

**Original**

**Molecular Characteristics of a Carbapenemase-producing  
*Enterobacter* Species and *Klebsiella* Species Outbreak  
in a Japanese University Hospital**

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**Abstract :** Carbapenemase-producing Enterobacteriaceae (CPE) have been reported worldwide, but the types of carbapenemase vary between countries. Metallo-beta-lactamase (MBL) IMP (*bla*<sub>IMP</sub>) is the most common type of carbapenemase in Japan. CPE outbreaks occurred in our hospital's neonatal intensive care unit (NICU) between October 2014 and October 2015. The aim of this study was to evaluate the results of active surveillance and to establish more effective CPE detection methods. We also investigated the molecular epidemiological characteristics of CPE in Japan. During the outbreak period, active surveillance was performed on a weekly basis by analyzing fecal cultures from all patients admitted to the NICU. Between January 2014 and December 2015, which included the outbreak period described above, Enterobacteriaceae strains isolated by fecal culture or from clinical specimens were analyzed by antimicrobial susceptibility testing and double-disc synergy testing (DDST) using ceftazidime or imipenem with sodium mercaptoacetic acid, polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE), and conjugal transfer experiments. During the outbreak period we analyzed 1073 isolates detected by fecal culture from 285 patients. Of these, we detected 64 *bla*<sub>IMP</sub>-producing Enterobacteriaceae from 52 patients, all of whom were asymptomatic carriers (52/285, 18.2%). *Klebsiella pneumoniae* (33/64 strains, 51.6%) and *Enterobacter cloacae* (20/64 strains, 31.3%) were the predominant species. Of the 64 *bla*<sub>IMP</sub>-producing Enterobacteriaceae, 7 strains were susceptible to imipenem and meropenem. Active surveillance by fecal culturing and DDST of carbapenem-resistant and third-generation cephalosporin-resistant pathogens may be an effective strategy for identifying CPE carriers. Therefore, this strategy may contribute to infection control and prevent further spread of disease in healthcare settings.

**Key words :** Carbapenemase-producing Enterobacteriaceae, outbreak, active surveillance, IMP, double-disc synergy test

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## Introduction

The spread of carbapenemase-producing Enterobacteriaceae (CPE) is a serious problem in healthcare settings. These strains are typically resistant to almost all available antimicrobials, increasing the risk of mortality following infection<sup>1, 2)</sup>. CPE-colonized patients can unwittingly spread infection and cause an outbreak of infectious disease. Detection of CPE is based on polymerase chain reaction (PCR) analysis. However, some CPE strains are susceptible to carbapenems and can remain undetected if antimicrobial susceptibility tests and PCR targeting carbapenem-resistant Enterobacteriaceae only are employed.

The most common carbapenemase in Japan is metallo-beta-lactamase (MBL) IMP (*bla*<sub>IMP</sub>), which was first detected in Japan in 1991<sup>3)</sup>. The gene encoding *bla*<sub>IMP</sub> is carried on a plasmid, which plays an important role in its transmission during healthcare-associated outbreaks<sup>4)</sup>. Thus, active surveillance of patients at high risk of CPE colonization, by taking stool or rectal swabs, is strongly recommended<sup>5)</sup>. In Japan, CPE outbreaks have been controlled by the careful use of antibiotics, strict hygiene practices, and strict compliance with contact precautions<sup>6, 7)</sup>.

Despite the precautions described above, there are insufficient data regarding infection control methodology, active surveillance, and the molecular epidemiological characteristics of CPE during outbreaks in Japan<sup>6-8)</sup>. In this study, we collected CPE isolates between January 1, 2014 and December 31, 2015, which included a year-long CPE outbreak at our hospital's neonatal intensive care unit (NICU). We performed active surveillance and investigated the drug-resistance, types of resistance genes, transmission mechanism, and plasmid content of CPE isolates to gain a better understanding of CPE outbreaks in Japan.

