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Note

Dissemination in Japan of multidrug-resistant *Pseudomonas aeruginosa* isolates producing IMP-type metallo-β-lactamases and AAC(6′)-Iae/AAC(6′)-Ib

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ABSTRACT

The spread throughout Japan of antibiotic-resistance factors in multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates was investigated epidemiologically, using immunochromatographic assays specific for IMP-type metallo-β-lactamases (IMPs) and aminoglycoside 6′-N-acetyltransferase [AAC(6′)]-Iae and -Ib. Three hundred MDR *P. aeruginosa* isolates were obtained during each of two years, 2011 and 2012, from 190 hospitals in 39 prefectures in Japan. The percentage of *P. aeruginosa* isolates producing IMPs, AAC(6′)-Iae or AAC(6′)-Ib increased significantly from 170/300 (56.7%) in 2011 to 230/300 (76.7%) in 2012, with 134/170 (78.8%) in 2011 and 179/230 (77.8%) in 2012 producing both IMP and either AAC(6′)-Iae or AAC(6′)-Ib. The MICs of antibiotics, including cephalosporins and carbapenems, were markedly higher for isolates that did than did not produce these resistance factors. These results indicated that MDR *P. aeruginosa* producing IMPs, AAC(6′)-Iae or AAC(6′)-Ib have spread throughout Japan and that these antibiotic-resistance factors are useful markers for monitoring MDR *P. aeruginosa* in Japan.

Although *Pseudomonas aeruginosa* is intrinsically sensitive to β-lactams (e.g., ceftazidime [CAZ] and imipenem [IPM]), aminoglycosides (e.g., amikacin [AMK] and tobramycin), and fluoroquinolones (e.g., ciprofloxacin [CIP] and ofloxacin [OFX]), *P. aeruginosa* resistant to these antibiotics has emerged and is widespread [1]. Nosocomial outbreaks of *P. aeruginosa* infection, particularly by multidrug-resistant (MDR) strains, have become more frequent in various countries [2–5], including Japan [1,6].

MDR *P. aeruginosa* isolates in Japan frequently produce IMP-type metallo-β-lactamases (MBLs) and/or aminoglycoside 6′-N-acetyltransferases [AAC(6′)s]-Iae and -Ib [1,7–9]. We recently designed immunochromatographic assay kits for the detection of IMP-type MBLs and AAC(6′)-Iae and -Ib [10–12]. Clinical assessment showed that the results of these immunochromatographic assays were fully consistent with those of PCR analyses [10–12]. The aim of the study is to elucidate the spread of antibiotic-resistance factors in MDR *P. aeruginosa* isolates throughout Japan.

Bacterial species were identified with the MicroScan WalkAway system and MicroScan breakpoint panels (Siemens Healthcare Diagnostics, Tokyo, Japan). Drug susceptibility was determined qualitatively as sensitive (S), intermediate (I) or resistant (R) using MicroScan breakpoint panels (Siemens Healthcare Diagnostics) consistent with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). MDR *P. aeruginosa* isolates were defined as isolates resistant to imipenem (IPM), amikacin (AMK) and ciprofloxacin (CPFX) using the breakpoint panels in the study. Minimum inhibitory concentrations (MICs) of CAZ, cefepime (CFPM), meropenem (MEMP), panipenem (PAPM), doripenem (DRPM), IPM, CPFX, levofloxacin (LVFX), AMK, and arbekacin (ABK) were determined by a broth microdilution method with dry plate (Eiken Chemical Co., Ltd., Tokyo, Japan). Values of MICs at which 50% and 90% of the isolates were inhibited (MIC_{50} and MIC_{90}, respectively) were determined.

Three hundred MDR *P. aeruginosa* isolates were obtained during each of two years (2011 and 2012) from single patients in 190

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Table 1
Regions and sample origins where MDR *P. aeruginosa* strains were obtained in 2011 and 2012.

A

Year	Hokkaido/ Tohoku	Kanto/ Koshinetsu	Tokai/ Hokuriku/Kinki	Chugoku/ Shikoku	Kyushu/ Okinawa	Total
2011	53 (17.7%)	136 (45.3%)	71 (23.7%)	17 (5.7%)	23 (7.7%)	300
2012	40 (13.3%)	162 (54%)	67 (22.3%)	19 (6.3%)	19 (6.3%)	300

B

Year	Respiratory tract	Urinary tract	Others	Total
2011	134 (44.7%)	148 (49.3%)	18 (6%)	300
2012	124 (41.3%)	164 (54.7%)	12 (4%)	300

Distributions about regions (A) or sample origins (B) were not significantly different between 2011 and 2012.

hospitals located in 39 of the 47 prefectures in Japan. IMP-type MBLs, AAC(6′)-Iae, and AAC(6′)-Ib produced by these isolates were detected with immunochromatographic assay kits (Mizuho Medy Co., Saga, Japan) [10–12], as described [10]. Chi square tests were performed to compare the differences between data in 2011 and 2012.

Regions where samples were obtained were shown in Table 1A, according to the five regional blocks: i.e., Hokkaido/Tohoku, Kanto/Koshinetsu, Tokai/Hokuriku/Kinki, Chugoku/Shikoku, and Kyushu/Okinawa. Sample origins were also shown in Table 1B. Distributions about regions or sample origins were not significantly different between 2011 and 2012.

The distributions of MDR *P. aeruginosa* isolates producing IMP, AAC(6′)-Iae and AAC(6′)-Ib in 2011 and 2012 are shown in Table 2. Of the 300 isolates obtained during 2011, 170 (56.7%) were positive for the production of an IMP, AAC(6′)-Iae or AAC(6′)-Ib. In comparison, 230 of the 300 (76.7%) isolates obtained during 2012 were positive for the resistance factors, which was a significant increase over the rate in 2011 ($P < 0.01$). Of these positive isolates with at least more than one resistance factor, IMP and AAC(6′)-Iae co-producers were the most prevalent in 2011 and 2012. In addition, these IMP and AAC(6′)-Iae co-producers significantly increased from 28.7% of all MDR *P. aeruginosa* isolates tested in 2011 to 41.7% in 2012 ($P < 0.01$). Producers with other combinations of resistance factors did not increase or decrease significantly between 2011 and 2012.

Since most of the MDR *P. aeruginosa* isolates produced both IMP and AAC(6′)-Iae or both IMP and AAC(6′)-Ib, we compared the drug susceptibility of these isolates in 2012 with that of the isolates not producing these factors (Table 3). The MIC_{50} and MIC_{90} of cephalosporins and carbapenems were markedly higher for the isolates that did than did not produce these factors. There were no marked between group differences in the MIC_{50} and MIC_{90} of fluoroquinolones. The MIC_{50} of AMK, but not ABK, was significantly higher for isolates producing both IMP and AAC(6′)-Iae than for other groups. Similar results were observed for strains isolated in 2011 (data not shown).

Our study found that, of MDR *P. aeruginosa* isolates in Japan, IMP and AAC(6′)-Iae co-producers increased from 2011 to 2012 and showed higher MICs of cephalosporins and carbapenems than other groups. These producers also showed higher MIC of AMK, not ABK. These results were supported by a previous report describing that *Escherichia coli* DH5α expressing AAC(6′)-Iae was resistant to AMK but not to gentamicin and ABK [9].

We recently isolated MDR *P. aeruginosa* strains producing the novel aminoglycoside enzymes, AAC(6′)-Iaf [13] and AAC(6′)-Iaj [14]. Thin-layer chromatographic assay demonstrated that these enzymes effectively hydrolyzed AMK more effectively [13,14]. It is necessary to carefully monitor MDR *P. aeruginosa* isolates producing IMP-type metallo-β-lactamases, including novel IMP variants. These variants have been detected in MDR *P. aeruginosa* isolates, with one, IMP-43, conferring greater resistance to doripenem and meropenem but not to imipenem [7].

Use of these immunochromatographic assays has revealed various aspects of MDR *P. aeruginosa* prevalence and provided epidemiological information about drug resistance factors

Table 2
Drug resistance factors in MDR *P. aeruginosa* isolates in Japan.

Drug resistant factors							
Year	IMP+ AAC(6′)-Iae	IMP+ AAC(6′)-Ib	IMP	AAC(6′)-Iae	AAC(6′)-Ib	Negative	Total
2011	86 (28.7%)	48 (16%)	5 (1.6%)	2 (0.6%)	29 (9.7%)	130 (43.3%)	300
2012	125 (41.7%)	54 (18%)	11 (3.6%)	10 (3.3%)	30 (10%)	70 (23.3%)	300

Three hundred isolates were analyzed in each of two years, 2011 and 2012. Analysis showed that 130 isolates (43.3%) obtained in 2011 and 70 isolates (23.3%) in 2012 were negative for all the three factors, while 170 isolates (56.7%) and 230 isolates (76.7%), respectively, harbored at least one of the three factors.

Table 3
Drug susceptibility test of MDR *P. aeruginosa* isolates in 2012.

Drug resistant factors						
	IMP+ AAC(6′)-Iae		IMP+ AAC(6′)-Ib		Negative	
Antibiotics	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Cephalosporin						
CAZ	>128	>128	>128	>128	8	64
CEPM	>128	>128	>128	>128	16	64
Carbapenem						
MEPM	>128	>128	128	>128	16	64
PAPM	>128	>128	>128	>128	32	128
DPRM	>64	>64	>64	>64	8	32
IPM	128	>128	128	>128	16	32
Fluorquinolone						
CPFX	64	>128	32	128	32	64
LVFX	64	>128	64	128	64	128
Aminoglycoside						
AMK	128	128	32	64	32	64
ABK	8	32	16	64	16	64

associated with MDR *P. aeruginosa*. These immunochromatographic assays are simple methods that can rapidly detect antibiotic-resistance factors and are a useful alternative to PCR analysis for nationwide surveillance.

Author contributions

Experimental design: MT (Tojo), TT, TMA, TK, NO; Collection of clinical isolates and determination of MIC: MS; Immunochromatographic assay: MT (Tanaka), KN; Data analysis and preparation of the manuscript: MT (Tojo), TK, NO.

Conflicts of interest

Masahiro Shimojima is an employee of BML Inc., Masashi Tanaka and Kenji Narahara are employees of Mizuho Medy Co., Ltd. R&D. Their involvement does not alter our adherence to all the Journal policies on sharing data and materials. This study was not funded by those companies. The remaining authors have no reported potential conflicts of interest.

