



Monitoring of hospital sewage shows both promise and limitations as an early-warning system for carbapenemase-producing Enterobacterales in a low-prevalence setting



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ABSTRACT

Carbapenemase-producing Enterobacterales (CPE) constitute a significant threat to healthcare systems. Continuous surveillance is important for the management and early warning of these bacteria. Sewage monitoring has been suggested as a possible resource-efficient complement to traditional clinical surveillance. It should not least be suitable for rare forms of resistance since a single sewage sample contains bacteria from a large number of individuals. Here, the value of sewage monitoring in early warning of CPE was assessed at the Sahlgrenska University Hospital in Gothenburg, Sweden, a setting with low prevalence of CPE. Twenty composite hospital sewage samples were collected during a two-year period. Carbapenemase genes in the complex samples were analyzed by quantitative PCR and the CPE loads were assessed through cultures on CPE-selective agar followed by species determination as well as phenotypic and genotypic tests targeting carbapenemases of presumed CPE. The findings were related to CPE detected in hospitalized patients. A subset of CPE isolates from sewage and patients were subjected to whole genome sequencing. For three of the investigated carbapenemase genes, *bla*_{NDM}, *bla*_{OXA-48-like} and *bla*_{KPC}, there was concordance between gene levels and abundance of corresponding CPE in sewage. For the other two analyzed genes, *bla*_{VIM} and *bla*_{IMP}, there was no such concordance, most likely due to the presence of those genes in non-Enterobacterales populating the sewage samples. In line with the detection of OXA-48-like- and NDM-producing CPE in sewage, these were also the most commonly detected CPE in patients. NDM-producing CPE were detected on a single occasion in sewage and isolated strains were shown to match strains detected in a patient. A marked peak in CPE producing OXA-48-like enzymes was observed in sewage during a few months. When levels started to increase there were no known cases of such CPE at the hospital but soon after a few cases were detected in samples from patients. The OXA-48-like-producing CPE from sewage and patients represented different strains, but they carried similar *bla*_{OXA-48-like}-harbouring mobile genetic elements. In conclusion, sewage analyses show both promise and limitations as a complement to traditional clinical resistance surveillance for early warning of rare forms of resistance. Further evaluation and careful interpretation are needed to fully assess the value of such a sewage monitoring system.

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1. Introduction

A significant contributing factor to the growing problem of antibiotic resistance is the continuous emergence of novel resistance factors in pathogens via horizontal gene transfer. One such ex-

ample is carbapenemase-producing Enterobacterales (CPE), which have spread globally during the 21st century (Logan and Weinstein, 2017; Pitout et al., 2019). While CPE are commonly detected in clinical samples in many countries, they are still rare in other parts of the world including the Nordic countries (ECDC, 2019).

Up-to-date local surveillance is a cornerstone for the management of antibiotic resistance, in particular for informing empirical treatment before the resistance profile of an infectious agent

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is determined. Resistance surveillance can also guide other management actions, for example by serving as an early warning system for emerging and still rare forms of resistance, including detection of asymptomatic carriage. To be informative surveillance data therefore needs to be based on samples from a large number of individuals, which not only include clinical cultures but also screening cultures to detect carriers. As this is highly resource-demanding and in the case of fecal screening can be questioned also from an ethical perspective (Nijsingh et al., 2020), sewage analyses have emerged as a possible attractive complement to traditional clinical surveillance since a single sewage sample can contain bacteria from thousands of individuals (Aarestrup and Woolhouse, 2020; Huijbers et al., 2019). Recently, resistance rates in sewage and clinical *Escherichia coli* isolates have been shown to correlate (Huijbers et al., 2020; Hutinel et al., 2019). In these studies, the isolation of sewage *E. coli* was conducted using non-selective agar (i.e. no addition of antibiotics). This gives the possibility to assess resistance rates for a wide panel of antibiotics, including various co-resistance patterns, in an un-biased way. The approach is, however, not suitable for studying rare forms of resistance. For these purposes, methodologies more efficient in screening large numbers of bacteria in the sewage samples could be applied, such as cultivation on selective plates or sensitive PCR analyses of total DNA extracted from the samples. In support of a PCR-based approach, a recent PCR array-based study showed that antibiotic resistance gene abundance in sewage could reflect differences in clinical resistance patterns across Europe (Parnanen et al., 2019).

To assess the value of sewage analyses in early warning of rare resistance threats we here aimed to analyze carbapenemase genes and CPE in hospital sewage and relate those observations to CPE in samples from the corresponding hospitalized population. We also aimed to investigate if PCR-based sewage analyses could provide similar information as the more resource-demanding culture-based approach.

2. Materials and methods

2.1. Study setting and CPE isolates from patients

Confirmed CPE cases are still rare in Sweden. CPE are notifiable according to Swedish law and during 2015–2017 the total number of detected cases nationwide was 413 and >85% were either *bla*_{OXA-48-like}- or *bla*_{NDM}-harboring bacteria, mostly imported cases detected through targeted fecal screening (SWEDRES-SVARM, 2019). The study was conducted at the Sahlgrenska University Hospital in Gothenburg, which is the largest hospital in Sweden (1950 beds) with approximately 450 000 patient days/year during the study period. Anonymized information regarding all confirmed cases with CPE at the hospital during the study period was provided by the infection control unit. CPE were detected either in screening samples or in samples taken due to clinical signs of infection. The Clinical Microbiology Laboratory analyzed approximately 14,000 urine samples and 11,000 blood cultures from patients at the main site of the hospital on a yearly basis. Approximately 2000 fecal/rectal screening samples were analyzed each year for the presence of multidrug resistant bacteria, including CPE. Fecal/rectal screening of patients is part of the hospital infection control program, stating that all patients hospitalized abroad within the preceding year and patients carrying certain risk factors (e.g. diarrhea or urinary catheter) in combination with a refugee status should be screened for CPE at or just prior to admission (screened once). In addition, patients at certain units, e.g. hematology and transplantation, are also screened on admission.

Patient samples received at the Clinical Microbiology Laboratory for diagnostic purposes of possible clinical infections were an-

alyzed according to routine clinical microbiology practice including the use of MALDI-TOF for species identification. For antibiotic susceptibility testing, the disk diffusion method and breakpoints according to EUCAST's recommendations at the time were used. Gram-negative isolates were subjected to meropenem susceptibility testing either directly or following detection of cephalosporin or amoxicillin-clavulanic acid resistance. Isolates non-susceptible to meropenem (using the EUCAST screening breakpoint for CPE detection) were screened for *bla*_{NDM}, *bla*_{OXA-48-like}, *bla*_{KPC}, *bla*_{IMP} and *bla*_{VIM} and *bla*_{GES} by PCR (Monteiro et al., 2012). Samples obtained through patient screening (stool or rectal swabs) were plated on Drigalski agar, a selective media for Gram-negative bacteria, in the presence of an ertapenem disk (placed in the primary streak). The plates were incubated at 37° up to 42 h (read daily). No prior enrichment culture was used. All colonies with differing morphologies in a sample with decreased susceptibility to ertapenem (zone diameter <28 mm) were tested further for the detection of CPE as outlined above.

