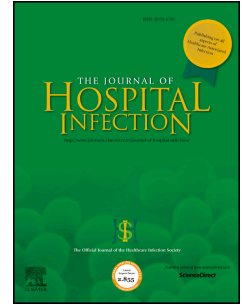


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Carbapenemase-producing Enterobacteriaceae in hospital wastewater: a reservoir that may be unrelated to clinical isolates[star]

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L. White *et al.*

Carbapenemase-producing Enterobacteriaceae in hospital wastewater: a reservoir that may be unrelated to clinical isolates^[star]

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SUMMARY

Background: Carbapenemase-producing Enterobacteriaceae (CPE) are an emerging infection control problem in hospitals worldwide. Identifying carriers may help reduce potential spread and infections.

Aim: To assess whether testing hospital wastewater for CPE can supplement patient-based screening for infection prevention purposes in a hospital without a recognized endemic CPE problem.

Methods: Wastewater collected from hospital pipework on 16 occasions during February–March 2014 was screened for CPE using chromID[®] CARBA agar and chromID[®] CPS agar with a 10 µg ertapenem disc and combination disc testing. Minimum inhibitory concentrations were determined using British Society for Antimicrobial Chemotherapy

methodology and carbapenemase genes detected by polymerase chain reaction or whole-genome sequencing. Selected isolates were typed by pulsed-field gel electrophoresis.

Findings: Suspected CPE were recovered from all 16 wastewater samples. Of 17 isolates sent to the Antimicrobial Resistance and Healthcare Associated Infections Reference Unit, six (four *Citrobacter freundii* and two *Enterobacter cloacae* complex) were New Delhi metallo- β -lactamase (NDM) producers and the remaining 11 (six *Klebsiella oxytoca* and five *Enterobacter cloacae* complex) were Guiana-Extended-Spectrum-5 (GES-5) producers, the first to be described among Enterobacteriaceae in the UK. The four NDM-producing *C. freundii*, two NDM-producing *E. cloacae* complex, and four out of five GES-5-producing *E. cloacae* complex were each indistinguishable isolates of the same three strains, whereas the six GES-5-producing *K. oxytoca* overall shared 79% similarity.

Conclusion: CPE are readily isolated from hospital wastewater using simple culture methods. There are either undetected carriers of CPE excreting into the wastewater, or these CPE represent colonization of the pipework from other sources. Surveillance of hospital wastewater for CPE does not appear helpful for infection control purposes within acute hospitals.

Keywords:

Guiana-Extended-Spectrum-5 (GES-5)

Metallo- β -lactamase

Carbapenem resistance

Surveillance

Introduction

The emergence of carbapenemase-producing Enterobacteriaceae (CPE) is a concern for hospitals worldwide.^{1,2} Isolation of an organism that exhibits carbapenem resistance from an infected site may require the use of less effective antibiotics and poses an infection control risk to other patients. Patients who are asymptomatic faecal carriers of these organisms also pose an infection control risk.³ In most UK hospitals, CPE are only isolated sporadically, most often from patients who have recently received healthcare in countries where CPE have become endemic. However, a few UK hospitals, most notably in the north-west of England, have had endemic CPEs for several years, which have not been eradicated despite strenuous screening and isolation programmes.⁴ National guidance has been issued by Public Health England (PHE) to reduce the risk of further spread. This advice rejects screening all admissions for the presence of CPE as this would be costly and time-consuming, and recommends that 'high-risk' patients, including those with a history of foreign travel and those transferred from hospital units with a known CPE problem, should be screened.⁵

Confirmation that this selective approach is adequate in any single hospital would require a prolonged period of comprehensive screening, to capture any cases missed by risk factor-based screening. Testing hospital wastewater for the presence of CPE offers a potential alternative approach, based on the assumption that carriers would excrete CPE into the hospital wastewater and that CPE would be present at a detectable level there with isolates not dissimilar to those from patients. Potentially this method could provide hospital infection control teams with assurance that a latent endemic CPE problem is not present if testing were negative, and do so at much lower cost than universal screening of all admissions.

In this study, samples of wastewater collected over a two-month period from a single UK hospital, without a known endemic CPE problem, were screened for CPE in order to determine whether there was an unrecognized CPE presence within the hospital.