## Materials and methods

### *Setting and outbreak details*

Showa University Hospital is an 815-bed tertiary care hospital in Tokyo, Japan. In October 2014 CPE was isolated from 3 NICU patients: we judged this to be an outbreak of CPE. An outbreak of CPE occurred in the hospital's NICU (comprising 38 beds) between October 2014 and October 2015. During this outbreak period, we routinely screened fecal cultures from NICU patients every Tuesday during admission. In this outbreak period, 313 patients were included in this study, and fecal culture was obtained from 285 patients. Sheep blood and Drigalski agar media were used for screening samples. When CPE were isolated from fecal cultures, the affected patients were spatially isolated in specific controlled areas within the NICU. Patients showing some clinical symptoms of infectious disease were regarded as infectious patients and those showing no symptoms were regarded as colonized patients. We discontinued acceptance of patients from other hospitals during the outbreak period. Hospitalization for in-hospital births was also restricted to a minimum to reduce the number of patients exposed to CPE. In addition, medical staff were repeatedly educated on contact prevention measures and hand hygiene.

*Bacterial isolates, susceptibility testing, and phenotypic assay*

All Enterobacteriaceae (6247 strains from 4434 patients) isolated at Showa University Hospital between January 1, 2014 and December 31, 2015 were included in this study. Strains isolated from the same patient and site of isolation were counted as a single strain. Identification and antimicrobial susceptibility testing were performed using a MicroScan WalkAway 96 (Siemens Healthineers and Siemens Medical Solutions USA, Inc., Malvern, PA, USA). We determined the minimum inhibitory concentration (MIC) for ampicillin, amoxicillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cefazolin, cefmetazole, cefotaxime, ceftazidime, imipenem, meropenem, gentamicin, amikacin, minocycline, sulfamethoxazole-trimethoprim, and levofloxacin. MIC values were interpreted as susceptible (S), intermediate (I), or resistant (R) according to the approved standard published in the Clinical and Laboratory Standards Institute (CLSI) M100-S22.

To detect MBLs, the double-disc synergy test (DDST) was performed using Kirby-Bauer discs containing ceftazidime or imipenem with sodium mercaptoacetic acid (Eiken Kagaku, Tokyo, Japan) on strains that were either resistant to third-generation cephalosporins (cefotaxime and ceftazidime) or non-susceptible (demonstrated full resistance or intermediate resistance) to carbapenems (imipenem and meropenem)<sup>9)</sup>. We defined a positive DDST result as one that displayed any slight enlargement in the diameter of the inhibition zone, which suggested that the strain produced an MBL.

*DNA extraction and PCR*

To detect carbapenemase genes, PCR analysis was performed on strains that were either non-susceptible to carbapenems (imipenem and meropenem), or resistant to third-generation cephalosporins (cefotaxime and ceftazidime) and DDST positive. Total bacterial DNA was extracted from colonies grown on Luria-Bertani (LB) medium agar plates. PCR was performed as described previously<sup>10)</sup>, using 50 pmol of the forward (F) and reverse (R) primers shown in Table 1. PCR products were electrophoresed on a 5% polyacrylamide gel and stained with SYBR Green<sup>®</sup> (Takara Bio Inc., Shiga, Japan).

*Pulsed-field gel electrophoresis (PFGE)*

PFGE was performed as described previously<sup>11)</sup>. Genomic DNA was digested with the restriction enzyme *Spe* I. Pulsing was performed by the lamp-pulse method at 60–90 s of switching for 22 h at 6 V/cm and at 14°C using the CHEF DR III system (Bio-Rad, Hercules, CA, USA). The PFGE patterns were analyzed using the Dendron computer software for Windows 3.1 (Soltech, Oakdale, CA, USA). The Dendron calculated the similarity value ( $S_{AB}$ ) for each pair of fingerprints on the basis of band position.

*Conjugal transfer experiments*

Conjugal transfer experiments were performed using rifampicin-resistant and cefotaxime-susceptible *E. coli* TUM2235 strains provided by Toho University (Tokyo, Japan) as the recipients. Eight strains of *bla*<sub>IMP</sub>-producing Enterobacteriaceae isolated from patients were used as