2.2. Sewage samples

A total of twenty composite sewage samples were collected from April 2015 to August 2017 at a sampling point (57°40'52.1"N 11°57'31.4"E) where raw sewage from the great majority of the main hospital site is collected. Each sample was collected over 24 h in a time-proportional manner, i.e. a subsample was collected every ninth minute. Samples were refrigerated during the sampling procedure and after being collected. The composite samples were processed within three hours from being collected.

2.3. Relative quantification of carbapenemase genes in sewage samples by quantitative PCR

Total DNA was isolated from each composite sewage samples by passing 40 mL through 0.45 µm pore size filters applying a vacuum manifold before using a PowerWater® DNA isolation kit (MO BIO Laboratories, Inc, Carlsbad, CA) according to the manufacturer's instructions. The DNA concentrations were measured with a Qubit fluorometer (Invitrogen, Carlsbad, CA). Using quantitative PCR (qPCR), the carbapenemase genes *bla*_{NDM}, *bla*_{OXA-48-like} (including e.g. *bla*_{OXA-48}, *bla*_{OXA-181} and *bla*_{OXA-244}), *bla*_{KPC}, *bla*_{IMP} and *bla*_{VIM} as well as the ESBL gene *bla*_{CTX-M} (group 1 including e.g. *bla*_{CTX-M-15}) were quantified, using 16S as a reference gene. All PCR reactions were run in 96-well plates by mixing 2 µl sewage DNA with 10 µl 2 × Power SYBR™ Green PCR master mix (Applied Biosystems, Carlsbad, CA) and 0.5 µM of PCR primers (Eurofins Genomics, Ebersberg, Germany) (Table S1) in a total volume of 20 µl. Standard amplification conditions described for the 7500 real-time PCR system were used and all reactions were run in duplicates. DNA from *K. pneumoniae* CCUG60138 (*bla*_{NDM}), *K. pneumoniae* CCUG60138 (*bla*_{NDM}), *K. pneumoniae* H080820413 (*bla*_{OXA-48}), *K. pneumoniae* CCUG56233 (*bla*_{KPC}), *P. aeruginosa* CCUG59626 or K44-24 (*bla*_{IMP}), *K. pneumoniae* CCUG58547 (*bla*_{VIM}) and *E. coli* 10-36051A (*bla*_{CTX-M-15}), prepared with the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany), was used as positive controls. The PCR efficiency for each primer pair was in the range of 1.9–2.0 when using standard curves prepared by DNA from positive controls alone and for *bla*_{NDM}, *bla*_{OXA-48-like}, *bla*_{KPC} and *bla*_{VIM} also when the standard curves were prepared by diluting the positive control DNA in complex DNA from a sewage sample negative for the carbapenemase genes. The latter test could not be performed for the other genes since they were ubiquitous in the sewage samples. To further ensure limited interference by PCR inhibitors, hospital sewage DNA was analyzed at three different quantities resulting in 10, 5 and 2.5 ng DNA in the final reactions. For the analysis of the 16S gene, the DNA was 64-fold more diluted than for

the analyses of carbapenemase genes due to the very high 16S abundance. The differences in the dilution of DNA were adjusted for before the formula $2^{CT(16S)-CT(target)}$ was used to calculate the abundance of the different target genes. Following detection in sewage samples, PCR amplicons from at least one selected sample per gene were purified by using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and confirmed to correspond to the target genes by Sanger sequencing (Eurofins Genomics).

2.4. Bacterial cultures

Sewage samples were serially diluted in 0.85% NaCl solution before 100 μ l aliquots of the 10^{-1} , 10^{-2} and 10^{-3} dilutions were used to inoculate CHROMagar™ ECC plates (CHROMagar, Paris, France) in triplicates. CPE-selective plates; ECC supplemented with meropenem (0.25 μ g/ml) and chromID® OXA-48 (Biomerieux, Marcy l'Étoile, France), were inoculated with 100 μ l and 500 μ l of the undiluted samples in triplicates. Samples from 2015 were not cultured on ECC-meropenem plates. All agar plates were incubated at 37 °C for 24 h before colonies were counted. Blue and pink colonies on ECC plates (non-selective) were counted to assess the total number of *E. coli* and other coliforms, respectively. Blue and pink colonies on ECC-meropenem and chromID® OXA-48 were regarded as presumptive CPE. Out of these, the blue colonies on ECC-meropenem and the pink colonies on chromID® OXA-48 plates were presumed to be *E. coli* whereas the remaining were presumed to be other coliforms.

2.5. Species determination of collected sewage isolates

From CPE-selective plates, up to twenty colonies of both presumed *E. coli* and presumed other coliforms per plate type and sample were randomly collected and subjected to species determination by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) using the VITEK® MS system (Biomerieux). In an earlier study, 99.7% of the blue colonies on ECC plates inoculated with the type of sewage samples used here were confirmed to be *E. coli* by MALDI-TOF MS (Hutinel et al., 2019). To assess the number of colonies corresponding to other species on CPE-selective plates, the total count of the non-*E. coli* coliform population was multiplied with the relative abundance of the species within that subpopulation according to MALDI-TOF MS analyses.

2.6. Detection of carbapenemase activity in collected sewage isolates by CARBA NP test

For each sample, if available, five to ten isolates of both *E. coli* and other coliforms (confirmed by MALDI-TOF MS) from both chromID® OXA-48 and ECC-meropenem plates were subjected to Carba NP test according to Dortet et al. (Dortet et al., 2014). *K. pneumoniae* CCUG60138 or CCUG68728 (both *bla*_{NDM}) and *K. pneumoniae* CCUG64452 (*bla*_{OXA-48}) were used as positive controls, whereas *K. pneumoniae* CCUG59346 (*bla*_{AmpC} overexpression) and *K. pneumoniae* CCUG59359 (*bla*_{TEM-52}) served as negative controls.

2.7. Detection of carbapenemase genes in collected sewage isolates by PCR

The isolates subjected to Carba NP test were also screened for carbapenemase genes by PCR. Total DNA was isolated from the strains using the DNeasy® Blood and Tissue Kit (Qiagen) and eluted in 100 μ l provided AE buffer. The PCR reactions consisted of 0.625 U AmpliTaq® polymerase, 2.5 μ l PCR buffer, 3 mM MgCl₂, 0.2 mM dNTPs (all from Applied Biosystems), 0.4 μ M PCR primers (Eurofins Genomics) (Table S1) and 2 μ l DNA in a total volume of 25 μ l. DNA targets were amplified via an initial activation step at

94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C (60 °C for *bla*_{KPC}) for 30 s and 72 °C for 40 s, before a final elongation step at 72 °C for 7 min using a GeneAmp® PCR System 9700 (Applied Biosystems). The same carbapenemase-harboring strains that served as positive controls for the real-time PCR assays were used as controls also here.