Methods

Study setting

The study was conducted at Royal Preston Hospital, a 709-bed hospital in north-west England offering secondary care to an immediate population of ~140,000 and a range of tertiary care services to the population of Lancashire and South Lakeland, ~1.5 million.⁶ There is substantial ethnic diversity within the catchment, with ~13% of the local population having family ties with the Indian subcontinent.⁶ Since May 2011 there has been a screening programme to detect carriage of CPE, with rectal swabs collected in all patients with a history of hospitalization overseas or within a healthcare facility in the UK with CPE problems. Patients who have had contact with a confirmed case, or who have previously been infected or colonized, are also screened. The programme was updated in line with PHE guidance issued in 2013.⁵ The hospital has comprehensive antibiotic guidance that imposes tight restrictions on the use of carbapenems and fluoroquinolones, and limits cephalosporin use.

Sample collection and processing

Wastewater samples were collected twice a week during February and March 2014, producing a total of 16 samples. Sampling was facilitated by the introduction of a tap into the wastewater pipework in the basement directly beneath the wards. The wastewater sampled was from operating theatres, critical care unit, paediatrics, orthopaedics, cardiac ward, cardiac catheter laboratory, oncology, and a staff toilet block. The wastewater had not undergone any treatment prior to the sampling point. The first 100 mL was run off and discarded to reduce risk of cross-contamination between samples. Fifty microlitres of each sample were inoculated on to chromID[®] CARBA agar and chromID[®] CPS agar (both bioMérieux, Basingstoke, UK) plus a 10 µg ertapenem disc (Mast Group Ltd, Bootle, UK). Cultures were incubated for 18–24 h at 35–37°C.

Isolate identification

Blue, green, or pink colonies growing within ≤ 27 mm of the ertapenem disc on the chromID CPS agar or on the chromID CARBA agar were presumed to be CPE isolates. All presumptive CPE colonies from both media were further analysed. Oxidase-negative, Gram-negative isolates were subcultured from both media on to cysteine lactose electrolyte-deficient (CLED) agar (E&O Laboratories Ltd, Bonnybridge, UK) and incubated at 35–37°C for 18–24 h. Bacterial identification was determined by matrix-assisted laser desorption–ionization mass spectrometry (MALDI-TOF MS; Bruker Daltonik GmbH, Bremen, Germany) as previously described.⁷ Isolates from the Enterobacteriaceae family were further characterized to determine carbapenemase production.

Antibiotic susceptibility determination

Isolates were tested for resistance to meropenem and ertapenem using the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method and zone sizes interpreted using BSAC guidelines.⁸ Isolates were also screened for synergy between meropenem and dipicolinic acid [for presumptive identification of metallo- β -lactamases (MBLs)], phenylboronic acid [*Klebsiella pneumoniae* carbapenemase (KPC)] and phenylboronic acid and cloxacillin (AmpC) using the KPC/MBL and OXA-48 confirmation kit (Bioconnections, Knypersley, UK). The first 13 isolates recovered in the study were referred to Public Health England's Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit as they showed resistance to ertapenem and meropenem with unclear but presumptive carbapenemase production using the methods described above. Additional carbapenem-resistant organisms were recovered as the study continued; however, only those that were presumptively identified as MBL producers were referred (due to limited resources) to AMRHAI in order to confirm the resistance mechanism. Therefore, 17 isolates were referred and minimum inhibitory concentrations (MICs) were determined by BSAC agar dilution using AMRHAI's standard Gram-negative antibiotic panel, including ertapenem, meropenem and imipenem (the latter tested with/without 320 mg/L EDTA to detect likely MBL producers). MICs were interpreted using BSAC breakpoints where available. Isolates were also screened for carbapenemase activity using the Rosco Rapid Carb test (Bioconnections) and the modified Hodge test.

Molecular detection of carbapenemases

In-house polymerase chain reaction (PCR) was used to screen for class A (KPC and IMI), class B (NDM, IMP, VIM, GIM, SIM, SPM), and class D (OXA-48-like) carbapenemase genes.^{9–13} Whole-genome sequencing (WGS) of three isolates with unexplained carbapenem resistance was performed using a HiSeq sequencer (Illumina, Little

Chesterford, UK) and data were analysed using an in-house bioinformatics pipeline.

Resistance genes were identified in WGS data by mapping reads against a library of known resistance genes curated in-house and assembled from publicly accessible databases.¹⁴

Typing

The 17 isolates submitted to the reference laboratory were typed by pulsed-field gel electrophoresis of *Xba*I-digested genomic DNA. Gel images were analysed and compared using Bionumerics software, version 6.1 (Applied Maths, Sint-Martins-Latem, Belgium).