Table 1. Primer sequences used in PCR analysis

Target genes		Primer sequence	Expected product size
<i>bla<sub>IMP</sub></i> *	forward	5'-CTACCGCAGCAGAGTCTTTG-3'	588 bp
	reverse	5'-GTGGCTTGAACCTTACCGTC-3'	
<i>bla<sub>VIM-2</sub></i>	forward	5'-TGACCGCGTCTATCATGGC-3'	768 bp
	reverse	5'-GAATGCGCAGCACCAGGAT-3'	
<i>bla<sub>KPC</sub></i>	forward	5'-ATGTCACTGTATCGCCGTCT-3'	893 bp
	reverse	5'-TTTTTCAGAGCCTTACTGCCC-3'	
<i>bla<sub>OXA-48</sub></i>	forward	5'-CCAAGCATTTTTTACCCGCATCKACC-3'	389 bp
	reverse	5'-GYTTGACCATACGCTGRCTGCG-3'	
<i>bla<sub>NDM-1</sub></i>	forward	5'-GGGCCGTATGAGTGA-3'	758 bp
	reverse	5'-GAAGCTGAGCACCGCATTAG-3'	
16SrRNA	forward	5'-AGGAGGTGATCCAACCGCA-3'	
	reverse	5'-AACTGGAGGAAGGTGGGGAT-3'	

\*The *bla<sub>IMP</sub>* primer can amplify IMP gene subtypes 1, 3-7, 9-11, 15, 16, 25, 28, and 29.

the donors. All of the donors were susceptible to rifampicin. Both donor and recipient isolates were incubated for 4 h in LB broth at 37°C and diluted to approximately  $1.5 \times 10^8$  CFU/ml using McFarland's nephelometer standard number of 0.5. Donor broth (1 ml) was mixed with 250 µl of recipient broth and incubated for 24 h at 35°C. The conjugation mixture was spread onto an LB agar plate containing 25 µg/ml of rifampicin and 2.5 µg/ml of cefotaxime to select transconjugants. Colonies were incubated overnight at 37°C and used for plasmid DNA extraction. MBL production was confirmed by DDST.

#### Plasmid DNA extraction and sequencing

We sequenced one of the transconjugant plasmid DNA. This strain was *Enterobacter cloacae* that was isolated from NICU patient's surveillance fecal culture, and fully resistant to imipenem and meropenem, and showed intermediate resistance to amikacin. Plasmid DNA was extracted from overnight cultures of 2×yeast extract tryptone broth using the QIAGEN Plasmid Maxi Kit® (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Sequencing reactions were performed using the BigDye Terminator v1.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, and products were sequenced on an ABI PRISM 310 Genetic Analyzer (Life Technologies, Gaithersburg, MD, USA). Database searches were conducted using the BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST>).

This was a retrospective study, and consent was obtained by opt-out. Approval for this study was obtained from the Ethics Committee at Showa University School of Health and Science (Kanagawa, Japan ; approval number 363).

Table 2. PCR analysis of 71 Enterobacteriaceae isolated from the NICU outbreak

Species	No. of isolates	No. of isolates positive for carbapenemases	
		IMP	KPC, VIM-2, NDM-1, OXA-48
<i>Klebsiella pneumoniae</i>	33	33	0
<i>Enterobacter cloacae</i>	24	20	0
<i>Klebsiella oxytoca</i>	5	5	0
<i>Escherichia coli</i>	2	2	0
<i>Citrobacter freundii</i>	2	2	0
<i>Enterobacter species</i>	3	1	0
<i>Enterobacter aerogenes</i>	2	1	0
Total	71	64	0

## Results

### *Bacterial isolates and detection of carbapenem-resistance genes*

During the whole study period (January 2014 to December 2015), 6247 strains (4434 patients) of Enterobacteriaceae were isolated from infected patients or by preventive screening of patients at Showa University Hospital. Of these, 151 strains were non-susceptible to carbapenems, resistant to third-generation cephalosporins, and gave a positive DDST result. PCR analysis revealed that 124 of these 151 strains carried the *bla*<sub>IMP</sub> gene. All 124 strains harboring the *bla*<sub>IMP</sub> gene gave a positive DDST result. Other types of carbapenemase, such as KPC, VIM-2, OXA-48, and NDM-1, were not detected (Table 2). Of the 124 *bla*<sub>IMP</sub>-producing Enterobacteriaceae, 117 strains were isolated from 91 patients who were colonized with these bacteria, and 7 strains caused infection in 6 patients.