2.8. Whole genome sequencing

Based on species and carbapenemase gene content, whole genome sequencing was conducted for sewage and patient isolates with similar profiles. The sequenced isolates, 33 from eight sewage samples and nine from six patients, were each given an ID that starts with an S (sewage) or P (patient) followed by the collection date (YYMMDD) and ends with a running number. Total DNA was prepared from the isolates using the DNeasy® Blood and Tissue Kit (Qiagen) and sent for sequencing at Science for Life Laboratories (Stockholm, Sweden). TruSeq DNA libraries were prepared and sequenced on Illumina MiSeq flowcells producing 2 × 250 bp paired-end reads. Sewage and patient isolates that were most similar to each other, based on sequence type (ST) and/or tentative carbapenemase-harboring plasmid content (information retrieved from Illumina sequencing), were additionally subjected to long-read sequencing in order to allow more complete assembly of the genomes. This was, after isolation of total DNA with Qiagen Genomic tips 500/G, conducted via either single molecule real-time sequencing technology (SMRT) on the PacBio platform (Pacific Biosciences, Menlo Park, CA) or Oxford Nanopore Technology on a MinION using the rapid barcoding kit SQK-RBK004 and a FLO-MIN106 flowcell.

The raw sequencing data was submitted to the sequence read archive and connected to BioProject PRJNA701733.

2.9. Sequence analyses

The short-reads from Illumina sequencing were used in the genome assembly of all isolates. First, the quality of the reads was assessed using FastQC (Andrews, 2010). Then, paired-end reads with low-quality bases were trimmed to reach a score of 20, and single reads with less than 20 bases in length were filtered using Trim Galore (Krueger, 2012). The remaining paired-end and single reads were assembled into longer contigs using SPAdes (with following parameters: -careful -k 21,33,55,77,99,127) (Bankevich et al., 2012). In order to assist the selection of isolates for long-read sequencing, the contigs were mapped to known sequenced plasmids from NCBI genome database (downloaded 13 July 2018) using MUMmer 3.0 (Delcher et al., 2003). The long reads generated subsequently for a subset of the isolates were combined with short reads to produce longer and more accurate contigs using a hybrid assembly approach. Long reads produced by MinION were basecalled using Guppy v3.4.5 in high accuracy mode. Guppy was also used to demultiplex and trim the barcodes from the reads. NanoPlot v1.29.1 was used to assess their quality (De Coster et al., 2018). The short and long reads (produced either on the PacBio or MinION platform) were combined into hybrid assemblies using Unicycler v0.4.8 in conservative mode (Wick et al., 2017).

The ST of the isolates were identified as follows: First, the data related to species of interest, i.e. *E. coli* (Achtman scheme) and *K. pneumoniae* (Pasteur scheme), were downloaded from the databases of allelic profiles and sequences on pubMLST (<https://pubmlst.org/data>, accessed 1 December 2020). Then, contigs were searched against them using the BLASTn algorithm in the BLAST+ package (Camacho et al., 2009). The presence of an exact and complete set of genes was assessed for each isolate and the corresponding ST was reported. Open reading frames (ORFs) on contigs were predicted using Prodigal (v2.6.3) (Hyatt et al.,

Table 1
CPE isolates from patients.

Patient	Specimen ^a	Collection date ^b	CPE ^c	Isolate ID ^d
1	Fecal screen	2015-05-12	<i>K. pneumoniae bla_{VIM}</i>	P-150512-1
2	Fecal screen	2015-06-24	<i>K. pneumoniae bla_{NDM}</i>	P-150624-1
3	Urine	2016-02-13	<i>E. coli bla_{OXA-48-like}</i>	P-160213-1
4 ^e	Wound	2016-02-17	<i>K. pneumoniae bla_{OXA-48-like}</i>	P-160217-1
	Fecal screen	2016-04-05	<i>E. coli bla_{OXA-48-like}</i>	P-160405-1
	Fecal screen	2016-04-05	<i>K. pneumoniae bla_{OXA-48-like}</i>	P-160405-2
5	Urine	2016-05-31	<i>K. pneumoniae bla_{OXA-48-like}</i>	P-160531-1
6	Fecal screen	2016-12-02	<i>E. coli bla_{NDM}</i>	P-161202-1
7	Fecal screen	2017-04-19	<i>E. coli bla_{NDM}</i>	P-170419-1
	Fecal screen	2017-04-19	<i>K. pneumoniae bla_{NDM}</i>	P-170419-2
8	Fecal screen	2017-07-07	<i>E. coli bla_{NDM} + bla_{OXA-48-like}</i>	P-170707-1

^a Fecal screen is either fecal or rectal swab.^b Dates are in the YYYY-MM-DD format and refer to collection of specimen.^c Carbapenemase gene content is based on phenotypic tests and subsequent PCR.^d Isolates in bold were subjected to whole genome sequencing.^e CPE from Patient 4 were isolated at a nearby hospital. Within two weeks before the isolation of the first CPE, the patient had been cared for at the studied hospital.

2010) before Diamond (v0.9.24) (Buchfink et al., 2015) was used to search ORFs against ResFinder database (downloaded 1 December 2020) (Zankari et al., 2012) in order to identify mobile antibiotic resistance genes. Insertion sequences were identified with ISFinder (Siguier et al., 2006). To identify plasmid replicons, contigs were searched against PlasmidFinder database (downloaded 13 July 2020) (Carattoli et al., 2014) using the BLASTn algorithm. Replicon sequences with greater than 95% identity and 60% coverage were reported. The relatedness of isolates/plasmids was also estimated by calculating the two-way Average Nucleotide Identity (ANI) (Goris et al., 2007) with ANI calculator (<http://enve-omics.ce.gatech.edu/ani>). BLASTn was used for the alignment of the nucleotide sequences (Altschul et al., 1990).

2.10. Statistical analysis

Pearson's coefficient was used to evaluate correlation between growth on chromID® OXA-48 plates and levels of *bla_{OXA-48-like}* in sewage. Significance of the correlation coefficient was assessed by *t*-test using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. CPE isolated from patients

During the more than two-year long study period, CPE were only isolated from seven patients hospitalized in wards contributing to the sewage at the sampling point. A patient, from which CPE was isolated at a nearby hospital within two weeks after the patient was cared, but not screened, at the studied hospital, was also included in the study. In total, *E. coli* and/or *K. pneumoniae* isolates positive for *bla_{OXA-48}*, *bla_{NDM}* and *bla_{VIM}* were detected in specimens from four, four and one patient/s, respectively, as shown in Table 1.

3.2. Carbapenemase genes in sewage samples

The *bla_{OXA-48-like}* gene was detected in 19 of 20 analyzed sewage samples using qPCR. After being detected at low levels in the first samples from 2015, a clear peak was seen in the end of that year and the beginning of 2016 (Fig. 1). Levels of *bla_{OXA-48-like}* varied more than 1000-fold. This should be compared to the relatively stable levels observed for *bla_{CTX-M}* (varied 15-fold), which was included as comparison. In contrast to *bla_{OXA-48-like}*, the *bla_{NDM}* gene was only detected in the sewage sample from April 2017 (Fig. 1).

Among the other investigated carbapenemase genes, *bla_{KPC}* was not detected in any of the samples, whereas *bla_{VIM}* and *bla_{IMP}* were detected in 18 and 19 samples, respectively (*bla_{IMP}* was not analyzed in one of the samples) (Fig. 1). The levels of *bla_{VIM}* varied more than 1000-fold, *bla_{IMP}* was consistently detected at relatively high levels and varied less (20-fold).