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Molecular and Epidemiological Characterization of IMP-Type Metallo-β-Lactamase-Producing *Enterobacter cloacae* in a Large Tertiary Care Hospital in Japan

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IMP-type metallo-β-lactamase enzymes have been reported in different geographical areas and in various Gram-negative bacteria. However, the risk factors and epidemiology pertaining to IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* (IMP-producing *E. cloacae*) have not been systematically evaluated. We conducted a retrospective, matched case-control study of patients from whom IMP-producing *E. cloacae* isolates were obtained, in addition to performing thorough molecular analyses of the clinically obtained IMP-producing *E. cloacae* isolates. Unique cases with IMP-producing *E. cloacae* isolation were included. Patients with IMP-producing *E. cloacae* were matched to uninfected controls at a ratio of 1 to 3. Fifteen IMP-producing *E. cloacae* cases were identified, with five of the isolates being obtained from blood, and they were matched to 45 uninfected controls. All (100%) patients from whom IMP-producing *E. cloacae* isolates were obtained had indwelling devices at the time of isolation, compared with one (2.2%) uninfected control. Independent predictors for isolation of IMP-producing *E. cloacae* were identified as cephalosporin exposure and invasive procedures within 3 months. Although in-hospital mortality rates were similar between cases and controls (14.3% versus 13.3%), the in-hospital mortality of patients with IMP-producing *E. cloacae*-caused bacteremia was significantly higher (40%) than the rate in controls. IMP-producing *E. cloacae* isolates were frequently positive for other resistance determinants. The MICs of meropenem and imipenem were not elevated; 10 (67%) and 12 (80%) of the 15 IMP-producing *E. cloacae* isolates had a MIC of ≤1 μg/ml. A phylogenetic tree showed a close relationship among the IMP-producing *E. cloacae* samples. Indwelling devices, exposure to cephalosporin, and a history of invasive procedures were associated with isolation of IMP-producing *E. cloacae*. Screening for carbapenemase production is important in order to apply appropriate clinical management and infection control measures.

The emergence of extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* has been observed globally in the health care-associated setting and in the community (1). The importance of carbapenem as a treatment option for ESBL-producing organisms, as well as chromosomal cephalosporinase-producing organisms, has been increasing (1). The recent spread of carbapenemase-producing Gram-negative bacteria is a significant public health problem. Class A enzymes, represented by *Klebsiella pneumoniae* carbapenemase (KPC)-type enzymes, and acquired class B metallo-β-lactamases (MBLs) are disseminated among bacteria internationally (2). The MBLs include various clinically and epidemiologically important types, such as VIM, NDM, and IMP types (3). IMP-type enzymes were first detected in Japan in the late 1980s (4). Since this time, IMP-type enzymes have been reported from different geographical areas, including Japan, in various Gram-negative bacteria (mostly in *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Serratia marcescens*) (3–6). IMP-type enzymes have broad substrate specificity that includes cephalosporins and carbapenems (7, 8).

Since 2011, metallo-β-lactamase-producing *Enterobacter cloacae* isolates have been obtained from multiple patients at the National Center for Global Health and Medicine in Tokyo, Japan (9). *E. cloacae* is a common nosocomial pathogen. *E. cloacae* is ubiquitous in the hospital environment and can survive on skin and dry surfaces (10). *E. cloacae* is known to possess inducible *ampC* chromosomal β-lactamase and may also carry plasmid-mediated ESBLs (11). Carbapenemase (e.g., IMP, VIM, KPC, and NDM)-producing *E. cloacae* isolates have been reported (2, 3, 9, 12). However, to the best of our knowledge, the risk factors, epidemiology, and clinical effects pertaining to IMP-type MBL-producing *E. cloacae* have not been systematically evaluated, in contrast to other carbapenemase-producing pathogens, such as KPC producers (13, 14).

Therefore, we conducted a case-control study of patients from whom IMP-type metallo-β-lactamase-producing *E. cloacae* (IMP-producing *E. cloacae*) isolates were obtained, in addition to thorough molecular analyses of the clinically obtained IMP-producing *E. cloacae* isolates.

MATERIALS AND METHODS

Study setting and design. A retrospective matched case-control investigation of risk factors and outcomes was conducted at the National Center for Global Health and Medicine (NCGM). NCGM has more than 800

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TABLE 1 Characteristics of patients from whom IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* was isolated

Patient	Age (yr)	Sex[a]	Isolation site	Infectious clinical syndrome associated with IMP-producing *E. cloacae*[b]	Reason for admission[c]	Underlying conditions[c]	Treatment for IMP-producing *E. cloacae*[d]	Outcome
1	91	M	Blood	CRBSI, peripheral line, polymicrobial (*P. vulgaris*)	CI	Hepatitis C virus-related cirrhosis, dementia, aspiration pneumonia	Peripheral line removal, Emp Tx with MEM, Tx with LVX for 7 days	Died 7 days after IMP-producing *E. cloacae* isolation, due to septic shock and pneumonia
2	77	M	Blood	CRBSI, central line	Esophageal cancer surgery	DM, ASO, HTN, CHF, CRF	Central line removal, Emp Tx with MEM, Tx with MEM + GEN for 5 days	Died 5 days after IMP-producing *E. cloacae* isolation, due to renal failure and respiratory failure
3	49	F	Urine	CA-UTI, polymicrobial (*E. faecalis*)	Subdural hematoma	SAH (status post-ventriculoperitoneal shunt placement)	Emp Tx with LVX, Tx with LVX for 7 days	Improved and discharged to subacute facility
4	88	M	Blood	SSI	Colon cancer surgery	AAA (status post-stent placement)	Emp Tx with MEM, Tx with LVX for 14 days	Improved and discharged to subacute facility
5	83	F	Urine	CA-ASB, polymicrobial (*E. faecium*)	CAPD peritonitis	DM, CRF, HTN, CI	No Tx for CA-ASB, but CAZ used for CAPD peritonitis	Improved and discharged home
6	64	M	Bile	Cholangitis, polymicrobial (*E. coli*, *K. pneumonia*, *E. agglomerans*)	Duodenal stenosis	Pancreatic cancer, DM	No Tx for IMP-producing *E. cloacae*, but MEM used for other organisms from bile	Improved and discharged to subacute facility
7	69	M	Bile	Cholangitis, polymicrobial (*E. faecium*, *E. casseliflavus*)	Cholangitis, pulmonary tuberculosis	DM	Emp Tx with PIP-TZB, VAN, Tx with LVX for 14 days, VAN and PIP-TZB also given	Improved and transferred back to another hospital
8	74	F	Urine	CA-ASB, polymicrobial (*P. rettgeri*)	DVT, CRBSI with *K. pneumonia*, *C. glabrata*, CoNS, *Rhodotorula* spp., also CDI	MCTD	No Tx against IMP-producing *E. cloacae*, but MEM, TEC, L-AMB used for CRBSI due to other organisms	Improved and discharged home
9	87	M	Stool	Colonization	CDI	Ileus	No Tx against IMP-producing *E. cloacae*	Transferred to another hospital
10	79	M	Bile	Cholangitis, polymicrobial (*P. aeruginosa*, *E. faecium*)	Cholangitis, choledocholithiasis, peritonitis	CRF, HTN, IHD (status post-stent placement)	Emp Tx with VAN, MEM, Tx with MEM for 14 days, VAN also given	Improved and discharged home
11	24	F	Sputum	Pneumonia, polymicrobial (*K. pneumonia*, α-hemolytic *Streptococcus* spp.)	SLE flare	SLE, schizophrenia	Emp Tx with VAN, MEM, Tx with MEM for 14 days	Improved and discharged to subacute facility
12	47	F	Wound, left leg	Colonization	Alveolar hemorrhage	SLE, CRF, DM, CDI, HTN	No antibiotic Tx	Still hospitalized

13	86	M	Blood	CRBSI, peripheral line	Duodenal stenosis	Duodenum papilla cancer (status post-stent placement)	Catheter removal, Emp Tx with VAN, MEM, Tx with LVX for 9 days	Improved and discharged home
14	42	F	Sputum	Colonization, polymicrobial (γ-hemolytic *Streptococcus* spp., CoNS)	Spinal injury	HIV	No antibiotic Tx	Improved and discharged to subacute facility
15	84	F	Blood	CA-UTI	Rectal ulcer	CHF	Emp Tx with CRO, Tx with LVX for 9 days	Improved and discharged to subacute facility

[a] F, female; M, male.
[b] CA-ASB, catheter-associated asymptomatic bacteriuria; CA-UTI, catheter-associated urinary tract infection; CoNS, coagulase-negative staphylococci; CRBSI, catheter-related bloodstream infection; IMP-producing *E. cloacae*, IMP-type metallo-β-lactamase-producing *Enterobacter cloacae*; polymicrobial, bacteria other than IMP-producing *E. cloacae* were isolated from the same culture specimen; SSI, surgical site infection.
[c] AAA, abdominal aortic aneurysm; ASO, arteriosclerosis obliterans; CAPD, continuous ambulatory peritoneal dialysis; CDI, *C. difficile* infection; CHF, congestive heart failure; CI, cerebral infarction; CRF, chronic renal failure; DM, diabetes mellitus; DVT, deep vein thrombosis; HTN, hypertension; IHD, ischemic heart disease; MCTD, mixed connective tissue disease; SAH, subarachnoid hemorrhage; SLE, systemic lupus erythematosus.
[d] Treatment (Tx) is defined as effective antimicrobial therapy provided from 24 h before to 28 days after IMP-producing *E. cloacae* isolation. Effective antimicrobial therapy was defined based on *in vitro* activity as reported by the NCGM clinical microbiology laboratory, in accordance with the Clinical and Laboratory Standards Institute (CLSI) criteria. Meropenem was considered effective definite therapy when the MIC of meropenem was <1 μg/ml. Empirical treatment (Emp Tx) is defined as antimicrobial therapy provided from 24 h before to 48 h after the IMP-producing *E. cloacae* culture. CAZ, ceftazidime; CRO, ceftriaxone; GEN, gentamicin; L-AMB, liposomal amphotericin B; LVX, levofloxacin; MEM, meropenem; PIP-TZB, piperacillin-tazobactam; TEC, teicoplanin; VAN, vancomycin.

inpatient beds and serves as a tertiary referral hospital for metropolitan Tokyo. Institutional review boards at the NCGM approved the study before its initiation.

Patients and variables. Patients from whom clinical isolates of IMP-producing *E. cloacae* were obtained from 1 October 2011 to 31 December 2012 were matched in a 1-to-3 ratio to uninfected controls who did not have *E. cloacae* isolated during the study period (15). The matching parameters for uninfected controls included (i) the hospital unit where the patient was being treated when the IMP-producing *E. cloacae* isolate was recovered, (ii) the calendar year and month, and (iii) the time at risk, i.e., time from admission to culture for patients with IMP-producing *E. cloacae*. For uninfected controls, the total duration of the hospital stay was considered to be the time at risk, and it had to be at least as long as the time at risk of the matched IMP-producing *E. cloacae* case. Once an eligible pool of controls was identified for each case, controls were randomly selected using the randomization function in Excel (Microsoft). For patients who had more than one strain of IMP-producing *E. cloacae* isolated during the study period, only the first episode was analyzed for the purpose of epidemiological analyses (i.e., the epidemiological part of the study included only unique patient episodes). Surveillance stool cultures were not routinely conducted at the NCGM during the study period.

