Clinical Microbiology and Infection 26 (2020) 1412.e7–1412.e12

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

Molecular characteristics of carbapenemase-producing *Enterobacterales* in the Netherlands; results of the 2014–2018 national laboratory surveillance

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ARTICLE INFO

Article history:
Received 11 October 2019
Received in revised form
17 December 2019
Accepted 21 January 2020
Available online 5 February 2020

Editor: P.T. Tassios

Keywords:
Carbapenemase activity
Carbapenemase genes
CPE
NGS
Surveillance

ABSTRACT

Objectives: Carbapenem resistance mediated by mobile genetic elements has emerged worldwide and has become a major public health threat. To gain insight into the molecular epidemiology of carbapenem resistance in The Netherlands, Dutch medical microbiology laboratories are requested to submit suspected carbapenemase-producing *Enterobacterales* (CPE) to the National Institute for Public Health and the Environment as part of a national surveillance system.

Methods: Meropenem MICs and species identification were confirmed by E-test and MALDI-TOF and carbapenemase production was assessed by the Carbapenem Inactivation Method. Of all submitted CPE, one species/carbapenemase gene combination per person per year was subjected to next-generation sequencing (NGS).

Results: In total, 1838 unique isolates were received between 2014 and 2018, of which 892 were unique CPE isolates with NGS data available. The predominant CPE species were *Klebsiella pneumoniae* ($n = 388$, 43%), *Escherichia coli* ($n = 264$, 30%) and *Enterobacter cloacae* complex ($n = 116$, 13%). Various carbapenemase alleles of the same carbapenemase gene resulted in different susceptibilities to meropenem and this effect varied between species. Analyses of NGS data showed variation of prevalence of carbapenemase alleles over time with $bla_{\text{OXA-48}}$ being predominant (38%, 336/892), followed by $bla_{\text{NDM-1}}$ (16%, 145/892). For the first time in the Netherlands, $bla_{\text{OXA-181}}$, $bla_{\text{OXA-232}}$ and $bla_{\text{VIM-4}}$ were detected. The genetic background of *K. pneumoniae* and *E. coli* isolates was highly diverse.

Conclusions: The CPE population in the Netherlands is diverse, suggesting multiple introductions. The predominant carbapenemase alleles are $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$. There was a clear association between species, carbapenemase allele and susceptibility to meropenem. **K. van der Zwaluw, Clin Microbiol Infect 2020;26:1412.e7–1412.e12**

Introduction

Carbapenems are often used to treat patients infected with bacteria that are resistant to cephalosporins, such as extended spectrum β-lactamase-producing strains [1,2]. Resistance to carbapenems is emerging worldwide and can be caused by different mechanisms, such as porin loss, increased efflux pump activity and carbapenemase production [3,4]. Carbapenemase-producing Enterobacterales (CPE) are a threat to public health as carbapenemase-encoding genes such as $bla_{\text{OXA-48}}$, bla_{NDM} and bla_{KPC} are predominantly located on plasmids, mobile elements that can easily be exchanged between species, leading to rapid spread of resistance [5]. Because different countries have found significant variation in prevalence and distribution of carbapenemase-encoding genes [6], the National Institute for Public Health and the Environment (RIVM) has started surveillance for CPE in 2012 to assess the molecular epidemiology of CPE in the Netherlands [7]. In this study, we describe the molecular

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https://doi.org/10.1016/j.cmi.2020.01.027

characteristics of CPE isolates obtained within the national surveillance system in the Netherlands between 2014 and 2018.

Materials and methods

Selection of bacterial isolates

For the national surveillance of CPE in The Netherlands, Dutch medical microbiology laboratories are requested to submit human *Enterobacterales* isolates with a reduced MIC for meropenem (>0.25 mg/L) or imipenem (>1 mg/L) to the RIVM using an online platform, Type-Ned. This low MIC threshold for submission was chosen to monitor CPE rather than carbapenems-resistant *Enterobacterales* (CRE), because CPE represent a reservoir for the spread of resistance genes. Of all submitted *Enterobacterales* isolates, only the first unique species-gene combination per person per year was included in this study. People from whom CPE were isolated are referred to as 'persons', because they include both patients and healthy individuals. Isolates from Dutch Caribbean territories were excluded.