3.3. CPE in sewage samples

Cultivation on two different selective plates revealed presence of presumptive CPE in all but two of the analyzed sewage samples (Fig. 2). Subsequent genotypic and phenotypic analyses of collected isolates detected OXA-48-like- and NDM-producing CPE, but no CPE producing KPC, VIM or IMP (Tables 2 and 3).

3.3.1. ChromID® OXA-48 plates

When cultured on chromID® OXA-48 plates, presumptive *E. coli* and non-*E. coli* coliforms were detected in all but three and two sewage samples, respectively (Fig. 2A and B). All presumed *E. coli* analyzed by MALDI-TOF MS were verified to be *E. coli* ($n = 92$) (Tables 2 and S2). Out of 45 *E. coli* isolates that were further analyzed, 44 were PCR positive for *bla_{OXA-48-like}* and 39 were positive in the Carba-NP test (Tables 3 and S3).

The great majority of the presumed non-*E. coli* coliforms were identified as *K. pneumoniae* (122/149). The remaining were identified as either *Raoultella* spp. or *Serratia* spp., which were detected in two sewage samples each (Tables 2 and S2). All 64 further tested *K. pneumoniae* and *Raoultella* isolates harbored OXA-48-like genes and 59 of them were also positive in the Carba-NP test. All ten further tested *Serratia marcescens* isolates were negative for the five investigated carbapenemase genes tested by PCR as well as in the CarbaNP test (Tables 3 and S3). In accordance with the qPCR analyses, a peak in growth on the chromID® OXA-48 plates (for both *E. coli* and other coliform) was observed in the beginning of 2016, with much higher levels than observed in the previous samples (Fig. 2A and B). Also in line with the qPCR data was the non-detection of OXA-48-like-producing CPE in the sewage sample from February 2017. Consequently a significant correlation was observed between *bla_{OXA-48-like}* abundance and the total growth of *E. coli*, *K. pneumoniae* and *Raoultella* spp. on chromID® OXA-48 plates (Fig. 3).

3.3.2. ECC-meropenem plates

Cultivation of sewage samples on ECC-meropenem plates showed the presence of presumptive CPE in all but one of the analyzed samples (samples from 2015 were not analyzed on this media) (Fig. 2C and D). Again it was the sample from February 2017

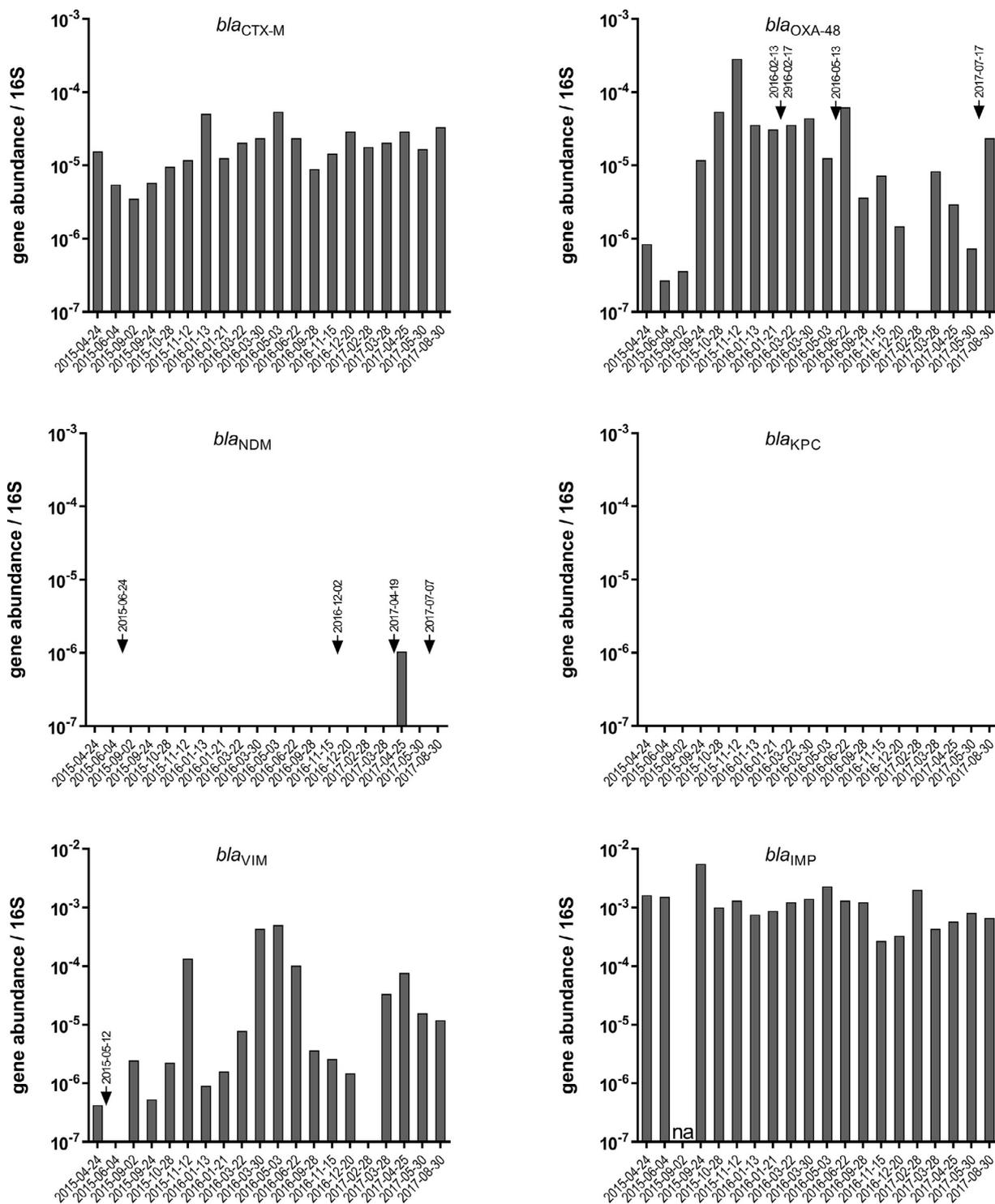


Fig. 1. Relative quantification of ESBL and carbapenemase genes in sewage samples collected on dates (YYYY-MM-DD format) indicated on the x-axis using qPCR. The arrows and dates next to them indicate when patients colonized/infected with CPE carrying the respective carbapenemase were detected. Out of the four NDM-producing CPE from patients, only one was detected close in time to a sewage sampling (2017-04-19). Further details about the patients and the isolated CPE are shown in Table 1. na, not analyzed.

that was negative. Also in parallel with what was seen on chromID® OXA-48 plates, the highest counts for presumptive *E. coli* was seen in the beginning of 2016 and all tested isolates were verified to be *E. coli* by MALDI-TOF MS ($n = 130$) (Tables 2 and S2). Of the 72 *E. coli* isolates subjected to further analyses, 60 were positive in the CarbaNP test and 63 were positive for any of the screened carbapenemase genes – 57 *bla_{OXA-48}*-positive and six

bla_{NDM}-positive (Tables 3 and S3). All *bla_{NDM}*-carrying *E. coli* were isolated from the April 2017 sample, the sample in which *bla_{NDM}* was detected by qPCR.