The parameters retrieved from patient records included the following: (i) demographics; (ii) background conditions and comorbid conditions (including Charlson scores [16]); (iii) recent health care-associated exposure, such as a stay in a health care facility, an invasive procedure, and the presence of an indwelling device; (iv) the severity of underlying disease, including the McCabe score (17); (v) recent (within 3 months) exposure to antimicrobials prior to isolation of IMP-producing *E. cloacae* (or prior to admission for controls); and (vi) outcome, including in-hospital and 90-day mortality, length of hospital stay, deterioration in functional status (defined as deterioration from admission to discharge in at least one activity of daily living according to the Katz criteria [18]), and discharge to a long-term facility after being admitted from home. Infectious clinical syndromes in patients from whom IMP-producing *E. cloacae* was isolated were determined according to the Centers for Disease Control and Prevention definitions (19) and, when present, according to consultation notes from the infectious diseases consult service. IMP-producing *E. cloacae* isolates were considered to be colonizers if patients did not have any sign of infection based on the above-described criteria and in cases of asymptomatic bacteriuria.

Antimicrobial susceptibility, detection of IMP-type metallo-β-lactamases, and bacterial strains. Bacteria were identified to the species level, and susceptibilities to predefined antimicrobials were determined by using an automated broth microdilution system (MicroScan WalkAway; Siemens AG, Germany) and in accordance with Clinical and Laboratory Standards Institute (CLSI) criteria (document M100–S19) (20). Clinical isolates of *E. cloacae* that were resistant to one or multiple agents in the extended-spectrum cephalosporin class and/or that demonstrated elevated MICs (>1 μg/ml) to imipenem and/or meropenem were screened for ESBL, MBL, and AmpC production using the Cica-Beta-Test I with HMRZ-86 (Kanto Chemical, Tokyo, Japan) (21). Subsequently, the isolates deemed positive for MBL production by the Cica-Beta-Test I were tested for IMP-type-metallo-β-lactamase production by using an immunochromatographic assay kit (Mizuho Medy Co., Saga, Japan) (22). The broth microdilution method was also performed manually according to the guidelines of the CLSI (document M100–S22) to determine the susceptibility of isolates included in the study (23). A total of 17 isolates (from 15 patients) were included in the molecular analyses. In addition, 2 *E. cloacae* isolates from NCGM in 2007 and 2 isolates of IMP-producing *E. cloacae* from other facilities in Japan were included in the phylogenetic analyses.

Detection of antibiotic resistance genes. The bla_{IMP} and *aac(6′)-Iae* genes were amplified using PCR primers as described previously (24). All of the PCR products were sequenced using an ABI Prism 3130 sequencer (Applied Biosystems, Foster City, CA). The class 1 integron was amplified

using the PCR primer set 5′CS and 3′CS (24). All of the PCR products were sequenced to identify the contents of the genes (25).

Multilocus sequence typing. Multilocus sequence typing was performed as described elsewhere (26). To analyze the clonality of the strains/isolates, phylogenetic analysis using the concatenated sequence comprising the loci was performed (26).

Statistical analysis. All analyses were performed using IBM SPSS Statistics 20 (2011) and SAS software, version 9.3 (SAS Institute). Matched bivariate analyses were conducted using a conditional logistic regression model. Matched multivariable models were constructed using Cox proportional hazards regression, accounting for clustering in matched pairs. All variables with a P value of <0.1 in the bivariate matched analyses were considered for inclusion in the multivariate matched analyses. A stepwise selection procedure was used to select variables for inclusion in the final model. The final selected model was tested for confounding. If a covariate affected the β-coefficient of a variable in the model by >10%, then the confounding variable was maintained in the multivariable model. The percentages reported are the "valid percentage," i.e., the percentage excluding data missing from the denominator, unless otherwise stated. A two-sided P value of <0.05 was considered statistically significant.

RESULTS

A total of 15 patients with IMP-producing *E. cloacae* were identified among 260 unique patients from whom *E. cloacae* was isolated during the study period. In these patients, IMP-producing *E. cloacae* isolates were identified from blood ($n = 5$), wounds ($n = 4$; 3 were intraabdominal), urine ($n = 3$), sputum ($n = 2$), and stool ($n = 1$). A patient who had IMP-producing *E. cloacae* isolated from stool was suspected as having infectious colitis. Therefore, a stool culture was performed, which grew IMP-producing *E. cloacae*. The characteristics of the 15 patients who had IMP-producing *E. cloacae* isolated are summarized in Table 1. The mean age of patients was 70.9 ± 19.4 years. Eight (53%) patients were admitted for diseases associated with the gastrointestinal tract (including the biliary tract), and 3 (20%) were admitted for neurological problems, including cerebral vascular accidents. With regard to infectious clinical syndromes associated with IMP-producing *E. cloacae*, 3 (20%) had catheter-related bloodstream infections (2 peripheral line associated and 1 central line associated), 3 (20%) had cholangitis, 2 (13%) had catheter-associated urinary tract infection, and 2 (13%) had catheter-associated asymptomatic bacteriuria. Overall, 10 cases were considered to have infection, and 5 (including 2 catheter-associated asymptomatic bacteriuria) cases had IMP-producing *E. cloacae* colonization. The median length of hospital stay prior to IMP-producing *E. cloacae* isolation was 47 days (interquartile range [IQR], 13 to 101 days).

Two of the 15 patients (13%; 40% of 5 patients with bacteremia and 20% of 10 patients with infection [not colonization]) died during their hospital stay despite receiving effective therapy. Five patients (33%) from whom IMP-producing *E. cloacae* isolates were obtained only had asymptomatic colonization, and therefore, no antibiotics targeting IMP-producing *E. cloacae* were given. Two patients (patients 3 and 13) did not receive appropriate antibiotics for IMP-producing *E. cloacae* based on *in vitro* susceptibility. However, both of these patients improved clinically, probably because of the infected site (the urinary tract, where high antibiotic concentrations can be expected) and removal of devices (urinary catheter and peripheral line). The rest of the patients received effective therapy. Nine (60%) patients had bacteria other than IMP-producing *E. cloacae* isolated from the same culture specimen (i.e., polymicrobial isolation).

Table 2 shows the susceptibility profiles and resistance genes of

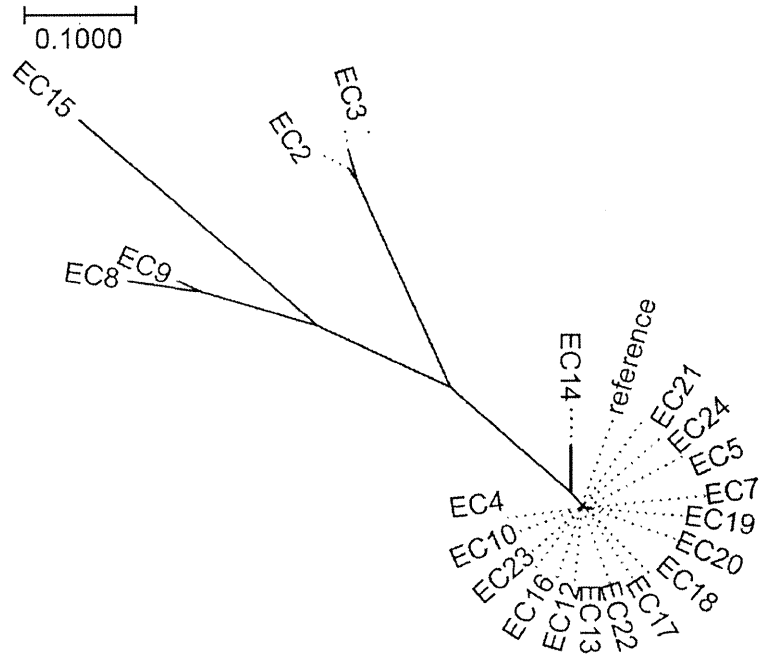
TABLE 2 Susceptibility profiles and resistance genes among IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* isolates

Patient	Isolate	IMP ICGA[a] result	Resistance gene						MIC[b] (μg/ml) and susceptibility interpretation																					
			bla_{IMP}	*aac(6′)*	*aac/aad*	*gyrA*	*qnrS*	bla_{TEM}	PIP-TZB		CTX		CAZ		FEP		IPM		MEM		CIP		AMK		GEM		ATM		CST	
1	EC4	+	1	*IIc*					128/4	R	512	R	512	R	32	R	1	S	2	I	0.5	S	1	S	0.5	S	64	R	0.5	S
2	EC5	+	11		*aacA1*	S83I[c]	+		32/4	I	32	R	128	R	8	S	1	S	1	S	32	R	16	S	≤0.125	S	≤4	S	1	S
3	EC7	+	11	*IIc*	*aacA1*	S83I	+	+	64/4	I	64	R	128	R	≤4	S	0.5	S	1	S	8	R	8	S	≤0.125	S	≤4	S	0.5	S
4	EC10	+	1	*IIc*					64/4	I	512	R	512	R	32	R	1	S	1	S	≤0.25	S	1	S	0.5	S	64	R	0.5	S
5	EC13	+	1	*IIc*					128/4	R	512	R	>512	R	32	R	1	S	2	I	4	R	0.5	S	0.5	S	512	R	0.5	S
6	EC14	+	11	*Ib*	*aacA4*			+	64/4	I	256	R	>512	R	256	R	32	R	32	R	≤0.25	S	16	S	0.25	S	512	R	0.25	S
7	EC15	+	1	*IIc*					8/4	S	128	R	512	R	16	I	2	I	4	R	≤0.25	S	1	S	0.5	S	≤4	S	0.5	S
8	EC16	+	1	*IIc*					128/4	R	512	R	512	R	32	R	1	S	1	S	≤0.25	S	0.5	S	1	S	64	R	0.5	S
9	EC17	+	1	*IIc*		S83Y[c]			≤4/4	S	512	R	512	R	8	S	4	R	4	R	1	S	1	S	0.5	S	≤4	S	0.25	S
10	EC18	+	11		*aacA1*	S83Y	+		≤4/4	S	64	R	128	R	≤4	S	1	S	1	S	1	S	16	S	0.25	S	≤4	S	0.5	S
11	EC19	+	11		*aacA1*	S83Y	+	+	32/4	I	32	R	64	R	≤4	S	0.5	S	0.5	S	8	R	8	S	≤0.125	S	≤4	S	0.5	S
12	EC20	+	1		*aacA1*	S83Y		+	32/4	I	128	R	256	R	8	S	0.5	S	1	S	8	R	8	S	0.5	S	≤4	S	0.5	S
13	EC21	+	1	*IIc*					64/4	I	512	R	512	R	16	I	1	S	1	S	4	R	4	S	0.5	S	64	R	0.5	S
14	EC22	+	1	*IIc*					128/4	R	512	R	512	R	32	R	1	S	1	S	≤0.25	S	0.5	S	0.5	S	128	R	0.5	S
15	EC24	+	1	*IIc*					64/4	I	512	R	512	R	16	I	1	S	1	S	≤0.25	S	1	S	0.5	S	64	R	0.5	S

[a] ICGA, immunochromatographic assay.