Results

Suspected CPE isolates were recovered from all 16 wastewater samples. The 55 colonies recovered (35 from chromID CARBA agar and 20 from chromID CPS agar + ertapenem disc) included 21 *Klebsiella oxytoca*, 21 *Enterobacter cloacae* complex, nine *Citrobacter freundii*, three *Citrobacter braakii*, and one *Citrobacter youngae*. BSAC disc diffusion determined that all 55 were intermediate or resistant to meropenem or ertapenem. Combination disc testing identified 16 presumptive KPC producers, six presumptive MBL producers, and seven isolates gave indeterminate results. Carbapenemase activity was not detected in the 26 isolates as determined by the interpretation of the combination discs. In total, 17 suspected CPE (as described earlier) were sent to AMRHAI for further characterization.

Minimum inhibitory concentrations are shown in Table I. All isolates were resistant to the three carbapenems tested (ertapenem MICs ≥ 8 mg/L; meropenem and imipenem MICs ≥ 32 mg/L). At least eight-fold synergy between imipenem and EDTA was noted for six isolates (four *C. freundii* and two *E. cloacae* complex), all of which had been identified as presumptive MBL-producing isolates by the KPC/MBL and OXA-48 confirmation kit, and *bla*_{NDM} genes were detected by PCR in these isolates. Typing of these isolates showed that both the two *E. cloacae* complex and four *C. freundii* isolates were each genetically indistinguishable within each group (data not shown).

The remaining six *K. oxytoca* isolates (four presumptively identified as KPC-producers and two as AmpC producers) and five *E. cloacae* complex isolates (three presumptively identified as KPC producers, and two in which no carbapenemase activity was detected) were highly carbapenem-resistant (ertapenem MICs ≥ 8 mg/L, meropenem; imipenem MICs ≥ 32 mg/L) with no significant imipenem/EDTA synergy (Table I). However, these 11 isolates were negative using in-house carbapenemase PCRs, and no carbapenemase activity was detected using the Rosco Rapid Carb test or modified Hodge test. Whole-genome sequencing of three of these isolates identified the non-metallo-carbapenemase *bla*_{GES-5}, which was subsequently identified through PCR and sequencing in the remaining eight

isolates. Typing of the six *K. oxytoca* isolates showed that they had similar but not identical profiles, sharing 79% genetic similarity. Four of the five *E. cloacae* isolates were representatives of a single strain, whereas the fifth had a distinct pattern (Table I).

Discussion

Carbapenem-resistant organisms were readily detected in the wastewater of the hospital. This was unexpected as only a small number of confirmed CPE had been detected between 2010 and 2014 from screening (430 screens) and clinical isolates within the hospital (six KPC, one NDM, and four OXA-48-like CPE since 2010; AMRHAI, unpublished data). The absence of KPC-producing organisms in the wastewater was also surprising given their relatively high incidence in north-west England.¹⁵ KPC-producing Enterobacteriaceae form the majority of the carbapenemase-producing organisms referred to PHE, with ~75% of KPC producers coming from clinical or screening specimens taken from hospitals in north-west England.^{5,16,17}

Local circumstances may affect the presence of CPE in the wastewater. Overuse of carbapenems may promote the recovery of CPE. Consumption of carbapenem antibiotics in 2013 within this hospital was 10.9 defined daily doses (DDD) per 100 admissions per day compared with 8.0 DDD per 100 admissions per day for England.¹⁸ Most carbapenem prescriptions require infection specialist approval, with only a few indications (e.g. post-neurosurgical meningitis) bypassing this stricture. In 2011, 13.7% of the local population had connections with the Indian subcontinent and therefore may have travelled frequently to areas with endemic CPE and become colonized. However, only 5% of admissions with recorded ethnicity data are within this group.⁶

This study has several limitations. The sampling window covered only two months. However, consistent recovery of CPE from all samples suggests that this is an ongoing problem, and, from a sample collected in March 2015, carbapenem-resistant organisms of the same species were isolated. The method used to collect and test the wastewater is not formally recognized, as this involves filtration. The aim, however, was to produce a simple and inexpensive method, so that any hospital's infection prevention team could realistically request monitoring of the wastewater for the presence of CPE. The small sample volume may reduce CPE but each sample recovered carbapenem-resistant organisms, indicating not only success but also potential underestimation. A further limitation is that only 17 out of 55 isolates were sent to AMRHAI for confirmatory testing. The remaining 38 isolates yielded varied results (extended-spectrum β -lactamase, KPC, AmpC, or undetermined) using the ROSCO discs, similar to those seen in 11 GES-5 positive isolates referred. We cannot confirm that these are further isolates of the same organisms; however, this seems likely given

their consistent detection in earlier samples, and the similar range of species and resistance profiles found.

