susceptibility profiles with high MICs of PIPC, CAZ, and CTX, not IPM. These results suggest that the BC-GN assay is useful for earlier administration of appropriate antibiotics and de-escalation. Whereas, no genotypic-phenotypic correlation was found in the *Acinetobacter* spp. isolate harboring bla_{OXA-58}. In that case, the BC-GN assay may lead to inappropriate use of antibiotic, and de-escalation is needed after conventional drug susceptibility testing.

Although bla_{CTX-M} is the most prevalent among genes of extended-spectrum β-lactamases (ESBLs)[26,27], the BC-GN assay cannot detect other ESBLs genes like bla_{TEM} and bla_{SHV}, and there remains concern of bacteria harboring drug resistance genes which were not detected by the assay. More than 200 bla_{TEM} and more than 100 bla_{SHV} genes are currently known in the Lahey clinic database [28]. Thus, it remains unfeasible to detect these variants in a rapid and simple manner. The emergence of various β-lactam resistance genes, including carbapenemases and ESBLs, have been extensively reported [6]. The timely direct detection of resistance genes from positive blood cultures will be very useful in clinical situations, since multidrug-resistant bacteria and polymicrobial infections are important causes of failure in the treatment of sepsis. Because drug resistance genes will continue to evolve and spread, it is necessary to carefully monitor the spread of drug resistance genes.

Of the 10 most frequently isolated microorganisms in ICU-acquired bloodstream infection in Europe, 7 were Gram-negative pathogens targeted by the BC-GN assay [29]. Similar results were obtained from monomicrobial nosocomial bloodstream infections both in ICU and non-ICU wards in the USA [30]. The National Healthcare Safety Network at CDC reported that a significant proportion of central line-associated bloodstream infections are caused by drug-resistant Gram-negative pathogens in the USA [31]. Of the 6 major pathogens other than coagulase-negative staphylococci associated with nosocomial blood stream infections in Japan, 3 were Gram-negative pathogens, 2 were Gram-positive ones, and 1 was *Candida* spp. [32]. The BC-GN assay, which targets 9 Gram-negative bacterial species, will contribute to the diagnosis and management of patients with Gram-negative sepsis.

A number of new diagnostic methods have been developed for rapid detection of pathogens in positive blood cultures. A real-time PCR-based assay (LightCycler SeptiFast Test) can detect a number of Gram-negative and Gram-positive bacteria and fungi in a single assay, although it cannot detect drug resistance genes [8]. PNA-FISH can detect 3 Gram-negative pathogens, including *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, but cannot detect drug resistance genes [9]. MALDI-TOF MS can detect bacterial isolates in blood culture, but cannot detect 2 or more bacterial species in polymicrobial samples or drug resistance gene products [33]. A DNA-based microarray platform, Probe-it sepsis assay, can detect various Gram-negative and Gram-positive pathogens in blood cultures. Nevertheless, it cannot detect drug resistance genes other than *mecA* [11].

To integrate the Verigene system into a laboratory workflow, Gram stain was needed after blood culture was positive to determine whether the BC-GN or BC-GP assay was used. The Verigene system cannot measure MICs of antibiotics, and thus should be used in combination with biochemical characteristics of bacteria obtained from conventional drug susceptibility testing.

This study has some limitations. Firstly, we could not fully evaluate the ability of the BC-GN assay to detect some drug resistance genes, such as $bla_{OXA-24/40}$ and bla_{OXA-48}. Secondly, to select samples containing organisms listed in the BC-GN panel, positive blood culture was stored for 3.1±1.4 days (maximum 5 days) before the BC-GN assay was done. The BC-GN assay should be done immediately after the culture becomes positive, although the storage of the culture in the study was unlikely to affect the assay results. A large-scale prospective clinical evaluation of the BC-GN assay is planned to determine the clinical impact of the assay including selection of antibiotics, length of stay, morbidity, and cost-effectiveness.

In conclusion, the Verigene system BC-GN assay accurately detects common Gram-negative bacterial isolates and their drug resistance genes from positive blood cultures in a rapid manner. The BC-GN assay demonstrated high sensitivity and specificity in the study, and it will generate significantly faster results compared to conventional methods in the medical settings. This new anitibioticroarray platform can be easily introduced into microbiological laboratories in various clinical settings. It will contribute to improved sepsis management as a result of earlier reporting of critical information leading to earlier administration of appropriate antibiotics and de-escalation.