[b] MIC interpretive criteria (I, intermediate; R, resistant; S, susceptible) are according to CLSI document M100–S22 (23), except for colistin. Colistin MIC interpretive criteria are according to EUCAST (27). AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; FEP, cefepime; CST, colistin; CIP, ciprofloxacin; CTX, cefotaxime; GEM, gentamicin; IPM, imipenem; MEM, meropenem; PIP-TZB, piperacillin-tazobactam.

[c] S-to-I or S-to-Y change at position 83 encoded by *gyrA*.



Patient no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Isolate no.	EC4	EC5	EC7	EC10	EC13	EC14	EC15	EC16	EC17	EC18	EC19	EC20	EC21	EC22	EC24

FIG 1 Phylogenetic tree of IMP-metallo-β-lactamase-producing *Enterobacter cloacae* isolates. The group of isolates included EC4, EC5, EC7, EC10, EC12, EC13, EC14, EC16, EC17, EC18, EC19, EC20, EC21, EC22, EC23, EC24, and the reference. Outliers included EC2, EC3, EC8, EC9, and EC15. EC2 and EC3 were *E. cloacae* isolates from NCGM in 2007. EC8 and EC9 were IMP-producing *E. cloacae* isolates from other facilities in Japan. EC15 was obtained from a patient who had been transferred from another hospital. EC10 (blood) and EC12 (wound) were IMP-producing *E. cloacae* isolates from the same patient (patient 4). EC15 (blood) and EC23 (urine) were IMP-producing *E. cloacae* isolates from the same patient (patient 15). Reference, de novo assembled contigs of all *E. cloacae* isolates used in this study were used as the reference.

the IMP-producing *E. cloacae* isolates. All 15 clinical isolates were susceptible to aminoglycosides (amikacin and gentamicin) (23) and colistin (27). The MICs of fluoroquinolone (ciprofloxacin) varied from ≤0.25 to 32 μg/ml; 7 (47%) of the 15 isolates were resistant per CLSI criteria (M100–S22) (23). The MICs of meropenem and imipenem were not elevated; 10 (67%) and 12 (80%) of the isolates, respectively, were categorized as susceptible (≤1 μg/ml) according to recent CLSI criteria (M100–S22) (23). Ten clinical isolates were positive for the bla_{IMP-1} gene, and 5 for the bla_{IMP-11} gene. IMP-producing *E. cloacae* isolates were positive for multiple resistance genes (Table 2).

A phylogenetic tree (Fig. 1) showed a close relationship among IMP-producing *E. cloacae* samples isolated from the NCGM during the study period, except for one isolate (EC15) obtained from a patient who had been transferred from another hospital. The time line of the hospital location of patients with IMP-producing *E. cloacae* is shown in Table 3. This time line suggested possible transmission of IMP-producing *E. cloacae* in particular wards (C, D, H, and K).

To further determine the risk factors for isolation of IMP-producing *E. cloacae*, 15 IMP-producing *E. cloacae* cases were matched to 45 uninfected controls. The overall mean age of the study cohort ($n = 60$) was 66 ± 18.7 years, and 28 (46.7%) of the patients were men. Bivariate analysis comparing IMP-producing *E. cloacae* cases and uninfected controls is shown in Table 4. Patients with isolation of IMP-producing *E. cloacae* were more likely to have health care-associated exposure, such as recent hospitalization, invasive procedures, and surgery within 3 months. Patients with isolation of IMP-producing *E. cloacae* had indwelling devices more frequently than uninfected controls. All patients with isolation of IMP-producing *E. cloacae* had at least 1 indwelling device, including a central line ($n = 4$, 27%), urinary catheter ($n = 9$, 60%), tracheostomy tube ($n = 2$, 13%), dialysis catheter ($n = 2$, 13%), biliary drainage tube/stent ($n = 4$, 27%), or nasogastric tube or percutaneous endoscopic gastrostomy ($n = 4$, 27%) at the time of IMP-producing *E. cloacae* isolation. Antibiotic exposure was more common in the IMP-producing *E. cloacae* group than in controls. All of the patients with isolation of IMP-producing *E. cloacae* had antimicrobial exposure within 3 months; the most frequent exposures were to cephalosporins ($n = 9$, 60%), followed by glycopeptides ($n = 6$, 40%), penicillins ($n = 6$, 40%), and carbapenems ($n = 6$, 40%).

Although in-hospital mortality was similar between cases and controls (14.3% versus 13.3%), the in-hospital mortality of patients with isolation of IMP-producing *E. cloacae* bacteremia was significantly higher (40%) than that in controls ($P = 0.014$). Functional deterioration was more common in the IMP-producing *E.*

TABLE 3 Time line of the hospital locations of patients from whom IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* was isolated

Patient	Isolate	Department[a]	Culture date (mo/yr)	Yr and mo of stay[b]: 2011, September	2011, October	2011, November	2011, December	2012, January	2012, February	2012, March	2012, April	2012, May	2012, June	2012, July	2012, August	2012, September	2012, October	2012, November	2012, December	2013, January
1	EC4	NE	10/2011	A B	B B B															
2	EC5	SU	3/2012	C	C I C	C C C	C C C	C C C	C D D	D D										
3	EC7	NS	3/2012						A	D D D	D D D									
4	EC10	SU	3/2012							C C	C C C	C C C	C C C	C C C	C C C					
5	EC13	Nep	4/2012							E	F F F	F								
6	EC14	GE	4/2012							C C	C C C	C								
7	EC15	PU	5/2012							G G G	G G G	G G G	G G G	G						
8	EC16	CT	6/2012							H H	H H H	H H H	H							
9	EC17	GE	7/2012											J						
10	EC18	GE	7/2012										C C	C C C	I C C	C C C	C C C	C C C		
11	EC19	CT	9/2012										E K K	K K K	K K K	K K H	H H H	H H H	H H H	H H H
12	EC20	CT	9/2012								K	K K I	I I H	H H H	H H H	H H K	K K K	K K K	K K K	K K K
13	EC21	GE	9/2012													E L	L			
14	EC22	ID	11/2012														M M M	M M		
15	EC24	GE	12/2012														A C C	C C C	C C C	C C

[a] CT, rheumatology; GE, gastroenterology; ID, infectious diseases; NE, neurology; Nep, nephrology; NS, neurosurgery; PU, pulmonology; SU, surgery.

[b] Each capital letter represents a hospital unit where the patient stayed. A boldface letter represents the unit where the patient was staying when the IMP-producing *E. cloacae* was isolated.

cloacae group than in uninfected controls (25% versus 2.6%, $P = 0.05$).

Independent predictors for the isolation of IMP-producing *E. cloacae* were determined by multivariate analyses (Table 5). Invasive procedures in the past 3 months and exposure to cephalosporins in the past 3 months were independently associated with isolation of IMP-producing *E. cloacae*, and thus, these were considered risk factors for the isolation of IMP-producing *E. cloacae*, in addition to the patients' locations in the hospital.

DISCUSSION

This study examined molecular and epidemiological characteristics of clinically obtained metallo-β-lactamase-producing *E. cloacae* isolates. To the best of our knowledge, this is the first study to systematically elucidate independent risk factors for the isolation of IMP-producing *E. cloacae*. For the risk factor analyses, we carefully included risk factors known to be associated with the isolation of multidrug-resistant *Enterobacteriaceae* species (28–30). In addition, two individuals (an infectious diseases specialist and an infection preventionist) independently reviewed the patients' medical records and visited each ward as often as possible to rule out any common source of infection other than those included in the risk factor analyses. We chose to collect samples from patients admitted to the hospital who were at risk of acquiring the antimicrobial-resistant organism (control type 2) rather than from patients with cultures positive for the antibiotic-susceptible form of the organism of interest (control type 1). This is because a previous study showed that the selection of control patients from the type 1 group can falsely identify certain antibiotics and overestimate the odds ratio (OR) of the use of antimicrobial agents as risk factors (15).

In our study, IMP-producing *E. cloacae* isolates were obtained from elderly, debilitated individuals whose length of hospital stay prior to the isolation of IMP-producing *E. cloacae* was long (median, 47 days; IQR, 13 to 101 days) (Table 1). Catheter-related bloodstream infections and cholangitis were two major infectious clinical syndromes caused by IMP-producing *E. cloacae*. Notably, all 15 patients with isolation of IMP-producing *E. cloacae* had exposure to antibiotics in the 3 months prior to IMP-producing *E. cloacae* isolation. Additionally, all patients with isolation of IMP-producing *E. cloacae* had at least one indwelling device at the time of IMP-producing *E. cloacae* isolation. These were apparent risk factors for the isolation of IMP-producing *E. cloacae*, although they could not be incorporated into the final multivariate models to identify independent risk factors for the isolation of IMP-producing *E. cloacae* because all (100%) IMP-producing *E. cloacae* cases had these exposures (exposures to antibiotics and/or indwelling devices). Other independent risk factors for the isolation of IMP-producing *E. cloacae* were invasive procedures and exposure to cephalosporins in the 3 months prior. Carbapenem and cephalosporin exposure has been reported as a risk factor for the isolation of carbapenem-resistant organisms, such as IMP-type metallo-β-lactamase-producing organisms (31), multidrug-resistant *P. aeruginosa* producing SPM-type metallo-β-lactamase (32), and carbapenem-resistant *K. pneumonia* (13). In our study, carbapenem exposure was much more common in the IMP-producing *E. cloacae* group than in uninfected controls, but this was not identified as an independent risk factor.