There have been several studies indicating that chromID CARBA agar is highly sensitive for the detection of CPE. Perry *et al.* determined 100% sensitivity and 93% specificity for a prototype of the CARBA agar; however, this was only for detection of NDM-1 carbapenemase producers whereas Vrioni *et al.* showed 92.4% sensitivity and 96.9% specificity for a prototype CARBA agar.^{19,20} A more recent study in Greece determined a sensitivity of 96.5% and specificity of 91.2% (before Gram staining) and 100% (after Gram staining) for the final chromID CARBA product, indicating that this is a good choice for a screening method.²¹ Unfortunately the chromID CARBA plate has been reported to not reliably detect OXA-48-like producers, which may correlate with the zero recovery in this study.²² Whereas OXA-48-like carbapenemases are becoming more widespread in the UK, until 2012 they were identified less frequently than KPC, NDM, and VIM carbapenemases.²³ Agar plates allowing more sensitive detection of OXA-48-like carbapenemases are now available and may need to be considered in future studies.^{24,25}

A total of 55 isolates were recovered across 16 samples over a two-month time period. It is possible that isolates were counted twice after being detected on both media. A biofilm may have built up in the tap with the repeat isolates recovered from here rather than a continuing presence in the wastewater itself. To minimize this, a 100 mL run-off was performed and discarded before sampling. In future studies a tap-cleaning brush could be used to reduce any physical build-up. However, the aim of this study was to determine whether carbapenem-resistant organisms could be identified using this method and whether CPE were present (rather than how many were present), which it has succeeded in doing. The protocol allows scope for further investigation using more quantitative methods to determine the extent of the presence of carbapenem-resistant organisms within our hospital.

The presence of GES-5-producing Enterobacteriaceae in the wastewater did not equate, in this hospital, with a clinical problem. Similarly, although NDM producers were recovered from the hospital wastewater, the only patient isolate with an NDM carbapenemase detected at the hospital's laboratory had been a *K. pneumoniae* isolated from a community urine specimen in 2010. We therefore found no link between isolates causing colonization or infection of inpatients and those present in wastewater. However, since screening is limited to those patients with risk factors, in accordance with current PHE guidance, the possibility that unidentified carriers within the hospital may be a reservoir for GES-5 and NDM-1 carbapenemase-producing organisms cannot be dismissed.

The typing results indicated that the same strains were recovered on several occasions over the two-month time period: the six NDM-positive isolates of *C. freundii* and the two *E. cloacae* complex isolates represented just two strains. In addition, four out of five *E. cloacae* GES-5-producing isolates were indistinguishable. This suggests that these particular strains may be persisting in the wastewater environment rather than having been excreted repeatedly by patients, and may not be of clinical significance. Prior to this study, GES-5-positive Enterobacteriaceae had not been described in the UK. Enterobacteriaceae producing the GES-5 carbapenemase have been isolated from clinical specimens in Korea and Southern Brazil.^{26,27} Wastewater is a potential habitat for the horizontal transfer of resistance genes, and the presumptive presence of excreted antimicrobials from patients into the wastewater allows for the selection of resistant bacteria.²⁸ Hospital wastewater is not routinely tested for CPE, so the prevalence of GES-5 or other carbapenemases in bacteria from this source is unknown. Manageiro *et al.* found GES-5-producing *K. pneumoniae* in water streams in Portugal, highlighting aquatic environments as a potential reservoir for resistance mechanisms.²⁹

This study also highlights the uncertainty as to whether GES-5-producing isolates can be reliably confirmed using the phenotypic methods used to confirm suspected CPE. As noted in this and previous studies, GES carbapenemases may not be reliably detected by colorimetric tests such as the Rosco Rapid Carb and some CarbaNP tests, and selected isolates from this study were negative in the modified Hodge test.³⁰ The identification of GES carbapenemase activity is not covered specifically by combination disc tests, although seven out of 11 were flagged as KPC producers, so would be further investigated. KPC and GES-5 are class A carbapenemases and thus both are inhibited by phenylboronic acid. All isolates were, however, highly resistant to carbapenems and thus would warrant sending to a reference laboratory, even if local testing failed to show carbapenemase production. Although there is potential for underdetection of GES-5, AMRHAI has had no previous isolates of Enterobacteriaceae from UK laboratories with gross carbapenem resistance in the absence of one of the more widespread carbapenemase genes (KPC, OXA, NDM, VIM, IMP).