Supporting Information

Table S1 MICs of antibiotics in clinical isolates harboring drug resistance genes. It was measured using the broth microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).
(XLSX)

Table S2 MICs of antibiotics in all clinical isolates determined by Microscan Walkaway.
(XLSX)

Acknowledgments

We thank Japanese Red Cross Blood Center for providing human whole blood. We also thank Yoshikazu Ishii (Toho university, Tokyo, Japan) and International Health Management Associates, Inc. for providing bacterial strains.

Author Contributions

Conceived and designed the experiments: MT KH HT TK NO. Performed the experiments: MT TF YA MN KH HT. Analyzed the data: MT TF YA TK NO. Contributed reagents/materials/analysis tools: MT TF YA KM AS YM HY TMA KT NO. Wrote the paper: MT TK NO.

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Biochemical Analysis of Metallo-β-Lactamase NDM-3 from a Multidrug-Resistant *Escherichia coli* Strain Isolated in Japan

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New Delhi metallo-β-lactamase-3 (NDM-3) was identified in a multidrug-resistant *Escherichia coli* isolate, NCGM77, obtained from the feces of a patient in Japan. The enzymatic activities of NDM-3 against β-lactams were similar to those of NDM-1, although NDM-3 showed slightly lower k_{cat}/K_m ratios for all the β-lactams tested except for doripenem. The genetic context for bla_{NDM-3} was *tnpA-*bla_{NDM-3}*-*ble_{MBL}*-trpF-dsbC-tnpA-sulI-qacEdeltaI-aadA2-dfrA1*, which was present on an approximately 250-kb plasmid.

Metallo-β-lactamases (MBLs) are produced by many species of Gram-negative bacteria and some species of Gram-positive bacteria, including *Bacillus* spp. (1, 2). MBLs can confer resistance or reduced susceptibility to carbapenems and, usually, to cephalosporins and to penicillins except for monobactams (3). New Delhi metallo-β-lactamase-1 (NDM-1), a recently discovered MBL, was initially found in Sweden from *Klebsiella pneumoniae* and *Escherichia coli* isolates that originated from India (4). Subsequently, at least 10 NDM variants (see www.lahey.org /studies) have been reported in several different countries (5–17).

Escherichia coli NCGM77 was isolated from the feces of a patient in a medical setting in Japan in 2013. The isolate was phenotypically identified, and species identification was confirmed by 16S rRNA sequencing (18). MICs were determined using the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (19). *E. coli* NCGM77 was resistant to various tested β-lactams (Table 1); the MICs of the other antibiotics were 32 μg/ml (amikacin), 8 μg/ml (arbekacin), 256 μg/ml (ciprofloxacin), <0.25 μg/ml (colistin), >1,024 μg/ml (fosfomycin), 64 μg/ml (gentamicin), 512 μg/ml (kanamycin), 32 μg/ml (levofloxacin), 1 μg/ml (minocycline), <0.25 μg/ml (tigecycline), and 64 μg/ml (tobramycin). PCR analysis for MBL genes of bla_{DIM}, bla_{GIM}, bla_{IMP}, bla_{NDM}, bla_{SIM}, bla_{SPM}, and bla_{VIM} was performed (20, 21). On the basis of the PCR results, the isolate was positive for bla_{NDM}. The DNA sequence of the PCR product revealed that the isolate had the bla_{NDM-3} gene (9). Multilocus sequence typing (MLST) of NCGM77 found it to be sequence type 88 (ST88) (*E. coli* MLST database [see http://www.pasteur.fr/recherche/genopole /PF8/mlst/EColi.html]). *Pseudomonas aeruginosa* IOMTU9 (17) was used as a source of the bla_{NDM-1} gene.

The bla_{NDM-3} and bla_{NDM-1} genes were cloned into the corresponding sites of pHSG398 (TaKaRa, Shiga, Japan) using the primer set EcoRI-NDM-F (5′-GGGAATTCATGGAATTGCCCA ATATTATG-3′) and PstI-NDM-R (5′-AACTGCAGTCAGCGC AGCTTGTCGGCCAT-3′). The *Escherichia coli* DH5α strain was transformed with pHSG398-NDM-3 or pHSG398-NDM-1 to determine the MICs of β-lactams.