The phylogenetic tree (Fig. 1) and time line of hospital locations of patients with IMP-producing *E. cloacae* (Table 3) sug-

TABLE 4 Bivariate analysis of risk factors and outcomes for isolation of IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* compared with uninfected controls

Parameter[a]	No. (%[b]) or value as indicated for:		Result for IMP-producing *E. cloacae* cases vs uninfected controls[c]	
	IMP-producing *E. cloacae* cases (n = 15)	Uninfected controls (n = 45)	OR (95% CI)	P value
Demographics				
Mean age ± SD (yr)	70.9 ± 19.4	64.4 ± 18.5	NA	0.249
Male	8 (53.3)	20 (44.4)	1.64 (0.41–6.56)	0.483
Non-home residence	3 (20)	3 (6.7)	6.46 (0.62–67.72)	0.119
Acute and chronic conditions on admission				
Dependent functional status	9 (60)	22 (48.8)	1.66 (0.48–5.78)	0.427
Impaired consciousness	6 (40)	14 (31.1)	1.64 (0.41–6.56)	0.483
Current use of H_2 blocker or PPI	13 (86.6)	19 (42.2)	**12.63 (1.57–101.35)**	**0.017**
Rapidly fatal McCabe score	1 (6.7)	6 (13.3)	0.39 (0.03–4.39)	0.446
Cerebrovascular accident	4 (26.6)	10 (22.2)	1.66 (0.23–12.09)	0.619
Congestive heart failure	3 (20)	2 (4.4)	4.5 (0.75–26.93)	0.099
Dementia	3 (20)	4 (8.9)	3 (0.47–19.04)	0.244
Connective tissue disease	3 (20)	10 (22.2)	0.81 (0.13–5.13)	0.819
Diabetes mellitus	4 (26.6)	6 (13.3)	2.56 (0.54–12.06)	0.234
Any liver disease	1 (6.7)	4 (8.9)	0.72 (0.07–7.35)	0.782
Any renal disease	8 (53.3)	2 (4.4)	**13.52 (3.07–59.58)**	**<0.001**
Active malignant disease	4 (26.6)	17 (37.7)	0.53 (0.13–2.16)	0.373
Median Charlson combined condition score (IQR)	5 (4–10)	5 (2–9)	NA	0.341
Immunosuppressive state[d]	6 (40)	14 (31.1)	1.69 (0.41–7.02)	0.468
Exposure to health care settings and environments before IMP-producing *E. cloacae* isolation				
Hospitalization in the past 3 mo	10 (66.6)	13 (28.8)	**5.35 (1.38–20.71)**	**0.015**
Median no. of days from last hospitalization (IQR)	10 (0–56)	15 (3–29)	NA	0.784
GI tract endoscopy in the past 3 mo	7 (46.6)	5 (11.1)	**14.65 (1.74–123.24)**	**0.014**
Invasive procedure in the past 3 mo[e]	6 (40)	6 (13.3)	**4.62 (1.11–19.31)**	**0.036**
Surgery in the past 3 mo	5 (33.3)	0	**21 (2.99–147.69)**	**<0.001**
GI tract endoscopy, invasive procedure, or surgery in the past 3 mo	11 (73.3)	7 (15.5)	**23.9 (3.02–189.32)**	**0.003**
Any permanent device[f]	15 (100)	1 (2.2)	**18.55 (6.09–56.51)**	**<0.001**
ICU stay in the past 3 mo	4 (26.7)	4 (8.9)	9 (0.94–86.52)	0.06
Antimicrobial exposure in the past 3 mo				
Any antibiotic	15 (100)	13 (28.9)	**6.02 (2.13–17.02)**	**<0.001**
Median no. of days from last hospitalization (IQR)	0 (0–10)	35 (0–65)	**NA**	**0.023**
Penicillins[g]	6 (40)	4 (8.9)	5.44 (1.34–22.01)	**0.018**
Oxyimino-cephalosporins[h]	4 (26.7)	1 (2.2)	**12 (1.34–107.36)**	**0.026**
Other cephalosporins	6 (40)	4 (8.9)	12.45 (1.45–106.64)	**0.021**
Cephalosporins	9 (60)	4 (8.9)	**21.27 (2.65–170.43)**	**0.004**
β-Lactam/β-lactamase inhibitors[i]	5 (33.3)	5 (11.1)	5.4 (0.99–29.46)	0.051
Imipenem or meropenem	6 (40)	1 (2.2)	**18 (2.17–149.51)**	**0.007**
β-Lactam antibiotics	12 (80)	6 (13.3)	**27.58 (3.53–215.3)**	**0.002**
Fluoroquinolones	5 (33.3)	7 (15.6)	2.79 (0.71–11)	0.144
Aminoglycosides	1 (6.7)	1 (2.2)	3 (1.89–47.96)	0.44
Glycopeptides	6 (40)	1 (2.2)	**15.54 (2.74–88.03)**	**<0.001**
Outcomes				
In-hospital mortality	2 (14.3)	6 (13.3)	1.26 (0.2–8.03)	0.809
Mortality within 3 mo	2 (14.3)	11 (24.4)	0.59 (0.11–3.32)	0.551
Functional status deterioration	3 (25)	1 (2.6)	7.24 (0.73–72.04)	0.091
Discharge to LTCF after being admitted from home	5 (55.6)	12 (32.4)	2.18 (0.47–10.05)	0.318
Additional hospitalizations within 6 mo following IMP-producing *E. cloacae* isolation[j]	3 (60)	6 (24)	9 (0.37–220.93)	0.16
Total length of hospital stay [median no. of days (IQR)]	93 (52–175)	57 (36–96)	NA	0.052
Total length of hospital stay excluding stays ending in death [median no. of days (IQR)]	83 (55–175)	56 (28–92)	NA	0.098

[a] GI, gastrointestinal; ICU, intensive care unit; IQR, interquartile range; LTCF, long-term care facilities; PPI, proton pump inhibitors.

[b] The percentage is of patients for whom data were available, i.e., excluding the missing cases.

[c] Boldface indicates statistically significant difference between groups ($P < 0.05$). CI, confidence interval; NA, data not available; OR, odds ratio.

[d] Includes one or more of the following: (i) neutropenia (<500 neutrophils) at time of culture, (ii) glucocorticoid/steroid use in the past month, (iii) chemotherapy in the past 3 months, (iv) radiotherapy in the past 3 months, (v) posttransplantation, (vi) anti-tumor necrosis factor alpha therapy in the past 3 months, or (vii) HIV.

[e] Includes percutaneous interventions, endoscopies, and biopsies.

[f] The presence of any indwelling device (e.g., tracheotomies, central lines, urinary catheters, orthopedic external fixators, percutaneous endoscopic gastrostomy, biliary stent/tube, ventriculoperitoneal shunt, nasogastric tube, continuous ambulatory peritoneal dialysis catheter, or hemodialysis catheter) (i) at the time of IMP-producing *E. cloacae* isolation or on admission for uninfected controls or (ii) at the time of IMP-producing *E. cloacae* isolation or on admission for uninfected controls.

[g] Includes β-lactam/β-lactamase inhibitor combinations.

[h] Includes ceftriaxone, cefepime, and ceftazidime.

[i] Includes ampicillin-sulbactam, piperacillin-tazobactam, ticarcillin-clavulanate, and amoxicillin-clavulanate.

[j] For uninfected controls, within 6 months following admission.

TABLE 5 Multivariate analysis of risk factors for the isolation of IMP-producing *E. cloacae*

Variable	IMP-producing *E. cloacae* cases vs uninfected controls	
	OR (95% CI)	*P* value
Invasive procedure in the previous 3 mo[a]	21.48 (1.88–246.18)	0.014
Exposure to cephalosporins in the previous 3 mo	19.1 (1.5–243.46)	0.023

[a] Includes percutaneous interventions, endoscopies, urological procedures, and biopsies.

gested possible transmission of IMP-producing *E. cloacae* in particular wards in our hospital. An infection control team emphasized the importance of infection control measures, especially strict compliance with contact isolation procedures in each ward. The incidence of new isolations of IMP-producing *E. cloacae* has eventually decreased to 2 cases over 5 months since March 2013.

IMP-producing *E. cloacae* possesses many other resistance genes (Table 2). Seven (47%) of the isolates were resistant to ciprofloxacin. This is in accordance with previous reports of metallo-β-lactamase-producing organisms with low susceptibilities to different classes of antibiotics (33). Isolates with *aacA1*/*aacA4* resistance genes had elevated MICs (8 to 16 μg/ml) to amikacin but not to gentamicin. The difference is probably related to the resistance mechanism of AAC(6′)-I, associated with the *aacA1*/*aacA4* gene, which is known to acetylate tobramycin and amikacin but not gentamicin.

As previously reported, the MICs of meropenem and imipenem were not elevated in our study. Bloodstream infections caused by IMP-type metallo-β-lactamase-producing *E. cloacae* isolates with a MIC of 2 μg/ml have been previously reported; however, in these reports, the exact methods of measuring the MIC were not described (9). In our study, even when using the revised CLSI criteria (defining susceptibility as a MIC of ≤1 μg/ml), 67% and 80% of IMP-producing *E. cloacae* isolates were categorized as susceptible to meropenem and imipenem, respectively (23). This finding underscores the difficulties in identifying metallo-β-carbapenemase-producing organisms solely based on MIC results, as previously reported (34–36). In geographical areas where the IMP-type carbapenemase is endemic, such as Asia (34–36), both ESBLs and metallo-β-lactamase might need to be considered when assessing a patient with infection due to *Enterobacteriaceae* species with elevated MICs to penicillins and cephalosporins, including oxyimino-cephalosporins (2, 37). This is particularly important for patients who fail to respond to carbapenem treatment despite the low MICs to carbapenems (9).

In our study, all of the bla_{IMP}-positive isolates were positive for IMP in the immunochromatographic assay, with 2 false-positive results during the study period. The immunochromatographic assay is technically easy to use as a screening method in hospital microbiology laboratories (22); further investigations are warranted to evaluate the diagnostic usefulness of detecting IMP-metallo-β-carbapenemase. The IMP-containing integron has been suggested to spread through horizontal transfer (38), so early recognition of IMP-producing organisms is of particular importance.

Two (patients 1 and 2) of the 15 patients (13%; 40% of 5 patients with bacteremia and 20% of patients with infection [not colonization]) died during their hospital stay. The IMP-producing *E. cloacae* isolates from these 2 patients had relatively low MICs to meropenem (MICs of 2 μg/ml and 1 μg/ml), and both patients received meropenem as empirical therapy; however, they both deteriorated clinically. Their multiple comorbid conditions and old age are likely to have contributed to their unfortunate clinical courses. However, our results raise some concern for relying on carbapenem as a treatment option in infections, especially for elderly individuals and/or those with comorbidities. Previous reports have suggested conflicting results (34, 39–41). Therefore, further studies are needed on this issue.

The majority of the isolates are clonally related, and thus, an outbreak might have occurred in the hospital. However, they possessed different resistance genes, as shown in Table 2. Although we suspected that mobile elements may have been transferred among strains that had the same drug resistance genes, the exact mechanisms for the closely related strains to acquire different drug resistance genes are not certain. Even though a close relationship among IMP-positive isolates was found by MLST, these isolates had two different types of bla_{IMP} genes, IMP-1 and IMP-11. Although IMP-type metallo-β lactamase enzymes are thought to be located within a variety of integron structures (38), in this study, we did not determine the exact mechanism of how each *E. cloacae* isolate acquired the IMP-type metallo-β-lactamase. Further studies are warranted to determine the exact mechanisms by which *E. cloacae* acquires IMP-type metallo-β-lactamase.