During the outbreak period (October 2014 to October 2015), 313 patients were admitted to the NICU. Fecal cultures from 285 patients (1073 samples) were screened. Of these 1073 samples, 63 strains (53 patients) were non-susceptible to imipenem or meropenem, and 8 carbapenem-susceptible strains (8 patients), which DDST confirmed to be MBL-producers, demonstrated resistance to cefotaxime or ceftazidime. PCR revealed that 64 of these 71 strains (isolated from 52 of the 58 patients) carried the *bla*<sub>IMP</sub> gene (Table 2). The predominant *bla*<sub>IMP</sub>-producing Enterobacteriaceae were *Klebsiella pneumoniae* (33/64 strains, 51.6%) and *Enterobacter cloacae* (20/64 strains, 31.3%). Each of these 64 *bla*<sub>IMP</sub>-producing Enterobacteriaceae were isolated from fecal culture samples following active surveillance of colonized patients, none of whom developed an infection. Of the *bla*<sub>IMP</sub>-producing Enterobacteriaceae, 18 strains (18/64, 28.1%) were susceptible to imipenem and 7 strains (7/64, 11.7%) were susceptible to meropenem (Table 3). Because meropenem susceptibility test was started using routinely from October 2014 in our hospital, the results of the meropenem susceptibility test could not be obtained for 4 cases (Table 3). Six *bla*<sub>IMP</sub>-producing Enterobacteriaceae were susceptible to both imipenem and meropenem (Table 4). One *bla*<sub>IMP</sub>-producing Enterobacteriaceae was susceptible to imipenem but was not

Table 3. Comparison of MIC values for carbapenems in the *bla*<sub>IMP</sub>-positive and -negative Enterobacteriaceae

		MIC (μg/ml)							
		Imipenem				Meropenem			
		≤ 1	= 2	≥ 4	Total	≤ 1	= 2	≥ 4	Total
<i>bla</i> <sub>IMP</sub> PCR status	+	18	35	11	64	7	7	46	60
	[n (%)]	(28.1)	(54.7)	(17.2)	(100)	(11.7)	(11.7)	(76.7)	(100)
	–	1	4	2	7	6	0	1	7
	[n (%)]	(14.3)	(57.1)	(28.6)	(100)	(85.7)	(0)	(14.3)	(100)

Table 4. Clinical and molecular characteristics of carbapenem-susceptible CPE

Species	Isolation site	DDST*	<i>bla</i> <sub>IMP</sub>	MIC (μg/ml)			
				Cefotaxime	Ceftazidime	Imipenem	Meropenem
<i>K. pneumoniae</i>	Stool	+	+	≥ 4	≥ 16	≤ 1	≤ 1
<i>K. pneumoniae</i>	Stool	+	+	≥ 4	≥ 16	≤ 1	N/A
<i>K. oxytoca</i>	Stool	+	+	≥ 4	≤ 4	≤ 1	≤ 1
<i>E. cloacae</i>	Stool	+	+	≥ 4	≥ 16	≤ 1	≤ 1
<i>E. cloacae</i>	Stool	+	+	≥ 4	≥ 16	≤ 1	≤ 1
<i>E. aerogenes</i>	Stool	+	+	≥ 4	≥ 16	≤ 1	≤ 1
<i>E. coli</i>	Stool	+	+	≥ 4	8	≤ 1	≤ 1

\*Double-disc synergy testing (DDST) was performed using sodium mercaptoacetic acid with ceftazidime and imipenem. Abbreviations : MIC, minimum inhibitory concentration ; N/A, not available.

available for susceptibility testing for meropenem (Table 4). Each of the 7 strains (4 *Enterobacter cloacae*, 2 *Enterobacter speices*, 1 *Enterobacter aerogenes*) identified by PCR as *bla*<sub>IMP</sub>-negative were identified as *Enterobacter* species : 4 were DDST positive, and 3 were DDST negative and resistant to carbapenems.

### PFGE

We used PFGE to analyze 31 strains of *K. pneumoniae*, 21 strains of *E. cloacae*, and 5 strains of *Klebsiella oxytoca* that were isolated from the NICU during the outbreak period. Several genome patterns were observed (Fig. 1). Samples 11, 12, 51, and 54 were categorized into group A (Fig. 1, 2). Sample 53 had a unique digestion pattern, and sample 55 was categorized into group B by subsequent PFGE analysis (Fig. 1, 2).