The open reading frames of NDM-1 and NDM-3 without signal peptide regions were cloned into the pET28a expression vector (Novagen, Inc., Madison, WI) using the primer set BamHI-TEV-NDM-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGCCAG CAAATGGAAACTGGCGAC-3′) and XhoI-NDM-R (5′-ATCT CGAGTCAGCGCAGCTTGTCGGCCATG-3′). Plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA). Recombinant NDM proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose according to the manufacturer's instructions (Qiagen, Hilden, Germany). His tags were removed by digestion with TurboTEV protease (Accelagen, San Diego, CA), and untagged proteins were purified by an additional passage over the Ni-NTA agarose. The purities of NDM-1 and NDM-3 were >90%, as estimated by SDS-PAGE. During the purification procedure, the presence of β-lactamase activity was monitored using nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). Initial hydrolysis rates were determined in 50 mM phosphate buffer (pH 7.0) containing 5 μM $Zn(NO_3)_2$ at 37°C, using a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m and k_{cat} values and the k_{cat}/K_m ratios were determined by analyzing β-lactam hydrolysis using a Lineweaver-Burk plot. Wavelengths and extinction coefficients for β-lactam substrates have been reported previously (22–24). Three individual experiments were performed to determine the K_m and k_{cat} values.

All cloned genes in the pHSG398 and pQE2 vectors were sequenced using an ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA).

The plasmid harboring bla_{NDM-3} was extracted (25) and sequenced by MiSeq (Illumina, San Diego, CA). The size of the plasmid harboring bla_{NDM-3} was determined using pulsed-field gel electrophoresis (PFGE) and Southern hybridization (12). A probe for bla_{NDM-3} was amplified by PCR by using the EcoRI-NDM-F and the PstI-NDM-R primers. Signal detection was carried out using the DIG High Prime DNA labeling and detection starter kit II (Roche Applied Science, Indianapolis, IN).

The bla_{NDM-3} probe hybridized to a 250-kb plasmid (Fig. 1). The sequence surrounding bla_{NDM-3} was *tnpA-*bla_{NDM-3}*-*ble_{MBL}*-trpF-dsbC-tnpA-sulI-qacEdeltaI-aadA2-dfrA1*. This plasmid showed more than 99.9% identity at the nucleotide sequence level from

Received 25 December 2013 Returned for modification 27 January 2014
Accepted 23 March 2014
Published ahead of print 31 March 2014
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doi:10.1128/AAC.02793-13

TABLE 1 MICs of various β-lactams for *E. coli* strains NCGM77 and DH5α transformed with plasmids carrying NDM-1 or NDM-3

Antibiotic	MIC (μg/ml) for:			
	NCGM77	pHSG398/ NDM-3	pHSG398/ NDM-1	pHSG398
Ampicillin	>1,024	256	256	4
Ampicillin-sulbactam	>1,024	128	128	2
Aztreonam	>1,024	0.063	0.063	0.063
Cefepime	>1,024	0.25	0.5	0.063
Cefoselis	—[a]	2	4	0.031
Cefotaxime	>1,024	4	8	0.031
Cefoxitin	>1,024	32	64	4
Cefpirome	—[a]	1	0.5	0.015
Ceftazidime	>1,024	256	256	0.25
Ceftriaxone	—[a]	8	8	0.031
Cephradine	>1,024	256	256	16
Doripenem	32	0.125	0.125	0.031
Imipenem	16	0.25	0.5	0.063
Meropenem	32	0.25	0.5	<0.015
Moxalactam	—[a]	8	8	0.125
Penicillin G	>1,024	128	256	32

[a] —, MICs of cefoselis, cefpirome, ceftriaxone, and moxalactam for the NCGM77 strain were not determined.