In conclusion, we identified the risk factors for isolation of IMP-producing *E. cloacae*, as well as molecular and microbiological characteristics of these isolates. Considering the clinical outcomes in our patient cohort, a lower threshold for screening for carbapenemase production is recommended in patients who have previous exposure to antimicrobials, indwelling devices, and recent invasive procedures and from whom *E. cloacae* that is resistant to one or multiple agents in the extended-spectrum cephalosporin class and/or shows elevated MICs (>1 μg/ml) to imipenem and/or meropenem has been isolated. Choosing an appropriate antimicrobial therapy, as well as applying strong infection control measures, are clinically important measures for patients from whom IMP-producing *E. cloacae* isolates have been obtained.

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Evaluation of an Automated Rapid Diagnostic Assay for Detection of Gram-Negative Bacteria and Their Drug-Resistance Genes in Positive Blood Cultures

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Abstract

We evaluated the performance of the Verigene Gram-Negative Blood Culture Nucleic Acid Test (BC-GN; Nanosphere, Northbrook, IL, USA), an automated multiplex assay for rapid identification of positive blood cultures caused by 9 Gram-negative bacteria (GNB) and for detection of 9 genes associated with β-lactam resistance. The BC-GN assay can be performed directly from positive blood cultures with 5 minutes of hands-on and 2 hours of run time per sample. A total of 397 GNB positive blood cultures were analyzed using the BC-GN assay. Of the 397 samples, 295 were simulated samples prepared by inoculating GNB into blood culture bottles, and the remaining were clinical samples from 102 patients with positive blood cultures. Aliquots of the positive blood cultures were tested by the BC-GN assay. The results of bacterial identification between the BC-GN assay and standard laboratory methods were as follows: *Acinetobacter* spp. (39 isolates for the BC-GN assay/39 for the standard methods), *Citrobacter* spp. (7/7), *Escherichia coli* (87/87), *Klebsiella oxytoca* (13/13), and *Proteus* spp. (11/11); *Enterobacter* spp. (29/30); *Klebsiella pneumoniae* (62/72); *Pseudomonas aeruginosa* (124/125); and *Serratia marcescens* (18/21); respectively. From the 102 clinical samples, 104 bacterial species were identified with the BC-GN assay, whereas 110 were identified with the standard methods. The BC-GN assay also detected all β-lactam resistance genes tested (233 genes), including 54 bla_{CTX-M}, 119 bla_{IMP}, 8 bla_{KPC}, 16 bla_{NDM}, 24 bla_{OXA-23}, 1 $bla_{OXA-24/40}$, 1 bla_{OXA-48}, 4 bla_{OXA-58}, and 6 bla_{VIM}. The data shows that the BC-GN assay provides rapid detection of GNB and β-lactam resistance genes in positive blood cultures and has the potential to contributing to optimal patient management by earlier detection of major antimicrobial resistance genes.

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Introduction

Sepsis caused by drug-resistant Gram-negative bacteria (GNB) often results in serious clinical outcomes in patients [1]. Inappropriate initial antibiotic treatment occurs in one third of patients with severe sepsis due to GNB, which is associated with increased hospital mortality and length of stay [2]. In contrast, early administration of appropriate antibiotics improves survival of sepsis patients [3]. Effective antibiotic administration within the first hour of documented hypotension was related to increased survival of patients with septic shock; however, 50% of septic shock patients did not receive effective antimicrobial treatment within 6 hours of documented hypotension [4]. To improve diagnosis of causative organisms of sepsis, automated continuous-monitoring blood culture systems were developed and introduced into clinical microbiological laboratories during the 1990s [5]. These early systems and subsequent generations of automated blood culture systems remain key diagnostic tools in the diagnosis of sepsis [5]. However, after a blood culture becomes positive, conventional bacteriological procedures still require 2 to 3 days for isolation, identification, and antimicrobial susceptibility testing. In addition to the time-consuming procedures, the emergence and spread of drug-resistant GNB producing various β-lactamases, including carbapenemase and extended spectrum β-lactamases (ESBLs), has been a serious problem for treatment of sepsis [6]. Thus, there has been an unmet need for rapid and automated technology to

identify bacterial species as well as detection of drug resistance genes.

Recently, several rapid molecular diagnosis assays for sepsis diagnosis have been introduced and evaluated [7]; including LightCycler SeptiFast Test [8], peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) [9], and matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) [10], and a DNA-based microarray platform [Prove-it sepsis assay [11] and the Verigene Gram-Positive Blood Culture (BC-GP) assay [12–19]].

The Verigene Gram-Negative Blood Culture (BC-GN) Nucleic Acid Test (Nanosphere Inc., Northbrook, IL) is a sample-to-result automated microarray-based, multiplexed assay for species identification of GNB and detection of their drug resistance genes in positive blood culture bottles. The Verigene BC-GN assay is designed to directly detect species of GNB from positive blood culture bottles with 5 minutes of hands-on and 2 hours of run time per sample. Recently, the BC-GN assay has been approved by the U.S. Food and Drug Administration (FDA), and the limit of detection, sensitivity, and specificity of the assay is shown on the FDA site (http://www.fda.gov/). In this study, we describe the performance of the BC-GN assay using simulated and clinical samples of positive blood culture bottles.

Materials and Methods

Bacterial strains

A total of 268 stored clinical isolates at the National Center for Global Health and Medicine (NCGM) were used in the study: 23 *Acinetobacter baumannii*; 1 *Acinetobacter oleivorans*; 4 *Citrobacter freundii*; 14 *Enterobacter cloacae*; 2 *Enterobacter hormaechei*; 1 *Enterococcus faecalis*; 30 *Escherichia coli*; 6 *Klebsiella oxytoca*; 43 *Klebsiella pneumoniae*; 1 methicillin-resistant *Staphylococcus aureus* (MRSA); 1 methicillin-sensitive *Staphylococcus aureus* (MSSA); 1 methicillin-resistant *Staphylococcus epidermidis* (MRSE); 1 *Morganella morganii*; 4 *Proteus mirabilis*; 4 *Proteus vulgaris*; 116 *Pseudomonas aeruginosa*; 15 *Serratia marcescens*; and 1 *Stenotrophomonas maltophilia*. Eighteen bacterial strains were donated by Yoshikazu Ishii (Toho University, Tokyo, Japan), including 2 strains of *A. baumannii* harboring $bla_{\text{OXA-23}}$; 1 *A. baumannii* harboring $bla_{\text{OXA-58}}$; 1 *Acinetobacter calcoaceticus* harboring $bla_{\text{IMP-1}}$; 1 *Acinetobacter nosocomialis* harboring $bla_{\text{IMP-1}}$; 1 *Acinetobacter pittii* harboring $bla_{\text{OXA-58}}$; 2 *E. cloacae* harboring $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-9}}$, respectively; 6 *E. coli* harboring $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-44}}$, respectively; 2 *K. pneumoniae* harboring $bla_{\text{CTX-M-2}}$; 1 *K. pneumoniae* harboring $bla_{\text{CTX-M-9}}$; and 1 *P. mirabilis* harboring $bla_{\text{CTX-M-2}}$. Bacterial identification of the 18 strains were determined as described previously [20]. Fifteen bacterial strains were obtained from International Health Management Associates, Inc. (Schaumburg, IL), including 3 strains of *A. baumannii* harboring $bla_{\text{OXA-23}}$; 2 *A. baumannii* harboring $bla_{\text{OXA-58}}$; 1 *A. baumannii* harboring $bla_{\text{OXA-24/40}}$; 1 *A. baumannii* harboring both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$; 2 *C. freundii* harboring $bla_{\text{KPC-2}}$ and $bla_{\text{KPC-3}}$, respectively; 1 *K. pneumoniae* harboring $bla_{\text{KPC-2}}$; 1 *K. pneumoniae* harboring $bla_{\text{KPC-4}}$; 2 *K. pneumoniae* harboring $bla_{\text{KPC-11}}$; 1 *K. pneumoniae* harboring both $bla_{\text{KPC-3}}$ and $bla_{\text{CTX-M-14}}$; and 1 *S. marcescens* harboring $bla_{\text{KPC-2}}$.

Preparation of simulated samples

Bacterial isolates were suspended in 10-ml Falcon tubes (Becton Dickinson, Tokyo, Japan) in phosphate-buffered saline, pH 7.4. The suspension was adjusted to McFarland standard 1, followed by a dilution of 10^6. A 0.1-ml aliquot was inoculated into 5 ml of human whole blood for blood transfusion that was scheduled for disposal (Japanese Red Cross, Kanto-Koshinetsu Block Blood Center, Tokyo, Japan). After mixing of the blood and bacterial inoculum, the sample was injected into a Bactec plus/F aerobic blood culture bottle (Becton Dickinson). A 0.1-ml aliquot from the diluted bacterial suspension was plated, and the colony-forming unit (CFU) was determined. The average CFU of the inoculum was 23.9 ± 22.6 CFUs per bottle (median: 16, range: 1 - 184). The inoculated blood culture bottles were incubated in the BACTEC 9050 automated blood culture system (Becton Dickinson) until positive. If positive blood culture bottles could not be tested by the BC-GN assay within 12 hours of blood culture positivity, positive bottles were refrigerated at 4°C for up to 48 hours. In some experiments, an equal volume from 2–3 positive blood culture bottles was mixed and the mixture was tested by the BC-GN assay.

Clinical samples

Blood culture bottles from suspected sepsis patients were collected from December 2012 to June 2013 at the 801 bed National Center for Global Health and Medicine (NCGM), from February to June 2013 at 572 bed NCGM Kohnodai Hospital, and from March to June 2013 at 1423 bed Tokyo Women's Medical University (TWMU) hospital. Bactec plus/F (Becton Dickinson) and BacT/Alert FA (bioMérieux, Tokyo, Japan) blood culture bottles were used at NCGM and TWMU, respectively. Of 102 clinical samples, 79 and 23 were collected at NCGM and TWMU, respectively. Hospital departments and wards were not specified in the study. The bottles were incubated in the automated blood culture system until positive. Positive blood cultures showing Gram-negative bacteria were tested with the BC-GN assay. To select samples containing organisms listed in the BC-GN panel, positive blood cultures were stored at room temperature and tested within 5 days of positivity. The average of storage periods was 3.1±1.4. Only one positive blood culture per patient was included in the study.