All tested presumed non-*E. coli* coliforms from the ECC-meropenem plates were confirmed by MALDI-TOF MS (Tables 2 and S2). Hence, only Enterobacterales spp. were identified among the isolates collected from both types of CPE-selective agar plates.

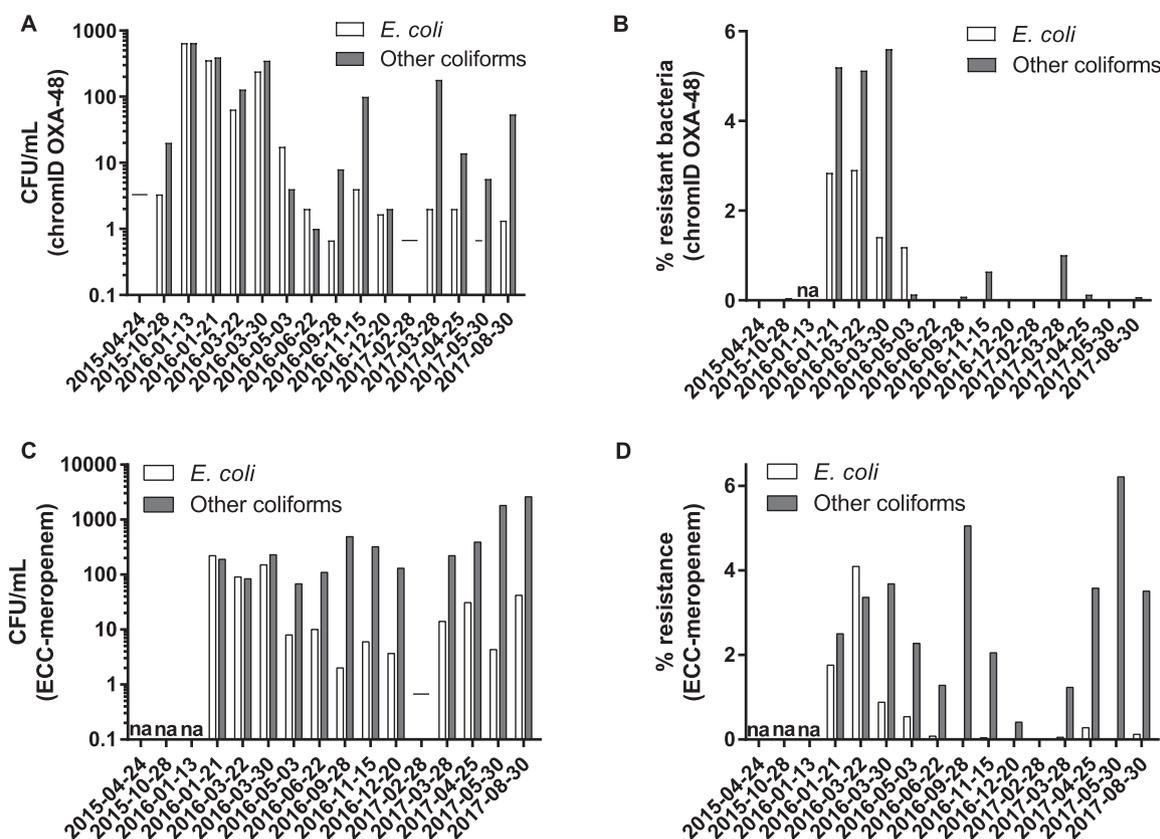


Fig. 2. Presumed CPE in hospital sewage as assessed by cultivation on chromID® OXA-48 plates (A and B) or ECC-meropenem plates (C and D) and expressed as colony forming units (CFU)/mL (A and C) or related to total number of *E. coli* and other coliforms (B and D) for each sampling occasion. The dates for sampling are indicated below the x-axis in the YYYY-MM-DD format. When no presumed CPE were detected, horizontal bars indicate detection limits. na, not analyzed.

Table 2
MALDI-TOF species determinations of collected sewage isolates.

Selective plate	No of presumed <i>E.coli</i> / other coliforms tested	<i>E. coli</i> , n (%) ^a	<i>K. pneum</i> , n (%) ^b	<i>K. oxytoca</i> , n (%) ^b	<i>Enterobacter</i> spp., n (%) ^b	<i>Raoultella</i> spp., n (%) ^b	<i>Serratia</i> spp., n (%) ^b
OXA-48	92/149	92 (100)	122 (82)			16 (11)	11 (7)
ECC-mer	130/173	130 (100)	39 (23)	3 (2)	113 (65)	18 (10)	

K. pneum; *K. pneumoniae*; OXA-48, chromID® OXA-48 plates; ECC-mer, ECC-meropenem plates.

^a Percentage represents the proportion of the tested presumed *E. coli*.

^b Percentage represents the proportion of the tested presumed non-*E. coli* coliforms.

Table 3
CarbaNP and PCR analyses of collected sewage isolates.

Selective plate	Species	No of tested isolates	No of <i>bla</i> _{OXA-48-like} positive	No of <i>bla</i> _{NDM} positive	No of Carbapenemase gene negative ^a	No of CarbaNP positive
OXA-48	<i>E. coli</i>	45	44	0	1	39
	<i>K. pneumoniae</i>	59	59	0	0	54
	<i>Raoultella</i> spp.	5	5	0	0	5
	<i>S. marcescens</i>	10	0	0	10	0
ECC-mer	<i>E. coli</i>	72	57	6	9	60
	<i>K. pneumoniae</i>	14	11	2	1	12
	<i>K. oxytoca</i>	1	0	1	0	1
	<i>Enterobacter</i> spp.	39	1	0	38	1
	<i>Raoultella</i> spp.	7	1	0	6	0

OXA-48, chromID® OXA-48 plates; ECC-mer, ECC-meropenem plates.

^a Negative for *bla*_{OXA-48-like}, *bla*_{NDM}, *bla*_{KPC}, *bla*_{VIM} and *bla*_{IMP}.

K. pneumoniae were, like on chromID® OXA-48 plates, commonly detected on ECC-meropenem media, not least in the beginning of 2016 as well as after cultivation of the sample from April 2017. However, in contrast to what was observed on chromID® OXA-48 plates, the non-*E. coli* coliform population on ECC-meropenem plates was dominated by other species than *K. pneumoniae* and

included *Enterobacter* spp., *Raoultella* spp., and *Klebsiella oxytoca*. Among the 61 isolates from the ECC-meropenem plates subjected to further analyses, a carbapenemase gene could be detected by PCR in almost all (13/14) of the *K. pneumoniae* and the single *K. oxytoca* isolate (Tables 3 and S3). Again *bla*_{OXA-48-like} was the most commonly detected gene. The *bla*_{NDM} gene was detected in

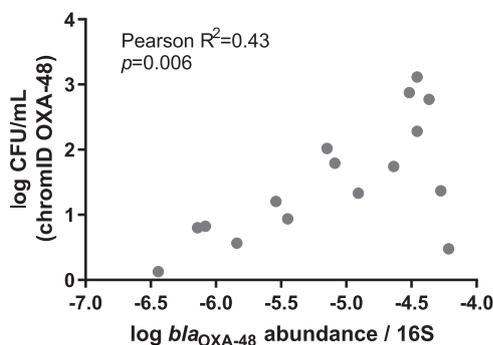


Fig. 3. Correlation between *bla*_{OXA-48-like} levels (assessed by qPCR) in hospital sewage samples and growth (*E. coli* + *K. pneumoniae* + *Raoultella*) on chromID® OXA-48 plates.