Dendrograms based on computer-assisted comparisons of the PFGE profiles of the 21 *E. cloacae* strains are shown in Fig. 2. The PFGE profiles could be divided into 3 clusters when the S<sub>AB</sub> cut-off value was set at 0.75. *K. pneumoniae* and *K. oxytoca* strains were divided into 9 and 2 clusters, respectively, using the same procedure. The results of the antimicrobial susceptibility tests, including those for carbapenems, varied among isolates within the same cluster. Of



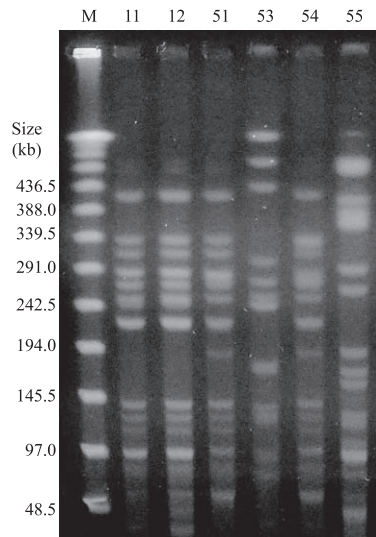


Fig. 1. Pulsed-field gel electrophoresis (PFGE) of 6 isolated CPE strains

Following digestion with *Spe* I, genomic DNA was analyzed by PFGE. The digestion patterns of samples 11, 12, 51, 53, 54, and 55 are shown. A lambda phage DNA concatemer was used as a size marker.

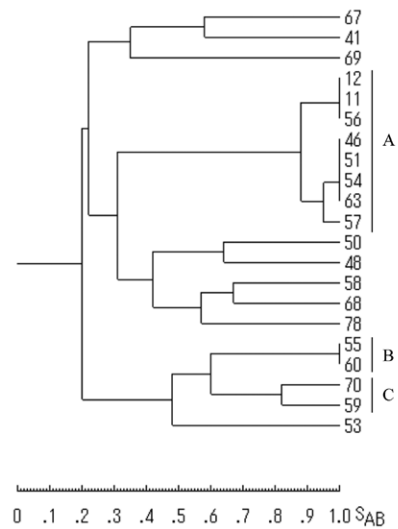


Fig. 2. A dendrogram of the pulsed-field gel electrophoresis (PFGE) profiles of 21 *Enterobacter cloacae* isolates

The PFGE profiles were divided into 3 clusters, Groups A, B, and C, when the cutoff of the  $S_{AB}$  (similarity value) was set at 0.75.

the 7 the *Enterobacter* species with a negative PCR result, 3 strains were analyzed by PFGE and were found to belong to different PFGE clusters.

#### Conjugal transfer experiment and sequencing

Eight strains of *bla*<sub>IMP</sub>-producing Enterobacteriaceae isolated from patients were used as donors. These included: 4 *E. cloacae* strains, 3 *K. pneumoniae* strains, and one *K. oxytoca* strain. They were selected based on the results of the PFGE. All 8 donors successfully transferred plasmids to recipients, and all of the resulting transconjugants were shown to produce MBL by DDST. A class I integron coding for integrase I, the att recombination site, *bla*<sub>IMP</sub>-11, and aac (6')-Ia was identified by sequencing the plasmid DNA extracted from one of the transconjugants (Fig. 3). *E. cloacae* carrying the transconjugant plasmid described above was fully resistant to imipenem and meropenem, and showed intermediate resistance to amikacin.

#### Discussion

In this study we describe a large, widespread outbreak of 64 *bla*<sub>IMP</sub>-producing Enterobacteriaceae in the NICU at a Japanese university hospital. Detecting CPE can be difficult if only a routine antimicrobial susceptibility test is used because the MIC values of carbapenems are variable<sup>12, 13</sup>. In this study, the MIC values ranged from  $\leq 1$  to  $\geq 4$   $\mu\text{g/ml}$ . Instead, we used a strategy in which MBL-producing pathogens were detected by DDST prior to genetic analysis. This may be an effective strategy because it reduces the time and cost required for detection