Confirmation, carbapenemase detection and PCR testing of bacterial isolates

All submitted isolates were characterized using the following methods: species identification was confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF; Bruker Daltonics GmbH, Bremen, Germany) and the MIC for meropenem of all isolates was confirmed by E-test (BioMérieux Inc., Marcy L'Étoile, France). Carbapenemase production was determined phenotypically by the Carbapenem Inactivation Method (CIM) [8]. Carbapenemase-encoding genes were assessed by two in-house developed multiplex PCR assays using primers targeting the following genes (see Supplementary material, Table S1): KPC, NDM, OXA-48-like, VIM, IMP [9], OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like, OXA-134-like and the bacterial 16S rRNA gene as an internal positive control [10]. PCR conditions were as described previously [8].

Next-generation sequencing of bacterial isolates

Next-generation sequencing (NGS) was performed on all CPE isolates included in this study, defined as being either CIM and/or PCR-positive. Nucleic acid extraction was done using an in-house protocol. Library preparation and sequencing of bacterial genomes were performed using the Illumina Nextera XT kit and the HiSeq 2500 with a paired-end 100 cycles protocol. Nucleic acid extraction and sequencing were performed by BaseClear (Leiden, the Netherlands). Resulting 125 base reads were trimmed and *de novo* assembly was performed using CLC Bio 9.5.3 (Qiagen Bioinformatics, Aarhus, Denmark). Samples that yielded over 200 contigs with *de novo* assembly were regarded as unreliable and omitted from the study. In addition, we excluded contigs shorter than 500 bp or with an average read coverage below 30 from further analyses. To identify carbapenemase alleles and assess the presence of other resistance genes, ResFinder 2.1 [11] was used with an identification threshold of 90% and a minimum length of 60%. PCR results were used to confirm ResFinder data and discrepant tests were repeated to rule out isolate mix-ups or contamination and to prevent detecting truncated genes. For *Klebsiella pneumoniae* and *Escherichia coli* isolates, multilocus sequence types (MLST) and whole genome MLST (wgMLST) were extracted from NGS data using SeqSphere 3.5.0 (Ridom GmbH, Münster, Germany). For MLST, existing schemes were used [12,13]. For wgMLST, in-house wgMLST schemes were designed. For the *K. pneumoniae* isolates, this scheme comprised 4978 genes (3471 core-genome and 1507 accessory-genome targets) using *K. pneumoniae* MGH 78578 (NC_009648.1) as a reference genome. For the *E. coli* isolates, the in-house wgMLST scheme was designed using *E. coli* strain CP000247.1 as a reference genome and included 4503 genes (3199 core-genome and 1304 accessory-genome targets). Sequence reads were deposited in the European Nucleotide Archive (ENA) under study accession number PRJEB35685 (http://www.ebi.ac.uk/ena/data/view/PRJEB35685).

Results

From 2014 until 2018, 1838 unique *Enterobacterales* isolates suspected of carbapenemase production were submitted to the RIVM. Of these, 892 (from 766 persons) were phenotypically positive for carbapenemase activity and/or genotypically positive for a carbapenemase-encoding gene and had reliable NGS data available. For 303 persons (40%), an association with a stay abroad was known (top countries: Turkey (n = 63), Morocco (n = 47), India (n = 26)). The annual number of CPE isolates increased from 49 to 304 (Table 1), meanwhile the number of participating laboratories increased from 40 to 51. During the study period, there were three known outbreaks with CPE: an outbreak in a hospital with 35 $bla_{\text{NDM-1}}$-positive *K. pneumoniae* in 2015 [14], an outbreak in a nursing home with eight $bla_{\text{VIM-1}}$-positive *E. coli* in 2017 and one in a hospital with 23 $bla_{\text{NDM-5}}$-positive *Citrobacter freundii* in 2018.