K. pneumoniae isolates from the sample that also harbored NDM-producing *E. coli* (April 2017). Twelve of the *Klebsiella* isolates were CarbaNP positive. In sharp contrast, almost all of the tested *Enterobacter* (38/39) and *Raoultella* (6/7) isolates were negative both in the CarbaNP test and the PCR-based carbapenemase screening indicating the carriage of other non-targeted resistance mechanisms, which allowed them to grow on ECC-meropenem plates (Tables 3 and S3). Although presumptive non-*E. coli* CPE were detected in all but one of the samples analyzed on ECC-meropenem plates, PCR and Carba-NP tests could not identify any CPE in five of the samples (May, June, September and December 2016 as well as May 2017). Only one of the five isolates analyzed from the August 2017 sample was confirmed to be CPE. For these six samples, none of the collected isolates were *K. pneumoniae*, whereas *K. pneumoniae* was always detected among collected isolates from the other samples. In summary, the detection of carbapenemase gene-carrying *K. pneumoniae* on ECC-meropenem plates followed the same pattern as observed for *E. coli* – highest levels in the beginning of 2016 (due to *bla*_{OXA-48-like}-positive strains) and in the sample from April 2017 (due to *bla*_{NDM}-positive strains). On the other sampling occasions, with the exception of March 2017, the non-*E. coli* coliform population on ECC-meropenem plates was dominated by strains that could not be confirmed to be CPE.

3.4. Comparisons of findings in sewage and patients

Carbapenemase genes detected in sewage CPE were restricted to *bla*_{OXA-48-like} and *bla*_{NDM}, which also were the most common carbapenemase genes in CPE from patients. When the levels of *bla*_{OXA-48-like} and corresponding CPE in sewage increased dramatically in the end of 2015, no patients with CPE were detected. However, during, or soon after, the observed peak in sewage, three patients (number 3, 4 and 5 in Table 1) with the same type of CPE regarding species and carbapenemase gene were identified (February–May 2016). One additional case was detected in July 2017. In close connection to the detection of *bla*_{NDM}-positive CPE in sewage samples, this type of CPE was detected in a patient sample (April 2017). CPE carrying *bla*_{NDM} were also detected in three additional patients, none of these samples were taken within two weeks of a sewage sampling.

3.4.1. Whole genome sequencing of *bla*_{OXA-48-like}-positive CPE

All sequenced *bla*_{OXA-48-like}-positive sewage isolates carried the *bla*_{OXA-48} gene, and all but one, collected on seven sampling occasions, were *E. coli* ST2450 ($n = 12$) or *K. pneumoniae* ST147 ($n = 12$) (Table S4). One *bla*_{OXA-48}-carrying sewage *E. coli* isolate (S-160622-2) was ST38. None of the sequenced *bla*_{OXA-48-like}-positive isolates from patients, of which three were shown to carry

*bla*_{OXA-48}, two to carry *bla*_{OXA-181} and one to carry *bla*_{OXA-244}, belonged to any of these STs (Table S4). However, the isolates from patient 4 as well as all the *E. coli* ST2450 and *K. pneumoniae* ST147 sewage isolates seemed to carry related plasmids since contigs from their short-read assemblies covered the majority of pE71T (GenBank KC335143, a 64-kilo base pair *bla*_{OXA-48}-harboring IncL plasmid) (Power et al., 2014) and other pOXA-48a-like plasmids (Pitout et al., 2019; Poirel et al., 2012).

Long-read sequencing confirmed that the patient and sewage isolates contained *bla*_{OXA-48}-plasmids closely related to pOXA-48a-like plasmids, including pE71T (99.95–99.98% ANI). The three isolates from patient 4 carried very similar IncL plasmids harboring the *bla*_{OXA-48} gene in a Tn1999.2 composite transposon (Table S4) (Pitout et al., 2019; Power et al., 2014). The two *K. pneumoniae* isolates (P-160217-1 and P-160405-2) collected on different dates from that patient were the same strain (100% ANI) and carried the exact same *bla*_{OXA-48} plasmid. The *E. coli* isolated from the same patient (P-160405-1) carried a *bla*_{OXA-48} plasmid with 99.98% nucleotide identity to the one of the *K. pneumoniae* isolates. Although overall very closely related to plasmids from patient 4 (99.96–99.98% ANI), the *bla*_{OXA-48}-carrying plasmids of sewage isolates subjected to long-read sequencing had slightly different structures. The *bla*_{OXA-48}-plasmids of two sewage isolates (S-160113-4 and S-160121-1) contained a 135 base pair segment in an already interrupted gene coding for a DNA primase, which was not present in the plasmids from patient 4. Additionally, the orientation of Tn1999.2 was inverted in S-160113-4 and in S-160121-1 the *bla*_{OXA-48} was in a Tn1999 composite transposon, which lacks the ISIR element present in Tn1999.2 (Pitout et al., 2019; Poirel et al., 2012).

Long-read sequencing also revealed another mobile genetic element (MGE) containing *bla*_{OXA-48-like} for which close resemblance between clinical and sewage isolates was evident. In S-160622-2, the only sewage *E. coli* isolate of a divergent ST (ST38) (Table S4), the *bla*_{OXA-48} gene was chromosomally located in a truncated Tn1999.2, which is embedded in the composite transposon Tn6237 (100% identity with GenBank KT444705) (Pitout et al., 2019; Turton et al., 2016). An almost identical chromosomally located composite transposon was detected in the clinical *E. coli* isolate P-160213-1. The transposon in the clinical isolate only differed by one nucleotide, which changed the *bla*_{OXA-48} gene to *bla*_{OXA-244}.

3.4.2. Whole genome sequencing of *bla*_{NDM}-positive CPE

All six sequenced *bla*_{NDM}-positive sewage *E. coli* isolates belonged to ST694, whereas the two sequenced *bla*_{NDM}-positive sewage *K. pneumoniae* isolates belonged to different STs (307 and unknown). Among the four sequenced *bla*_{NDM}-positive isolates from patients, two were of the same STs as the sewage isolates (Table S4). These strains were isolated from the same patient in close connection to the collection of the *bla*_{NDM}-positive sewage isolates and were *E. coli* ST694 (P-170419-1) and *K. pneumoniae* ST307 (P-170419-2).

All five *bla*_{NDM}-positive isolates subjected to long-read sequencing carried the carbapenemase gene on very similar 83-kilo base pair IncM2 plasmids (Table S4). The sewage *E. coli* S-170425-2 and S-170425-6 were the same strain as the patient isolate P-170419-1 (100% ANI) and carried identical *bla*_{NDM1}-harboring plasmids. The sewage *K. pneumoniae* S-170425-8 was the same strain as the patient isolate P-170419-2 (100% ANI) and carried a *bla*_{NDM1}-harboring plasmid differing from the one of the *E. coli* isolates by only six nucleotides, including four nucleotides changing *bla*_{CTX-M-28} found on the *E. coli* plasmid into *bla*_{CTX-M-3} in *K. pneumoniae* (Table S4).