In conclusion, a simple culture method was able to isolate CPE from hospital wastewater. However, there appears to be little correlation between the carbapenemases found and the hospital's experience of CPE-positive samples from patients. This suggests that the isolates may be adapted to the environment and consistently present within the pipework. Whereas comprehensive screening of both patients and staff would be needed to accurately describe the correlation between human isolates and presence of CPE in wastewater, the possible presence of CPEs of environmental origin severely limits the role of this approach for CPE surveillance at a hospital level.

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Conflict of interest statement

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Table I

Susceptibility patterns and genes identified for the 17 isolates referred to the Antimicrobial Resistance and Healthcare Associated Infections Reference Unit, UK

	Isolate sample date (all 2014)																
	Feb 3rd	Feb 6th	Feb 6th	Feb 10th	Feb 10th	Feb 10th	Feb 10th	Feb 14th	Feb 14th	Feb 17th	Feb 17th	Feb 20th	Feb 20th	Feb 27th	Feb 18th	Mar 18th	Mar 24th
Bacterial ID	KOX	ECL	KOX	ECL	ECL	ECL	CFR	KOX	ECL	KOX	KOX	KOX	ECLO	CFR	ECL	CFR	CFR
		O		O	O	O			O						O		
Carbapenemas	GES-	GES-	GES-	NDM	GES-	GES-	NDM	GES-	GES-	GES-	GES-	GES-	GES-	ND	ND	NDM	NDM
e	5	5	5		5	5		5	5	5	5	5	5	M	M		
PFGE profile	P1K	P1EB	P1K	P1EB	P1EB	P1EB	P1CB	P1K	P1EB	P1K	P1KL	P1K	Uniqu	P1C	P1E	P1CB	P1CB
	L-1	-3	L-1'	-2	-3	-3	-1	L-1	-3	L-1	-1''	L-1'	e	B-1	B-2	-1	-1
ATM	8	8	8	16	8	16	0.5	0.25	4	8	8	8	>64	≤0.1	1	0.25	0.25
														25			
CTX-CLOX	16	0.5	32	64	1	32	256	1	0.25	32	32	32	64	256	128	256	256
CTX	64	4	64	>256	32	128	256	2	4	128	128	64	64	256	128	256	256
CTX-CLA	2	8	2	>32	>32	16	>32	2	16	2	2	2	8	>32	>32	>32	>32
CAZ	16	16	16	>256	64	32	>256	4	8	16	16	16	256	>256	>256	>256	>256
CAZ-CLA	8	8	16	>32	8	8	>32	2	16	16	8	8	8	>32	>32	>32	>32
FEP	16	0.25	32	32	1	16	64	0.5	0.25	32	32	16	16	64	64	>64	64
FEP-CLA	1	0.25	2	>32	1	0.5	>32	0.5	0.25	1	1	1	1	>32	>32	>32	>32
IPM-EDTA	>16	16	>16	2	>16	>16	1	>16	>16	>16	>16	>16	>16	1	2	1	1
IPM	128	32	128	32	64	64	64	128	64	128	128	128	128	64	32	64	64

MEM	>32	32	>32	32	>32	32	32	>32	32	>32	>32	>32	>32	>32	>32	>32	>32
ETP	>16	8	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
COL	≤0.5	2	≤0.5	>32	>32	16	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	>32	1	1
AMK	4	2	4	1	4	4	2	4	4	8	8	4	2	2	2	2	2
GEN	>32	>32	>32	1	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	0.5	>32

KOX, *Klebsiella oxytoca*; ECLO, *Enterobacter cloacae* complex; CFR, *Citrobacter freundii*; NDM, New Delhi metallo- β -lactamase; PFGE, pulsed-field gel electrophoresis; ATM, aztreonam; CTX-CLOX, cefotaxime/cloxacillin (100 mg/L); CTX, cefotaxime; CTX-CLA, cefotaxime/clavulanate (4 mg/L); CAZ, ceftazidime; CAZ-CLA, ceftazidime/clavulanate; FEP, cefepime; FEP-CLA, cefepime/clavulanate; IPM-EDTA, imipenem/EDTA (320 mg/L); IPM, imipenem; MEM, meropenem; ETP, ertapenem; COL, colistin; AMK, amikacin; GEN, gentamicin. P1KL-1, P1KL-1' and P1KL-1'', shared 79% genetic similarity.