Characteristics of bacterial isolates

Among the 892 CPE isolates, the predominant species were *K. pneumoniae* (n = 388, 43%), *E. coli* (n = 264, 30%) and *Enterobacter cloacae* complex (n = 116, 13%) (Table 1). Overall, 275 (31%) were phenotypically resistant for meropenem (EUCAST clinical breakpoint, MIC >8 mg/L), 445 isolates (50%) were susceptible (MIC

Table 1
Distribution of species among unique[a] Dutch CPE isolates analysed by NGS, 2014–2018

	2014 *n* (%)	2015 *n* (%)	2016 *n* (%)	2017 *n* (%)	2018 *n* (%)	**Total *n* (%)**
No. of CPE with NGS	49	147	160	232	304	**892** 66[b]
Klebsiella pneumoniae	21 (43)	75 (51)	73 (46)	98 (42)	121 (40)	**388 (43)** 35[b]
Escherichia coli	14 (29)	34 (23)	47 (29)	80 (34)	89 (29)	**264 (30)** 8[b]
Enterobacter cloacae complex	8 (16)	22 (15)	16 (10)	32 (14)	38 (13)	**116 (13)**
Citrobacter freundii	2 (4)	8 (5)	4 (3)	9 (4)	33 (11)	**56 (6)** 23[b]
Klebsiella oxytoca	1 (2)	2 (1)	5 (3)	4 (2)	6 (2)	**18 (2)**
Proteus mirabilis	2 (4)	3 (2)	5 (3)	1 (0)	5 (2)	**16 (2)**
Other (15 species)	1 (2)	3 (2)	10 (6)	8 (3)	12 (5)	**34 (4)**

[a] One species/carbapenemase gene combination per person per year, excluding Dutch Caribbean territories.
[b] Isolates belonging to a known outbreak.

≤2 mg/L) and the remaining 172 isolates (19%) had intermediate resistance levels. Sixty-four persons simultaneously carried two to four distinct species with the same carbapenemase-encoding allele, 31 carried multiple species with different carbapenemase-encoding alleles and eight carried multiple strains of the same species with distinct carbapenemase-encoding alleles. Seven persons persistently carried the same species–allele combination over a period of 2 or 3 years.

Carbapenemase-encoding alleles

The most prevalent alleles found were *bla*$_{OXA-48}$ (38%, 336/892) and *bla*$_{NDM-1}$ (16%, 145/892) (Fig. 1). In total, 26 different carbapenemase alleles were identified, including several alleles not reported before in the Netherlands (see Supplementary material, Table S2). In 41 isolates, 34 of which belonged to *Enterobacter cloacae* complex, no carbapenemase allele could be identified despite their phenotypic carbapenemase activity as measured by CIM.

The alleles *bla*$_{OXA-48}$ and *bla*$_{NDM-1}$ were found across all species, whereas >90% of *bla*$_{KPC-3}$ and *bla*$_{OXA-244}$ alleles were found in *K. pneumoniae* and *E. coli* isolates, respectively. The *bla*$_{OXA-23}$ allele only occurred among *Proteus mirabilis* isolates.

Thirty-five CPE isolates carried a *bla*$_{NDM}$ allele simultaneously with another carbapenemase allele. The number of isolates with multiple carbapenemase alleles increased over time from a single isolate in 2014 to 20 in 2018.