TABLE 2 Kinetic parameters of NDM-3 and NDM-1 enzymes[a]

β-Lactam	NDM-3			NDM-1		
	K_m[b] (μM)	k_{cat}[b] (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
Ampicillin	228 ± 35	73 ± 7	0.32	500 ± 14	255 ± 8	0.51
Aztreonam	NH[c]	NH[c]	NH[c]	NH[c]	NH[c]	NH[c]
Cefepime	103 ± 10	17 ± 1	0.16	147 ± 18	41 ± 3	0.28
Cefotaxime	28 ± 3	24 ± 1	0.9	36 ± 4	63 ± 2	1.7
Cefoxitin	17 ± 1	1.6 ± 0.2	0.10	20 ± 3	4.4 ± 0.2	0.22
Ceftazidime	64 ± 9	11 ± 1	0.17	233 ± 35	58 ± 5	0.25
Cephradine	12 ± 3	26 ± 1	2.2	13 ± 2	66 ± 1	5.0
Doripenem	92 ± 2	34 ± 1	0.37	116 ± 18	41 ± 4	0.35
Imipenem	148 ± 13	25 ± 1	0.17	123 ± 21	59 ± 3	0.48
Meropenem	81 ± 4	32 ± 1	0.40	78 ± 6	74 ± 2	0.95
Penicillin G	42 ± 3	47 ± 1	1.1	24 ± 4	99 ± 4	4.3

[a] The proteins were initially modified by a His tag, which was removed after purification.
[b] K_m and k_{cat} values represent the means of 3 independent experiments ± the standard deviations.
[c] NH, no hydrolysis was detected under conditions with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

69,229 to 78,275 bp of the pGUE plasmid (GenBank accession no. JQ364967) from the *E. coli* strain GUE, which was isolated in India (26). The entire sequence of the plasmid was not determined with the sequence data generated by MiSeq (Illumina).

Expression of the bla_{NDM-3} and bla_{NDM-1} genes in *E. coli* DH5α conferred resistance or reduced susceptibility to all cephalosporins, moxalactam, and carbapenems (Table 1). The MIC of cefpirome was 2-fold higher for *E. coli* expressing NDM-3 than for *E. coli* expressing NDM-1. In contrast, those of cefepime, cefoselis, cefotaxime, cefoxitin, imipenem, meropenem, and penicillin G were 2-fold lower for NDM-3 than for NDM-1.

As shown in Table 2, recombinant NDM-3 and NDM-1 hydrolyzed all β-lactams tested except for aztreonam. The profiles of the enzymatic activities of NDM-3 against β-lactams tested were similar to those of NDM-1, although NDM-3 had slightly but significantly lower k_{cat}/K_m ratios for all β-lactams tested except for doripenem. The lower k_{cat}/K_m ratios were mostly caused by the lower k_{cat} values of NDM-3 compared with those of NDM-1, i.e., the values of NDM-3 were 19.0 to 47.5% of those of NDM-1 (Table 2). In fact, the substitution from Asp to Asn at position 95 of NDM appeared to decrease the hydrolysis rate of all β-lactams tested except for doripenem (Table 2). An amino acid substitution at position 95 from Asp to Asn decreased the k_{cat} values of NDM-3 compared to those of NDM-1 (Table 2). Residue 95 is in α1, which is located on the protein surface. The crystal structure of NDM-1 revealed that the active site of NDM-1 is located at the bottom of a shallow groove enclosed by 2 important loops, L3 and L10 (27–30). Residue 95 in α1, however, was not located in these loops. This residue may indirectly affect the interaction of the substrate with the active site. Among all 9 NDM variants, amino acid substitutions were identified at 7 positions (28, 88, 95, 130, 152, 154, and 233). It remains unclear which position(s) plays a critical role in the enzymatic activities. Relative to IMP-1 and VIM-2, NDM-1 does not bind to carbapenems as tightly, but it turns over carbapenems at a rate similar to that of VIM-2 (4). NDM-4 with an amino acid substitution at position 154 (Met to Leu) showed greater hydrolytic activity toward carbapenems, cephalotin, cefotaxime, and ceftazidime than that shown by NDM-1 (12). NDM-5 with substitutions at positions 88 (Val to Leu) and 154 (Met to Leu) resulted in reduced susceptibilities of *E. coli* transformants to cephalosporins and carbapenems (12). NDM-8 with substitutions at positions 130 (Asp to Gly) and 154 (Met to Leu) resulted in enzymatic activities against β-lactams that were similar to those of NDM-1 (17). The drug susceptibilities of *E. coli* transformants with bla_{NDM-2}, bla_{NDM-3}, bla_{NDM-6}, bla_{NDM-7}, and bla_{NDM-9} have not been reported.