Identification of bacterial species and detection of resistance genes

Bacterial isolates were phenotypically identified using the MicroScan WalkAway™ system (Siemens Healthcare Diagnostics, Tokyo, Japan). Isolates generating identification discrepancies between the MicroScan WalkAway system and the BC-GN assay were analyzed by 16S ribosomal RNA (rRNA) sequencing [21]. DNA sequences were determined using an ABI PRISM3130 sequencer (Applied Biosystems, Foster City, CA). The sequence similarity was determined using the EzTaxon server (http://eztaxon-e.ezbiocloud.net/ezt_identify). The presence of 9 genes listed in the BC-GN panel was examined using PCR in simulated and clinical samples tested, regardless of the results with the BC-GN assay. The primers for PCR were shown in Table 1. The DNA sequences of the drug-resistant genes were determined when isolates generated discrepant results between the BC-GN assay and PCR.

Minimum inhibitory concentrations of antibiotics

Minimum inhibitory concentrations (MICs) of amikacin (AMK), ampicillin (ABPC), amoxicillin/clavulanate (AMPC/CVA), aztreonam (AZT), cefazolin (CEZ), cefepime (CFPM), cefmetazole (CMZ), cefotaxime (CTX), cefotiam (CTM), ceftazidime (CAZ), ciprofloxacin (CPFX), colistin, gentamicin (GM), imipenem (IPM), levofloxacin (LVFX), minocycline (MINO), meropenem (MEMP), piperacillin (PIPC), piperacillin-tazobactam (PIPC/TAZ), sulfamethoxazole-trimethoprim (ST), and tigecylcine (TGC) were determined by the Microscan Walkaway and/or the broth

Table 1. Primers used in this study.

Detection for antibiotic resistance genes		16S rRNA gene sequencing	
Primers	**Sequence(5′-3′)**	**Primers**	**Sequence(5′-3′)**
CTX-M-F	CGTTGTAAAACGACGGCCAGTGAA	5F	TTGGAGAGTTTGATCCTGGCTC
	TGTGCAGYACCAGTAARGTKATGGC	341F	CTACGGGAGGCAGCAGTGGG
CTX-M-R	TGGGTRAARTARGTSACCAGAAYCAGCGG	810R	GCGTGGACTTCCAGGGTATCT
IMP-F	GGAATAGAGTGGCTTAAYTCTC	1194R	ACGTCATCCCCACCTTCCTC
IMP-R	GGTTTAAYAAAACAACCACC	1485R	TACGGTTACCTTGTTACGAC
KPC-F	CGTCTAGTTCTGCTGTCTTG		
KPC-R	CTTGTCATCCTTGTTAGGCG		
NDM-F	GGTTTGGCGATCTGGTTTTC		
NDM-R	CGGAATGGCTCATCACGATC		
OXA-23like-F	GATCGGATTGGAGAACCAGA		
OXA-23like-R	ATTTCTGACCGCATTTCCAT		
OXA-24/40like-F	GGTTAGTTGGCCCCCTTAAA		
OXA-24/40like-R	AGTTGAGCGAAAAGGGGATT		
OXA48-F	GCGTGGTTAAGGATGAACAC		
OXA48-R	CATCAAGTTCAACCCAACCG		
OXA-58like-F	CCCCTCTGCGCTCTACATAC		
OXA-58like-R	AAGTATTGGGGCTTGTGCTG		
VIM-F	GATGGTGTTTGGTCGCATA		
VIM-R	CGAATGCGCAGCACCAG		

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microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [22]. Values of MICs at which 50% and 90% of *E. coli* isolates from clinical samples were inhibited (MIC_{50} and MIC_{90}, respectively) were determined in β-lactams.

Verigene System

The Verigene BC-GN assay detects 9 bacterial species and 9 drug resistance genes (Table 2). The Verigene System consists of the Verigene Reader and the Processor SP. Following loading of the extraction tray, utility tray and test cartridge into the Verigene Processor SP, 700 µl of positive blood culture was added to the extraction tray sample well. The Verigene Processor SP extracted nucleic acids from positive blood culture media. The extracted nucleic acids were automatically transferred to the test cartridge and hybridized to synthetic specific oligonucleotides attached to the microarray slide. The nucleic acids bound on the microarray slide were further hybridized to the second specific oligonucleotides with gold nanoparticles. After 2 hours, the microarray slide was manually removed and inserted into the Verigene Reader for analysis. The Verigene system contains internal controls, including negative controls, hybridization controls, and extraction controls. When any internal control do not generate correct results, or target signals were not adequately higher than the negative control signals, the Verigene Reader reported "No Call" meaning a technical error. When the Verigene Reader reported "No Call", the BC-GN assay was repeated until results other than "No Call" were generated. Once the result was obtained, the BC-GN assay was not repeated.

Statistical analysis

The concordance rate between the BC-GN assay and standard laboratory methods was examined, and the 95% confidence interval of the rate was calculated with the R Software (http://www.r-project.org/).

Ethical considerations

The study protocol was carefully reviewed and approved by the ethics committee of the National Center for Global Health and Medicine (No. 1268) and Tokyo Women's Medical University hospital (No. 2740-R), respectively. Individual informed consent was waived by the ethics committee listed above because this study used currently existing sample collected during the course of routine medical care and did not pose any additional risks to the patients.

Results

Bacterial identification of simulated samples

Of the 397 positive blood culture samples in the study, 295 were simulated samples prepared by inoculating an isolate of one of the Gram-negative bacterial species listed in Table 2. All the samples became culture-positive. Of the 295 samples, 289 generated a result of the BC-GN assay on the first attempt (98.1%) (data not shown). The remaining 6 generated "No Call " results meaning technical errors. When retested, all the 6 samples generated results (data not shown). The concordance rate of the 295 simulated samples between the BC-GN assay and the MicroScan WalkAway system for bacterial identification were as follows: *Acinetobacter* spp., *Citrobacter* spp., *Enterobacter* spp., *E. coli*, *K. oxytoca*, *Proteus* spp., and *P. aeruginosa*: 100%; *K. pneumoniae*: 88.2%; and *S. marcescens*: 81.3% (left columns in Table 3).

Table 2. Bacterial species and Antimicrobial resistance genes identified by the BC-GN assay.

Gram-negative species
Acinetobacter spp.
Citrobacter spp.
Enterobacter spp.
E. coli
K. oxytoca
K. pneumoniae
Proteus spp.
P. aeruginosa
S. marcescens
Antimicrobial resistance genes
bla_{CTX-M}
bla_{IMP}
bla_{KPC}
bla_{NDM}
bla_{OXA-23}
$bla_{OXA-24/40}$
bla_{OXA-48}
bla_{OXA-58}
bla_{VIM}

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Of 51 samples inoculated with stored *K. pneumoniae* isolates (left columns in Table 3), 45 were detected with the BC-GN assay, but the remaining 6 were not correctly detected. Of these 6 samples, 5 were reported as "Not detected", and 1 was reported as *Enterobacter* spp. The MicroScan WalkAway system reported these 6 samples as *K. pneumoniae*. The 16S rRNA sequences of these samples showed more than 99.3% similarity to both *K. pneumoniae* and *K. variicola*. Of 16 samples inoculated with stored *S. marcescens* (left columns in Table 3), 3 were not detected with the BC-GN assay. The MicroScan WalkAway system reported these 3 samples as *S. marcescens*. The 16S rRNA sequences of these samples showed more than 99.5% similarity to both *S. marcescens* and *Serratia nematodiphila*. Their biochemical profile based on carbohydrates utilization corresponded to those of *S. marcescens* [23].

The BC-GN assay was tested on blood culture bottles inoculated simultaneously with 2 or 3 clinical isolates. The following combinations of bacteria species were tested: *A. baumannii* (harboring bla_{OXA-23}) and *K. pneumoniae* (bla_{CTX-M} and bla_{NDM}); *A. baumannii* (bla_{OXA-23}) and MRSA; *C. freundii*, *K. pneumoniae* (bla_{KPC}) and *P. mirabilis*; *C. freundii* (bla_{KPC}), *E. faecalis* and MSSA; *E. cloacae* (bla_{IMP}) and *S. marcescens*; *E. faecalis* and *E. coli* (bla_{CTX-M}); *E. coli* (bla_{CTX-M}) and *P. aeruginosa* (bla_{VIM}); *P. aeruginosa* (bla_{VIM}) and MRSE. All Gram-negative bacterial isolates were correctly detected by the BC-GN assay regardless of the presence of other bacterial species, including Gram-positive strains. The BC-GN assay also detected all drug resistance genes under these conditions (data not shown). The BC-GN assay was also tested on 2 blood culture bottles inoculated with *M. morganii* and *S. maltophilia*, respectively, which are Gram-negative pathogens but are not among the targets of the BC-GN assay. As expected, the BC-GN assay did not detect these species (data not shown).

Bacterial identification of clinical samples

A total of 102 blood culture-positive samples obtained from sepsis patients, and 101 of them generated a result of the BC-GN assay on the first attempt (99.0%) (data not shown). From the 102 blood culture-positive samples, a total of 110 Gram-negative bacterial species were isolated (middle columns in Table 3). The concordance rate of 110 bacterial species between the BC-GN assay and the MicroScan WalkAway system for bacterial identification were 94.5% (NCGM: 82/86; TWMU: 22/24), and the rate of each bacterial species were as follows: *Acinetobacter* spp., *Citrobacter* spp., *E. coli*, *K. oxytoca*, *Proteus* spp., and *S. marcescens*: 100%; *Enterobacter* spp.: 91.7%; *K. pneumoniae*: 81.0%; and *P. aeruginosa*: 88.9% (middle columns in Table 3). Of all the clinical samples, 8 were obtained from anaerobic bottles, and all the 8 results of the BC-GN assay agreed with those of the MicroScan WalkAway system (data not shown).

Of the 110 bacterial isolates, 6 isolates (5.5%) were not detected with the BC-GN assay (middle columns in Table 3). Of these 6 isolates, the MicroScan WalkAway system reported 1 isolate as *Enterobacter* spp., 4 isolates as *K. pneumoniae* and 1 isolate as *P. aeruginosa* (middle columns in Table 3). The sample reported as *Enterobacter* spp. by the MicroScan WalkAway system was reported as "No Call" with the BC-GN assay on two tries. The 16S rRNA sequence of the isolate reported as *Enterobacter* spp. by the MicroScan WalkAway system showed more than 99.9% similarity to *Enterobacter agglomerance*. Of the 4 samples reported as *K. pneumoniae* by the MicroScan WalkAway system and not detected by the BC-GN assay, 2 were from polymicrobial bacteremia cases: *E. coli* and *K. pneumoniae* was isolated from one positive bottle, and *K. pneumoniae* and *P. aeruginosa* was isolated from another. The 16S rRNA sequences of the 4 isolates reported as *K. pneumoniae* by the MicroScan WalkAway system showed more than 99.2% similarity to both *K. pneumoniae* and *K. variicola*. One *P. aeruginosa* sample not detected with the BC-GN was from a polymicrobial bacteremia case of *K. pneumoniae* and *P. aeruginosa*. The 16S rRNA sequence of the isolate reported as *P. aeruginosa* by the MicroScan WalkAway system showed more than 99.9% similarity to *P. aeruginosa*.