Association between carbapenemase allele and susceptibility to meropenem

There was a marked association between susceptibility to meropenem and the carbapenemase allele. Of all isolates carrying *bla*$_{OXA-48}$, only 18% (62/336) were resistant, whereas among isolates with *bla*$_{NDM-5}$ this proportion was 68% (62/91). Different alleles of the same carbapenemase gene do not result in similar susceptibility distributions either. For instance, of the isolates harbouring allele *bla*$_{NDM-1}$ only 31% (45/145) were resistant. Also, the relationship between susceptibility to meropenem and the carbapenemase allele is different between *K. pneumoniae* and *E. coli* isolates (Fig. 2). Interestingly, 98% of *K. pneumoniae* carrying *bla*$_{OXA-48}$ (168/172) or *bla*$_{NDM-1}$ (90/92) also carried an extended-spectrum β-lactamase. For *E. coli* carrying *bla*$_{OXA-48}$ or *bla*$_{NDM-1}$, these values were 80% (77/96) and 62% (16/26), respectively. The globally occurring extended-spectrum β-lactamase gene *bla*$_{CTX-M-15}$ [15] was found in 106/172

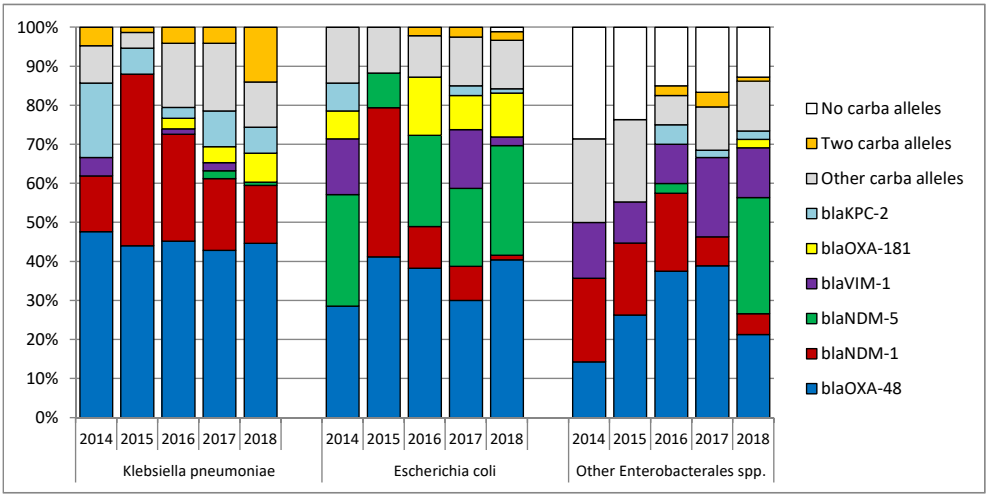

Fig. 1. Distribution of carbapenemase alleles in Dutch carbapenemase-producing *Enterobacterales* isolates for *Klebsiella pneumoniae*, *Escherichia coli* and other *Enterobacterales*.

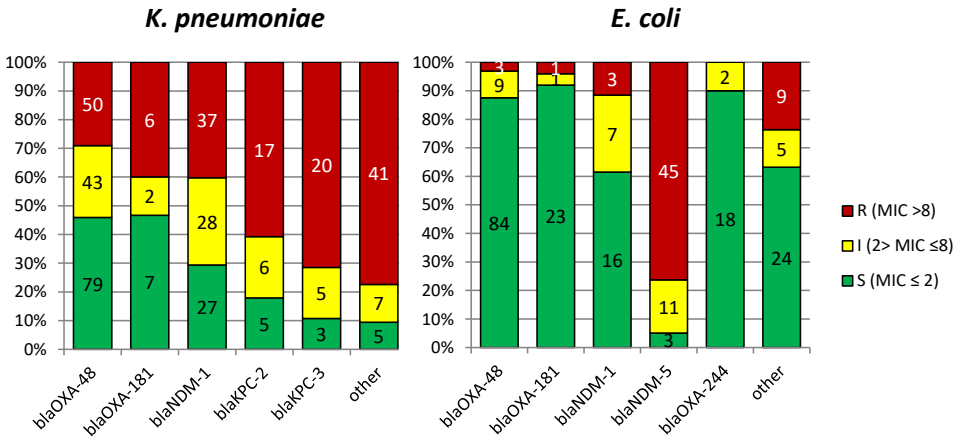

Fig. 2. Distribution of susceptibility to meropenem, according to EUCAST guidelines for clinical breakpoints, among the five most frequently found carbapenemase alleles in Dutch *Klebsiella pneumoniae* (left) and *Escherichia coli* (right) isolates 2014–2018.