4. Discussion

This study investigates if detection of carbapenemase genes and CPE in hospital sewage samples can provide information on the presence of such bacteria among admitted patients. The majority of the characterized sewage CPE or the carbapenemase-harboring MGEs they carried showed apparent relation to CPE detected in patient samples. For three of the five investigated carbapenemase genes, qPCR analyses provided similar information regarding abundance of corresponding sewage CPE as culture-based analyses. We will below scrutinize the results and discuss the evidence for using sewage analyses as an early warning and surveillance system for CPE.

Over the study period there was detection of *bla*_{OXA-48-like}-positive CPE in most sewage samples, of *bla*_{NDM}-positive CPE on a single occasion and no detection of *bla*_{KPC}-positive CPE. This qualitatively reflected what was observed in patient samples from the studied hospital, *i.e.* detection of CPE producing OXA-48-like and/or NDM but none producing KPC. The findings of CPE in sewage were also clearly matched by the carbapenemase gene-specific qPCR analyses of the complex sewage samples. Hence, there was a good accordance between abundance of genes and corresponding types of CPE in the sewage samples for the three carbapenemase genes most frequently detected in isolates from patients in all of Sweden (SWEDRES-SVARM, 2019). The two additional carbapenemase genes included in this study, *bla*_{VIM} and *bla*_{IMP}, were detected in the majority of sewage samples, but not in any of the collected CPE isolates although high qPCR signals were detected for both genes. In contrast to the other three, these two genes are often carried in the form of integron gene cassettes and are as such widespread in several non-Enterobacterales species (Logan and Weinstein, 2017). Hence, the qPCR detection of these genes in the present study is likely due to the presence of *bla*_{VIM}/*bla*_{IMP}-harboring strains of non-Enterobacterales species in the sewage and would thus be less informative regarding the presence and early warning of CPE. However, it should be acknowledged that non-Enterobacterales hosts have been described also for *bla*_{NDM} (Walsh et al., 2011), *bla*_{KPC} (Villegas et al., 2007) and *bla*_{OXA-48-like} genes (Meunier et al., 2016; Tacão et al., 2018).

In addition to having the potential to provide qualitative information about CPE in the contributing population, the sewage analyses also provide quantitative measurements that might inform about CPE prevalence. In order to serve as an appropriate surveillance and early warning system, an increased sewage signal should indicate increased prevalence in the contributing population. This was clearly seen for *bla*_{NDM}-positive CPE when the single sewage detection coincided with detection of such CPE in a patient sample. In addition, three of the four patients known to carry/be infected with *bla*_{OXA-48-like}-positive CPE were detected during the beginning of 2016 when there was a dramatic peak of such CPE in the sewage. Other studies have also shown similar repertoires of CPE in sewage and patient samples. A screening of samples from twenty wastewater treatment plants in the East of England could detect CPE that based on species and carbapenemase genes largely mirrored what was found in samples from patients (Ludden et al., 2017). In addition, an analysis of sewage sampled across Israel could observe that the most commonly detected CPE types matched those in the clinic (Meir-Gruber et al., 2016). In neither of these two studies a comparison between wastewater and patient isolates beyond species and carbapenemase genes was conducted.

In the present study, whole genome sequencing showed that *bla*_{NDM}-positive strains isolated from sewage were almost identical to CPE strains from a patient, emphasizing that what we observe in the sewer can inform about the situation in the clinical setting. Several earlier studies have also shown identically typed CPE

in patients and sewage/sewage-contaminated water (Khan et al., 2018; Lepuschitz et al., 2019; Mahon et al., 2017). However, the sequenced *bla*_{OXA-48-like}-positive sewage strains in the current study differed from those identified in patients, although they carried closely related *bla*_{OXA-48-like}-harboring mobile genetic elements. All of the *bla*_{OXA-48-like}-harboring elements detected in sewage were related to such found in CPE from patients.

A likely, at least partial, explanation for finding some CPE strains in sewage but not in patients could be that, although a screening program is in place, CPE carriers might remain undetected. Only a minor fraction of the patients are screened for CPE and generally only once, *i.e.* at or just prior to admission. None of the visitors and staff are screened. If carriers do not develop subsequent infection with CPE, which based on studies of patients carrying ESBL-producing Enterobacterales at the hospital can be presumed to be very rare (Lindblom et al., 2018), it would further prevent detection of CPE in the clinical setting. Thus, the *bla*_{OXA-48-like}-carrying strains in the sewage might represent a silent outbreak at the hospital; the marked increase during a few consecutive months fits this hypothesis. The dramatic increase in *bla*_{OXA-48-like}-positive CPE and *bla*_{OXA-48-like} genes in the sewage during the end of 2015 and beginning of 2016 also matches the evident increase in number of such CPE cases in patients on a national level in Sweden during those years (SWEDRES-SVARM, 2019).

Repeated shedding into the sewage from another source, such as environmental biofilms containing CPE, could possibly also contribute to the observed peak in sewage *bla*_{OXA-48-like}-positive CPE. In a recent investigation of biofilms in the main sewer at a French hospital, carbapenemase-producing bacteria were detected but no CPE (Ory et al., 2019). In contrast, Weingarten and co-workers isolated CPE from hospital wastewater pipe sludge and multiple studies have shown that hospital sinks can harbor CPE (Clarivet et al., 2016; Decraene et al., 2018; Kizny Gordon et al., 2017; Regev-Yochay et al., 2018; Weingarten et al., 2018). However, even if the commonly detected OXA-48-producing sewage CPE in the present study represent strains thriving in the draining/sewer systems it is likely that they, or at least their plasmids, have been introduced by the hospital population at an earlier time-point. It is striking that all but one of the sequenced OXA-48-producing sewage CPE isolates were either *K. pneumoniae* ST147 or *E. coli* ST2450, a finding that could support either of the abovementioned suggested explanations (silent outbreak or repeated shedding from a source in the wastewater network). Similar explanations were recently suggested, *i.e.* residency in the wastewater system and/or circulation in the contributing community, when two ESBL-positive *E. coli* clones were repeatedly isolated from a wastewater pump station in a Norwegian suburb (Paulshus et al., 2019).

On occasions when there is a non-complete match between patient and sewage isolates but still detection of closely related MGEs carrying the relevant antibiotic resistance gene, it could be a result of horizontal gene transfer between strains detected in patient and sewage samples. The sharing of genetic material could, in addition to within the patient's microbiome, occur in the sewers or in the hospital environment connected to the sewage system. Such an event has earlier been postulated to have occurred in a sink drain at the hospital studied by Weingarten et al. (Weingarten et al., 2018). In the present study, closely related *bla*_{OXA-48-like}-carrying MGEs were detected in patient and sewage isolates. However, since those MGEs represent groups of closely related MGEs, acting as main drivers behind the global spread of *bla*_{OXA-48-like} genes (Pitout et al., 2019), it is quite plausible that individuals other than the patients detected in this study were sources of the MGEs observed in sewage. Importantly, the initial detection in sewage isolates occurred when there were still no known CPE cases at the hospital and thus preceded the detection in isolates from patient

samples. This suggests a value of sewage monitoring as an early warning system.