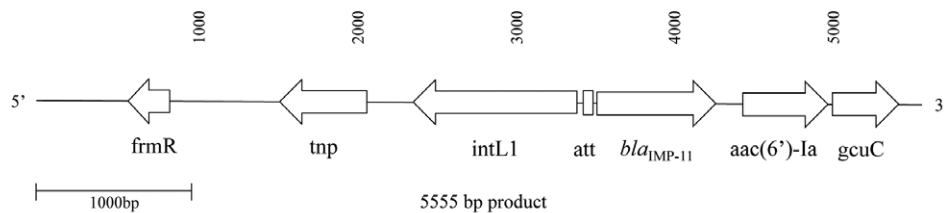


Fig. 3. Structure of the integron region in plasmid DNA isolated from a transconjugant. The plasmid DNA was extracted from a *bla<sub>IMP</sub>*-producing *E. cloacae* strain and sequenced. This class I integron structure contained carbapenem and aminoglycoside resistant gene *bla<sub>IMP-11</sub>* and *aac(6')-Ia*. The sequence was submitted to GenBank with the accession number LC179842.

especially in regions where MBL-producing pathogens are predominant, such as Japan<sup>14)</sup>.

Using our strategy, we analyzed isolates that were non-susceptible to carbapenem and isolates that produced MBL and were resistant to third-generation cephalosporins to detect carbapenem-susceptible CPE. We identified 7 *bla<sub>IMP</sub>*-positive isolates with carbapenem susceptibility. We also detected 7 *bla<sub>IMP</sub>*-negative strains by PCR. However, 4 of the *bla<sub>IMP</sub>*-negative strains had positive DDST results, and may encode other IMP subtypes that could not be determined in this study. The remaining 3 strains had negative DDST results and were resistant to carbapenem, which may be the result of a decrease in porin expression and beta-lactamase expression but not MBL. Various subtypes of IMP-type metallo- $\beta$ -lactamases have been reported, such as IMP-1 and IMP-6 that were reported in Japan<sup>6, 8, 14)</sup>. In this study, the transconjugant plasmid DNA we sequenced possessed the *bla<sub>IMP-11</sub>* gene; however, we did not identify the IMP subtypes in the other strains.

For effective infection control, it is important to understand how CPE spreads in healthcare settings. MBL genes are harbored by integrons in a transferable plasmid and can be easily transferred from one bacterium to another<sup>15, 16)</sup>. We observed various clusters within each bacterial strain and many unique patterns among the isolates in our PFGE analysis. This may suggest that our outbreak was not only caused by the spread of a pathogen from a single clone, but also by horizontal transfer via a plasmid. Our conjugal transfer experiments also indicated that *bla<sub>IMP</sub>*-producing Enterobacteriaceae isolated from patients can transfer plasmids to another strain of Enterobacteriaceae, *Escherichia coli*. Sequencing the transconjugant plasmid DNA revealed that the transferable plasmid contained a class I integron harbouring the *bla<sub>IMP-11</sub>* and *aac(6')-Ia* genes.

As Enterobacteriaceae are a normal part of the intestinal flora, CPE can colonize the human colon over a long time period. Therefore, a delay in detecting CPE-colonized patients can result in the spread of CPE. The United States Centers for Disease Control and Prevention recommend active surveillance testing of individuals with epidemiologic links to a patient with CPE infection in acute care facilities, especially those located in endemic areas<sup>5)</sup>. In this study, 285 out of 313 (91.1%) patients admitted to the NICU during the outbreak period were subjected to active surveillance by fecal culture. Out of 1073 samples obtained from the 285 patients, 64



strains (64/1073, 6.0%) of CPE were isolated and 52 colonized patients (52/285, 18.2%) were detected. Active surveillance identified more CPEs in the NICU during the outbreak period compared to the 124 CPE isolated from 6247 Enterobacteriaceae strains (2.0%) during the whole study period. Since many CPE carriers were identified at the NICU, strict control measures were implemented, such as contact prevention measures, limiting the number of hospitalizations, and isolating carriers. In doing so, we succeeded in terminating the CPE outbreak at the NICU.

We have shown that active surveillance by fecal culturing and DDST of carbapenem-resistant and third-generation cephalosporin-resistant pathogens is an effective strategy for identifying CPE carriers. We believe this strategy may contribute to infection control and prevent further spread of disease in healthcare settings.

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### Conflict of interest disclosure

No potential conflicts of interest were disclosed.

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