(62%) of *K. pneumoniae* carrying bla_{OXA-48} and 76/92 (82%) of *K. pneumoniae* carrying bla_{NDM-1}, among *E. coli* carrying bla_{OXA-48} or bla_{NDM-1}, these values were 15% (14/96) and 19% (5/26) respectively.

An increasing number of isolates carried two carbapenemase alleles. Of these, 30/35 isolates (86%) had a resistant phenotype of which 29 had a MIC of ≥32 mg/L. Compared to isolates carrying a single carbapenemase allele, isolates harbouring two carbapenemase alleles were significantly more often resistant to meropenem ($p < 0.00001$, Fisher's exact test).

Population diversity among K. pneumoniae and E. coli isolates

MLST inferred from the NGS data of 388 *K. pneumoniae* isolates showed that the isolates belonged to 96 different classical MLST sequence types (STs). The predominant STs were ST307 ($n = 47$), ST873 ($n = 37$) and ST101 ($n = 35$) accounting for 31% of all submitted carbapenemase-producing *K. pneumoniae* isolates. The inclusion of 35 isolates from an outbreak of NDM-1-producing ST873 *K. pneumoniae* in a Dutch hospital in 2015 caused the abundance of ST873 isolates [14]. ST258, known for its worldwide presence [16], was only found in 11 isolates (3%) in our selection. Among 264 *E. coli* isolates, there were 87 different classical MLSTs, top three being ST38 ($n = 46$), ST167 ($n = 22$) and ST405 ($n = 16$), accounting for 32% of all *E. coli* isolates. ST131, known for its global occurrence [17], was found eleven times, nine of which were isolated in 2017 (see Supplementary material, Table S3). The Simpson's index of diversity for both the *K. pneumoniae* and *E. coli* selections based on classical MLST was 0.948.

Whole genome MLST based on two schemes of 4978 and 4503 genes, respectively, resulted in the identification of 365 different wgMLST profiles for *K. pneumoniae* and 264 for *E. coli*. A minimum spanning tree based on these profiles showed a high degree of genetic diversity within these populations with an average genetic distance between neighbouring isolates of 730 genes for *K. pneumoniae* and 771 genes for *E. coli* (Fig. 3). Plotting bla_{OXA-48} and bla_{NDM-1} on this minimum spanning tree showed that the distribution of these carbapenemase alleles was not restricted to isolates with related genetic background. In Fig. S1 (see Supplementary material), classical MLST types have been indicated as colours in this minimum spanning tree to facilitate comparison of wgMLST with classical MLST.

Discussion

In the Netherlands, CPE isolates occur in many variations of both carbapenemase alleles and genetic composition. Over the years 2014–2018, bla_{OXA-48} was the most predominant allele (40%), followed by bla_{NDM-1} with 19%. Other European countries such as France, also identified these alleles the most frequently [18,19]. During the study period, there were three known CPE outbreaks (bla_{NDM-1}-positive *K. pneumoniae* in 2015, bla_{VIM-1}-positive *E. coli* in 2017 and bla_{NDM-5}-positive *C. freundii* in 2018), which can be found reflected in the results. Even so, there was still considerable variation in carbapenemase allele distribution among CPE species collected in the Netherlands, indicating that the CPE population in the Netherlands is dynamic. Also, wgMLST showed the *K. pneumoniae* and *E. coli* CPE populations were highly diverse. Especially the increasing occurrence of CPE isolates harbouring two distinct carbapenemase alleles combined with the observed higher level of resistance to meropenem among these isolates, may represent an emerging threat.