This is the first report describing NDM-3-producing Gram-negative pathogens in Japan. It appears that NDMs have recently begun evolving; therefore, careful monitoring of NDM-producing pathogens is required.

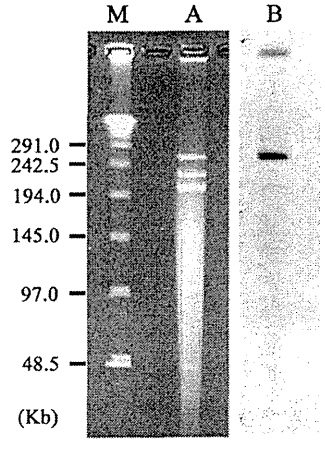

FIG 1 Localization of the bla_{NDM-3} gene on the plasmid of *E. coli* strain NCGM77 separated by PFGE. Lane M, midrange PFG marker (New England BioLabs, Tokyo, Japan); lane A, plasmids of *E. coli* strain NCGM77; lane B, hybridization of the plasmid with a probe specific for the bla_{NDM-3} gene.

Plasmid sequence accession number. The plasmid sequence including the bla_{NDM-3} gene has been deposited in GenBank under the accession number AB898038.

ACKNOWLEDGMENTS

This study was supported by International Health Cooperation Research (grant 23-A-301), the Ministry of Health, Labor and Welfare of Japan (grant H24-Shinko-Ippan-010), and the JSPS KAKENHI (grant 24790432).

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Antimicrob. Agents Chemother. doi:10.1128/AAC.01212-13

Dissemination of 16S rRNA Methylase ArmA-Producing *Acinetobacter baumannii* and Emergence of OXA-72 Carbapenemase Coproducers in Japan

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Running title: ArmA producing *A. baumannii* in Japan

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Abstract

Forty-nine clinical isolates of multidrug-resistant *Acinetobacter baumannii* were obtained from 12 hospitals in 7 prefectures throughout Japan. Molecular phylogenetic analysis revealed the clonal spread of *A. baumannii* ST208 and ST455 isolates harboring *armA* and ST512 harboring *armA* and bla_{OXA-72}. These findings show that *A. baumannii* isolates harboring *armA* are disseminated throughout Japan, as well as being the first report to show that *A. baumannii* strains harboring bla_{OXA-72} and *armA* are emerging in hospitals in Japan.

TEXT

Multidrug-resistant *Acinetobacter baumannii* has become a threatening nosocomial pathogen worldwide (1). Most of these strains develop resistance to carbapenems by mechanisms associated with carbapenem-hydrolyzing class D OXA-type β-lactamases (CHDLs) (2). The overproduction of intrinsic chromosomal OXA-51-like enzymes, and the production of acquired OXA-23-, OXA-24/40-, OXA-58-, OXA-143- and OXA-235-like enzymes, have been associated with carbapenem-resistant *A. baumannii* isolates (3-5). The gene $bla_{\text{OXA-72}}$, one of the $bla_{\text{OXA-40-like}}$ genes, was first identified in an *A. baumannii* strain isolated in 2004 in Thailand (GenBank Accession no. AY739646). Since then, *Acinetobacter* spp. harboring $bla_{\text{OXA-72}}$ have been reported in Brazil (6), China (7), Colombia (8), Croatia (9), France (10), Italy (11), Lithuania (12), South Korea (13), Spain (14), Taiwan (15) and the United States of America (16).

The *armA* gene, encoding a 16S rRNA methylase that confers aminoglycoside resistance, was initially identified in *Citrobacter freundii* in 2002 in Poland (17), and later detected in several Gram-negative bacterial spp., including *A. baumannii*, in Africa, Asia, Europe and North America (18).

