{OXA-48}	79721	80518	79502	99900	20398	5	2
cRIVM_C014045	127	<i>bla</i> _{OXA-48}	nd	nd	nd	nd	20673	3	2
cRIVM_C017886	127	<i>bla</i> _{OXA-48}	4003811	4004608	4003592	4023990	20398	4	2
cRIVM_C018149	127	<i>bla</i> _{OXA-48}	nd	nd	nd	nd	nd	8	2
cRIVM_C019118	127	<i>bla</i> _{OXA-48}	3939883	3940680	3939664	3960068	20404	4	2
cRIVM_C019384	127	<i>bla</i> _{OXA-48}	3968348	3969145	3968129	3988533	20404	3	2
cRIVM_C019437	127	<i>bla</i> _{OXA-48}	3884417	3885214	3884198	3904602	20404	2	2
cRIVM_C019469	127	<i>bla</i> _{OXA-48}	4000159	4000956	3999940	4020344	20404	6	2
cRIVM_C019658	127	<i>bla</i> _{OXA-48}	3910758	3911555	3910539	3930943	20404	2	2
cRIVM_C018248	349	<i>bla</i> _{OXA-244}	89993	90790	89454	91619	2165	15	2
cRIVM_C011531	361	<i>bla</i> _{OXA-244}	80789	81568	80376	100968	20592	36	2
cRIVM_C018403	940	<i>bla</i> _{OXA-48}	3841159	3841956	3840907	3859484	18577	25	2
cRIVM_C019699	1722	<i>bla</i> _{OXA-244}	102501	103298	101585	115864	14279	2	2
CP38505_1	nd	<i>bla</i> _{OXA-244}	102956	103753	102737	123135	20398	10	2
CP050382_1	nd	<i>bla</i> _{OXA-244}	80907	81704	80688	82533	1845	25	2
<i>K. pneumoniae</i>									
cRIVM_C014072	45	<i>bla</i> _{OXA-48}	4405354	4406151	4404860	4425910	21050	6	2
cRIVM_C018499	45	<i>bla</i> _{OXA-48}	4348845	4349642	4348626	4369024	20398	7	2
cRIVM_C015656	101	<i>bla</i> _{OXA-48}	262228	263025	262095	264541	2446	0	0
cRIVM_C015042	377	<i>bla</i> _{OXA-48}	2534772	2535569	2535788	2515390	-20398	9	2
NZ-CP040023_1	nd	<i>bla</i> _{OXA-48}	1406533	1407330	1405017	1407743	2726	1	1

Size of the *bla*_{OXA-48}-like insertion element is excluding the IS1R-IS1D elements.

If a number denotes a “-”, the *bla*_{OXA-48}-like element is in reverse orientation.

Table S1. CPE analyzed in this study.

Year	<i>E. coli</i>	<i>K. pneumoniae</i>	Total
2014	7	13	20
2015	15	36	51
2016	30	43	73
2017	38	51	89
2018	58	87	145
2019	82	77	159
Total	230	307	537

479 **Table S2. Classical MLST sequence types of the isolates analyzed in this study.**

<i>E. coli</i>					<i>K. pneumoniae</i>				
ST	<i>bla</i> _{OXA-48}		<i>bla</i> _{OXA-244}		ST	<i>bla</i> _{OXA-48}		<i>bla</i> _{OXA-181}	
	Chromosome	Plasmid	Chromosome	Plasmid		Chromosome	Plasmid	Plasmid	Plasmid
10					14				
23					15				
38					16				
46					17				
69					35				
70					36				
99					45				
127					101				
131					133				
164					147				
167					219				
205					231				
216					294				
224					307				
297					320				
349					361				
354					377				
361					383				
362					391				
372					392				
405					395				
410					405				
448					437				
453					449				
540					628				
648					661				
746					784				
939					792				
940					873				
963					985				
1049					1207				
1056					1243				
1064					1537				
1722					1540				
2521					1824				
5552					2004				
6262					2096				
6691					2497				
7480					2499				
8346					2502				
					2633				
					2731				
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C

b

Variable region

a

E. coli (n= 230)

b

K. pneumoniae (n= 307)

c

E. coli *K. pneumoniae*