We identified numerous alleles of carbapenemase-encoding genes that to our knowledge have not been reported in the Netherlands before. Some of these alleles were only found once, but others such as $bla_{OXA-181}$ were increasingly found after their first detection. The predominant classical MLST type among $bla_{OXA-181}$-carrying *K. pneumoniae* and *E. coli* isolates were ST147 and ST410, respectively. These sequence types are known to circulate around the world [20,21], which might be reflected by the increasing occurrence of $bla_{OXA-181}$ in the Netherlands. Another carbapenemase allele worth noting is bla_{VIM-1}, which until 2016 was found rarely among *Enterobacterales* in the Netherlands and globally [22], but was found 25 times in 2017, 11 of which belonged to an outbreak.

Isolates with different carbapenemase alleles had different meropenem susceptibility levels, indicating that each allele does not result in the same resistance phenotype. Differences in the level of expression of the carbapenemases or their affinity for meropenem are most likely responsible for this phenomenon [23,24]. The effect of the

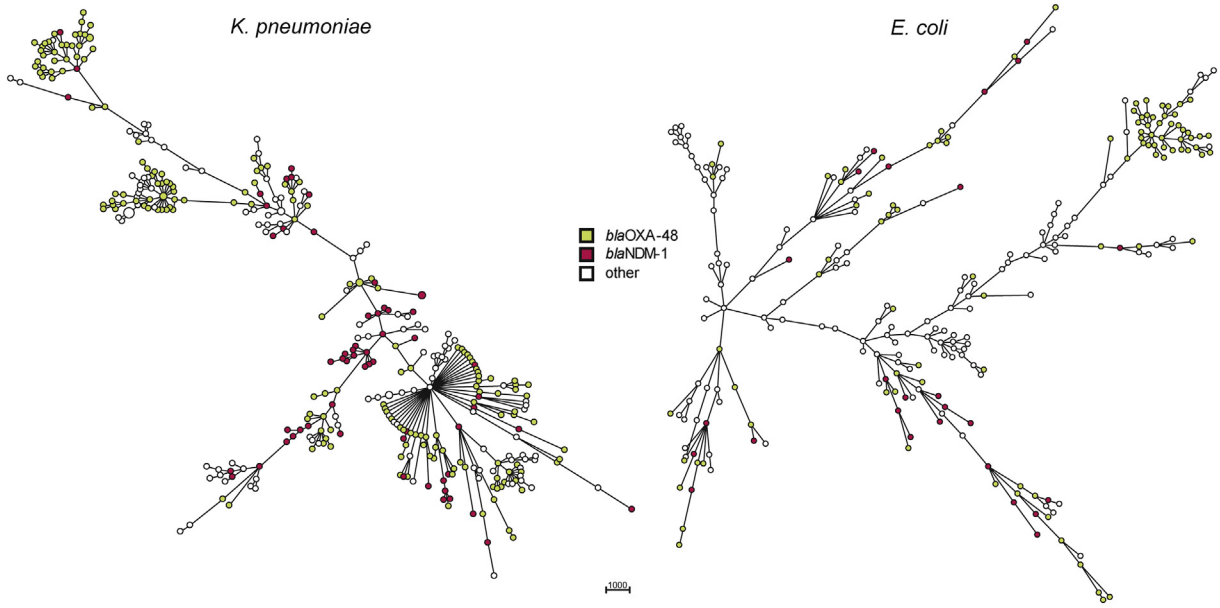

Fig. 3. Minimum spanning tree of *Klebsiella pneumoniae* (left) and *Escherichia coli* (right) carbapenemase-producing *Enterobacterales* isolates with bla_{OXA-48} and bla_{NDM-1} indicated with colours.

carbapenemase allele on the meropenem susceptibility also differed between *K. pneumoniae* and *E. coli* isolates. This may be caused by other factors influencing the susceptibility such as porin loss or efflux pumps [25] or the presence of extended-spectrum β-lactamases, which may be more common and/or more efficient among *K. pneumoniae* than among *E. coli* isolates [26]. Some carbapenemase alleles are primarily found among certain species, which is in concordance with previous findings showing that some of the plasmids on which these alleles are located have a narrow host range [27].