Seven of the 102 clinical samples were polymicrobial. In 4 of the 7 samples, the BC-GN assay detected all of the multiple bacterial species, even in a sample containing 3 different Gram-negative species. In a sample containing *Enterococcus casseliflavus* and *E. coli*, the BC-GN assay detected only *E. coli* but not *E. casseliflavus*, since it detects only Gram-negative bacteria but not Gram-positive ones. In the remaining 3 polymicrobial positive blood culture samples, the BC-GN assay did not detect one of the multiple pathogens (*K. pneumoniae* in 2 samples and *P. aeruginosa* in 1 samples). The bacterial identification of the samples not detected by the BC-GN assay was described above.

Identification of drug resistance genes

With respect to drug resistant genes, the BC-GN assay reported that, of the 295 simulated samples tested, 184 were positive for one of the resistance gene targets detected by the BC-GN assay and 18 were positive for two of the targets. These results agreed with those of PCR and/or DNA sequencing. As shown on left columns in Table 4, 42 bla_{CTX-M} (2 *E. cloacae*, 19 *E. coli*, 2 *E. hormaechei*, 18 *K. pneumoniae*, and 1 *P. mirabilis* isolates), 119 bla_{IMP} (1 *A. calcoaceticus*, 1 *A. nosocomialis*, 1 *A. pittii*, 9 *E. cloacae*, 4 *K. pneumoniae*, and 103 *P. aeruginosa*), 8 bla_{KPC} (2 *C. freundii*, 5 *K. pneumoniae*, and 1 *S. marcescens*), 16 bla_{NDM} (2 *A. baumannii*, 1 *E. hormaechei*, 1 *E. coli*, 11 *K. pneumoniae*, and 1 *P. aeruginosa*), 24 bla_{OXA-23} (24 *A. baumannii*), 1 $bla_{OXA-24/40}$ (1 *A. baumannii*), 1 bla_{OXA-48} (1 *K. pneumoniae*), 3 bla_{OXA-58} (2 *A. baumannii* and 1 *A. pittii*), and 6 bla_{VIM} (6 *P. aeruginosa*) were detected by the BC-GN assay.

Table 3. Identification of bacterial isolates in blood culture samples with the BC-GN assay.

	Simulated samples[b] (n = 295)				Clinical samples[c] (n = 102)			Total (n = 397)
Bacterial species[a]	Inocultaed isolates	Correctly identified isolates[d] (%)	Not detected isolates[e] (%)	Incorrectly identified isolates[f] (%)	Identified species[g]	Correctly identified isolates[d] (%)	Not detected isolates[e] (%)	Concordance rate[h] (95% CI[i])
Acinetobacter spp.	37	37 (100%)	0	0	2	2 (100%)	0	100% (91.0–100)
Citrobacter spp.	6	6 (100%)	0	0	1	1 (100%)	0	100% (59.0–100)
Enterobacter spp.	18	18 (100%)	0	0	12	11 (91.7%)	1 (8.3%)	96.7% (82.8–99.9)
E. coli	36	36 (100%)	0	0	51	51 (100%)	0	100% (95.8–100)
K. oxytoca	6	6 (100%)	0	0	7	7 (100%)	0	100% (75.3–100)
K. pneumoniae	51	45 (88.2%)	5 (9.8%)	1[j] (2.0%)	21	17 (81.0%)	4 (19%)	86.1% (75.9–93.1)
Proteus spp.	9	9 (100%)	0	0	2	2 (100%)	0	100% (91.0–100)
P. aeruginosa	116	116 (100%)	0	0	9	8 (88.9%)	1 (11.1%)	99.2% (95.6–99.9)
S. marcescens	16	13 (81.3%)	3 (18.7%)	0	5	5 (100%)	0	85.7% (63.7–97.0)
Total	295	286 (96.9%)	8 (2.7%)	1 (0.3%)	110	104 (94.5%)	6 (5.5%)	96.3% (94.0–97.9)

a Bacterial species inoculated into blood culture bottles or bacterial species identified in clinical samples of blood culture bottles.
b Blood culture samples into which bacterial isolates were inoculated.
c Clinical blood culture samples obatined from sepsis patients.
d Numbers of isolates which were correctly identified with the BC-GN assay.
e Numbers of isolates which were not detected with the BC-GN assay.
f Numbers of isolates which were incorrectly identified with theBC-GN assay.
g Numbers of bacterial species which were identified in clinical samples with the conventional methods. Some of the samples contained 2 or 3 bacterial species.
h Concordance rate between the BC-GN assay and the conventional methods for bacterial identification.
i CI indicates confidence interval
j A sample inoculated with K. pneumoniae was identified as Enterobacter spp. with the assay.
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Table 4. Identification of resistance genes in blood culture samples with the BC-GN assay.

Resistance genes[a]	Simulated samples[b] (n = 295)			Clinical samples[c] (n = 102)			Total (n = 397)
	Positive genes for PCR/DNAsequences	Correctly identified genes[d] (%)	Not detected genes[e]	Positive genes for PCR/DNAsequences	Correctly identified genes[d] (%)	Not detected genes[e]	Concordance rate[f] (95% CI[g])
bla_{CTX-M}	42	42 (100%)	0	12	12 (100%)	0	100% (93.3–100)
bla_{IMP}	119	119 (100%)	0	0	0	0	100% (96.9–100)
bla_{KPC}	8	8 (100%)	0	0	0	0	100% (63.1–100)
bla_{NDM}	16	16 (100%)	0	0	0	0	100% (79.4–100)
bla_{OXA-23}	24	24 (100%)	0	0	0	0	100% (85.8–100)
$bla_{OXA-24/40}$	1	1 (100%)	0	0	0	0	100% (2.5–100)
bla_{OXA-48}	1	1 (100%)	0	0	0	0	100% (2.5–100)
bla_{OXA-58}	3	3 (100%)	0	1	1 (100%)	0	100% (39.8–100)
bla_{VIM}	6	6 (100%)	0	0	0	0	100% (54.1–100)
Total	220	220 (100%)	0	13	13 (100%)	0	100% (98.4–100)

a Resistance genes which were harbored by bacterial species inoculated into blood culture bottles or isolated from sepsis patients.
b, c See footnotes b and c in Table 3.
d Numbers of genes which were correctly identified with the BC-GN assay.
e Numbers of genes which were not detected with the BC-GN assay.
f Concordance rate between the BC-GN assay and the conventional methods for resistance-genes identification.
g CI indicates confidence interval
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Table 5. Drug susceptibility of β-lactams of E. coli harboring or not harboring.

	β-lactams									
	Number of isolates	PIPC		CAZ		CTX		IMP		
		MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
E. coli (bla_{CTX-M} -positive)	8	>512	>512	8	>16	>32	>32	≤1	≤1
E. coli (bla_{CTX-M} -negative)	43	≤8	>64	≤1	≤1	≤8	≤8	≤1	≤1

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Of the 102 clinical samples tested, 13 were positive for one of the 9 drug resistance genes. As shown on middle columns in Table 4, 12 bla_{CTX-M} (2 *E. cloacae*, 8 *E. coli*, and 2 *K. pneumoniae*), and 1 bla_{OXA-58} (1 *A. baumannii*) were detected by the BC-GN assay. These results were completely agreed with those of our laboratory methods.

MICs of *E. coli* isolates from patients harboring or not harboring bla_{CTX-M} were determined (Table 5). The *E. coli* isolates harboring bla_{CTX-M} showed higher MIC_{50} and MIC_{90} of β-lactams tested than the isolates not harboring bla_{CTX-M}. MICs of IMP were ≤ 1 μg/ml in *E. coli* isolates harboring or not harboring bla_{CTX-M}. An isolate of *Acinetobacter* spp. harboring bla_{OXA-58} was susceptible to all β-lactams tested (Table S1). MICs of all clinical isolates were shown in Table S1, 2.

Discussion

We determined the overall concordance rate, 96.3%, between the BC-GN assay and standard laboratory methods (Table 3). All bacterial species tested except for *K. pneumoniae* and *S. marcescens* showed the concordance rate of over 95%. In addition to the good performance, the BC-GN assay can be completed within 2 h with less than 5 min of hands-on time, enabling same day analysis and starting on appropriate treatment. The concordance rate of the BC-GN assay was comparable to that of the BC-GP assay. However, the BC-GN assay will need to be evaluated using various blood culture systems, since the BC-GP assay have already evaluated as well [12–19].

The BC-GN assay did not accurately identify a total of 15 isolates from 9 simulated samples and 6 clinical samples (Table 3). Of the 15 isolates, 10, 3, 1 and 1 were reported as *K. pneumoniae*, *S. marcescens*, *Enterobacter* spp., *P. aeruginosa* by the MicroScan WalkAway system, respectively. Relatively higher rates of misidentification in *K. pneumoniae* and *S. marcescens* isolates were likely to be due to the probes used in the BC-GN assay. These probes will be improved in the further study. The concordance rates of Gram-positive bacteria were comparable in the BC-GP assay [12–19]. The lower accuracy results of *K. pneumoniae* were shown in both the spiked and clinical observations. The 16S rRNA sequence revealed that the 10 misidentified isolates shared over 99% similarity to both *K. pneumoniae* and *K. variicola*. Previous studies have reported that routine methods may identify *K. variicola* as *K. pneumoniae* [24]. Further studies are required to clarify the clinical significance of *K. variicola* in sepsis patients. As described above, three samples containing *S. marcescens* were not detected with the BC-GN assay, which were observed only in the simulated samples. It is unclear whether clinical samples containing *S. marcescens* were not detected accurately with the BC-GN assay, since the number of samples tested was low.

As for the detection of polymicrobial samples, the BC-GN assay performed well in the simulated samples, but not in the clinical samples. All isolates were not detected completely in 3 of 7 clinical samples containing polymicrobial organisms. Although fungal species like *Candida albicans* were not tested, these results can have significant clinical implications. Rapid molecular-based assays including the BC-GP assay and the FilmArray blood culture identification assay showed relatively low sensitivity in polymicrobial infections [14,25].

We evaluated the performance of the BC-GN assay in detecting various drug resistance genes using simulated samples. The BC-GN assay correctly detected all the drug resistance genes tested, even when multiple drug resistance genes were present in a sample. In addition, MICs of *E. coli* isolates from clinical samples indicated that the presence of bla_{CTX-M} was related to drug