The evident increase in *bla*_{OXA-48-like}-positive CPE in the sewage during a few consecutive months also warrants some reflection with regard to the magnitude of that peak. Measured levels of *bla*_{OXA-48-like}-positive *E. coli* were as high as a few percent of total *E. coli* in the sewage samples and both qPCR signals and relative colony forming unit counts were up to 1000-fold higher than observations at other time-points. Even though both constant changes in the hospitalized population and the risk for transmission of CPE between individuals are factors that could contribute to fluctuating levels of CPE prevalence, it seems unlikely that the number of carriers/infected at the hospital should vary that much. A possible explanation could be that the low levels observed are due to bacteria released from biofilms in the sewers creating a background signal and that it is only the higher levels that correspond to direct detection of carriers at the hospital. Since we saw a perfect match between NDM-producing strains in the sewage and strains from the single carrier detected in the clinic in April 2017, it is tempting to speculate that the NDM signals (e.g. 0.3% of total *E. coli*) in the sewage on that occasion were due to the contribution of a single or a small group of individuals. If so, the magnitude of those NDM measurements could give a hint about which signals could be directly impacted by carriers/infected at the hospital and which are due to background (that indeed could be a result of indirect, delayed impact by carriers/infected individual as discussed above). The highest observed levels of *bla*_{OXA-48-like}-positive *E. coli* were on par to what one can expect to find for CTX-M (group1)-producing *E. coli* in the sewage samples. A previous analysis of the same sewage samples from 2016 showed 5.5% of the *E. coli* in the sewage to be ESBL-producers (Hutinel et al., 2019) and a preceding study showed that 75% of the ESBL-producing *E. coli* from urine samples in the local region were positive for *bla*_{CTX-M} group1 (Heldal et al., 2013). Our findings of similar levels of OXA-48-like and CTX-M-producers in the sewage is also supported by the relatively similar levels of *bla*_{OXA-48-like} and *bla*_{CTX-M} genes as measured by qPCR in the current study. The carrier rate for ESBL-producing *E. coli* is around 5% in Sweden (Ny et al., 2017), but *bla*_{OXA-48-like}-positive CPE are reported to be much less common (SWEDRES-SVARM, 2019). The sewage levels of *bla*_{OXA-48-like}-positive CPE during the peak could thus represent rather significant undetected transmission of CPE at the hospital. Alternatively, and as discussed above, it cannot be excluded that repeated shedding from an environmental biofilm is responsible for the observed peak.

There might also be other explanations behind the high CPE levels in the sewage samples. A sampling bias and/or presence of a selective pressure could possibly skew the bacterial populations in the sewage samples (Kraupner et al., 2021). Since the distance between the sewage sampling point and the source is relatively short, there is limited time for the fecal material to be properly suspended and thus a risk for collecting lumps containing many bacteria from the same individual. This would lead to overrepresentation of certain strains in the composite sample. Although this most likely happens from time to time when hospital sewage is collected like in the present study (Hutinel et al., 2019), there is no obvious reason why it should happen on several consecutive occasions involving the same or closely related bacterial strains. A selection pressure promoting antibiotic resistance and thus the detected CPE could also have explained the high levels during the peak. However, in a previous study where resistance to a broad panel of antibiotics was determined for *E. coli* isolates from the very same sewage samples included in the present study, none of the samples from the beginning of 2016 displayed exceptionally high resistance rates (Hutinel et al., 2019). In fact, one of the samples with high CPE abundance (March 22) was actually one of two

samples where the overall lowest resistance rates were observed for collected *E. coli* isolates, including low resistance to beta-lactam antibiotics.

Several earlier studies at hospitals have also identified sewage CPE strains not detected in patients. A study at a US hospital included whole genome comparisons between CPE isolates (as well as other carbapenemase-producing bacteria) from patient and environmental (including sewage) samples (Weingarten et al., 2018). Although closely related CPE (>99.9% ANI) were detected in patient and sewage samples, the great majority of detected sewage CPE strains were not detected in samples from patients. Similar observations that majority of hospital sewage CPE are not matched by strains from patients have also been done by White et al. and Koh et al. (Koh et al., 2015; White et al., 2016). In these studies the sewage CPE collections have been dominated by species not representing the most commonly detected CPE species in patients, where *E. coli* and *K. pneumoniae* usually dominate. By focusing on the most clinically important CPE species, the sewage monitoring might be more relevant from a clinical perspective. Such more species-restricted comparisons have shown clear resemblance between ESBL-producing strains, which have a similar epidemiology as corresponding CPE, from sewage and patients (Drioux et al., 2016; Jørgensen et al., 2017; Zarfel et al., 2013). On the other hand, narrowing the scope might imply a risk of neglecting something that could be a threat, albeit an unusual one.

Apart from when sewage CPE are not detected in samples from patients, the present and previous studies (Koh et al., 2015; Weingarten et al., 2018) also include reversed observations – CPE strains detected in the clinic but not observed in the sewage samples. The major reason for this is probably shortcomings in the frequency of sewage sampling, with regard to collection of both composite samples and subsamples. Although the present study included sewage sampling during more than two years, none of the samplings, apart from when NDM-producing CPE matching patient isolates were detected (April 2017), occurred in close connection to (less than two weeks after) any of the CPE detections in the clinic. Other reasons could be 1) that the colonized patient does not defecate during hospitalization or 2) that the nature of infection does not lead to release of CPE to sewage. Given that CPE infections often originate from the gut flora and most patients can be expected to defecate during hospitalization, it should be possible to capture a large proportion of the CPE strains in admitted patients by applying frequent sewage sampling (continuous collection of composite sewage samples).

5. Conclusions

This study shows connections between both carbapenemase genes and CPE detected in hospital sewage and CPE identified in patients. By combining culture- and gene-based analyses, we conclude that *bla*_{OXA-48-like}, *bla*_{NDM} and *bla*_{KPC} could be indicative of corresponding CPE in sewage, whereas the link between *bla*_{IMP} and *bla*_{VIM} detection and presence of CPE is much more uncertain. Detection of the two latter genes can still indicate presence of carbapenemases in other relevant pathogens though. Since also the former three genes can be found in non-Enterobacterales bacteria, PCR detections needs to be confirmed by cultivation data or another methodology that can assign the detected gene to its host, especially if assays are applied to samples from a new site. Overall, the results of the present study at a single site shows promise for using sewage monitoring, which has the capacity to screen bacteria from considerably more individuals compared to classical clinical surveillance, as an early warning and surveillance system for rare forms of resistance. Yet, additional validation is needed – ideally through studies including culture- and gene-based approaches as well as extensive whole genome sequencing at hospitals where dif-

ferent abundance of carriers/infected individuals is to be expected and where continuous and frequent sampling of sewage as well as patients can be conducted during the study period. After such successful validation, the resource-efficient sewage-based surveillance approach could have a significant value not only in the large parts of the world where continuous surveillance of antibiotic resistance is lacking, but also in settings with relatively extended surveillance including fecal screening programmes of selected patient groups.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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