Unfortunately, the NGS data available were based on short-read sequencing, making them unsuitable to infer conclusions on plasmid content or whether resistance genes are chromosomally located or not. This is a limitation of this study.

The occurrence of CIM-positive isolates for which no carbapenemase-encoding gene was identified, might be explained by either false-positivity of the CIM, or presence of a currently unknown carbapenemase-encoding gene. The fact that these were primarily *Enterobacter cloacae* complex might suggest false positivity due to overexpressed AmpC [28,29]; however, among these isolates, 37% (15/41) carried an acquired AmpC gene, not more frequently compared with overall (37%, 326/892). Other mutations or differences in permeability may play a role in their CIM positivity. In addition, these isolates could be tested for carbapenemase activity with a different test such as MALDI-TOF mass spectrometry meropenem-hydrolysis assay [30].

The majority of the CPE isolates (69%) had MICs below the clinical breakpoint for resistance, underlining the distinction between CPE and CRE. Even though CRE might be considered more clinically relevant than CPE, the latter represent a reservoir for spread of resistance genes. To monitor these isolates, our surveillance system uses the low threshold (MIC >0.25 mg/L for meropenem) for submission of isolates. For the national carbapenemase surveillance, we only performed susceptibility testing with meropenem to confirm this threshold. The lack of data on susceptibility of other carbapenems and other antibiotics is another limitation of this study.

Between 2014 and 2018, the number of submitted CPE isolates increased each year. Whether this increase reflects the increased commitment of medical microbiology laboratories to submit samples or is also an actual increase of the occurrence of CPE in the Netherlands is unknown, because the surveillance system in the Netherlands is based on voluntary submission of isolates, which is a third limitation of this study. The implementation of Type-Ned in 2016, where medical microbiology laboratories can register an isolate online, monitor its progress and view results and basic statistics, may have contributed to an increased willingness to submit isolates. In addition, if the occurrence of CPE has indeed increased in the Netherlands, it has not caused an increased proportion of *E. coli* and *K. pneumoniae* isolates with elevated carbapenem MIC values (https://www.rivm.nl/bibliotheek/rapporten/2019-0038.pdf).

Of the 766 persons included in this study, 303 (40%) had a known association with a stay abroad. However, such additional information was only available for 551 persons, resulting in a higher percentage (55%).

In conclusion, during the study period 2014–2018 the number of isolates submitted for the national CPE surveillance increased each year and the majority of the CPE isolates harbour either $bla_{\text{OXA-48}}$ or $bla_{\text{NDM-1}}$ and belong to the species *K. pneumoniae* and *E. coli*. However, we observed an increasing number of CPE isolates with other or multiple carbapenemase alleles. Also, we noticed variations in susceptibility to meropenem between different carbapenemase alleles and species. The prevalence of the carbapenemase alleles varied over time and the genetic backgrounds of both the *K. pneumoniae* and *E. coli* isolates were highly diverse, suggesting multiple introductions of CPE in the Netherlands. This dynamic CPE population in the Netherlands justifies a continued close monitoring using extensive surveillance as described here.

Funding

No external funding was received for this study.

Authors' contributions

All authors have made substantial contributions to and have approved the final manuscript. KZ, LW, LS and TB have been involved in the conception and design of the study. KZ, SW, MS, FL and AH have contributed to the acquisition and analysis of data. KZ, LW, LS and TB have contributed to the interpretation of data.

Transparency declaration

The authors have nothing to disclose.

Acknowledgements

The authors would like to express their gratitude to colleagues of the Department of Bacterial Determinations at RIVM for technical support and to Sabine de Greeff, Annelot Schoffelen, Antoni Hendrickx and Marcel Mennen for critically reviewing the manuscript. Part of these results has also been presented as a poster at the ECCMID in April 2018 in Madrid and as an oral at the KNVM Scientific Spring Meeting in March 2018.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.01.027.

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