From July to December 2012, BML Biomedical Laboratories R&D Center (Kawagoe, Saitama, Japan) acquired 16,343 isolates of *Acinetobacter* spp. from 3,015 medical settings

45 located in 47 prefectures throughout Japan. These included 49 isolates of multidrug-resistant
46 *A. baumannii*, obtained from 49 patients in 12 hospitals located in 7 prefectures in Japan (Fig.
47 S1). Of these 49 isolates, 41 were from respiratory tracts, 7 from urinary tracts and 1 from
48 blood. Multidrug-resistant *A. baumannii* strains were defined as having MICs ≥16 μg/ml to
49 imipenem/meropenem, ≥32 μg/ml to amikacin, and ≥8 μg/ml to levofloxacin/gatifloxacin or
50 ≥4 μg/ml to ciprofloxacin, according to the criteria of the Japanese Nosocomial Infection
51 Surveillance System (JANIS) of the Japanese Ministry of Health, Labour and Welfare
52 (MHLW). Species were determined by the VITEK system (bioMérieux SA, Marcy l'Etoile,
53 France) and by the sequences of the 16S rRNA, *gyrB*, and $bla_{\text{OXA-51-like}}$ genes.
54 The MICs were determined using MicroScan WalkAway™ (Siemens Japan K.K.,
55 Tokyo, Japan) and the microdilution method, as described by the guidelines of the Clinical
56 Laboratory Standards Institute (19).
57 Whole genomes of the 49 multidrug-resistant isolates were extracted by DNeasy
58 Blood & Tissue kits (QIAGEN, Tokyo, Japan) and sequenced by MiSeq (Illumina, San Diego,
59 CA). More than 10-fold coverage was archived for each isolate. To identify SNPs among
60 these genomes, the sequence of *A. baumannii* MDR-TJ (Accession no. CP003500) (20) was
61 used as a control, with all reads of each isolate aligned against the MDR-TJ sequence using
62 CLC genomics workbench version 5.5 (CLC bio, Tokyo, Japan). SNP concatenated sequences

were aligned by MAFFT (http://mafft.cbrc.jp/alignment/server/). A maximum-likelihood phylogenetic tree was constructed from SNP alignment with PhyML 3.0 (21). The probability of node branching was evaluated with 100 bootstrappings. Multilocus sequence typing (MLST) was deduced, as described by the protocols of the Institut Pasteur MLST (IP-MLST) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html) and PubMLST (http://pubmlst.org/abaumannii/) databases. Clonal complexes were determined by eBURST version 3 (http:/eburst.mlst.net). Sequences of 916 drug-resistance genes, including β-lactamase encoding genes at the website (www.lahey.org/studies), aminoglycoside resistance genes (22, 23) and quinolone resistance genes (24), were determined using CLC genomics workbench version 5.5. Pulsed-field gel electrophoresis (PFGE) analysis was performed as described (25).

A genome of *A. baumannii* strain NCGM237, one of the 49 multidrug-resistant isolates, was sequenced using a PacBio™ RSII platform (Pacific Biosciences of California, Inc., Menlo Park, CA).

All 49 isolates were resistant to most of the antibiotics tested (Table 1), including to imipenem and meropenem with MICs ≥16 μg/ml. Five isolates showed higher MICs to imipenem and meropenem, of 64 and 128 μg/ml, respectively, than the other 44 isolates. All 49 isolates were resistant to amikacin, arbekacin and gentamicin, with MICs ≥512 μg/ml; and

to ciprofloxacin, with MICs 32-1,024 μg/ml. Of the 49 isolates, 45 were susceptible to colistin.

IP-MLST showed that all isolates belonged to ST2. According to the MLST scheme from www.pasteur.fr, all isolates belonging to ST2 will belong to the worldwide clonal lineage II (European Clone II) (26), indicating that *A. baumannii* European Clone II isolates were spreading in Japan. PubMLST showed that 23, 21 and 5 isolates belonged to ST455, ST208 [clonal complex (CC) 92] and ST512 (CC92), respectively. CC 92 is the most widely disseminated complex worldwide (27). ST455 does not belong to CC92. Molecular phylogenetic analysis based on SNP concatenation showed that the 49 isolates could be clustered into 3 clades (Fig. 1). PFGE pattern analysis showed 2 clusters (Cluster I and II) (Fig. S2). Cluster I had the isolates belonging to ST455 and Cluster II had the isolates belonging to ST208 and ST512.

The 23 isolates belonging to the ST455 clade had both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-66}}$ (Table 2). Of the 21 isolates belonging to the ST208 clade, 17 had $bla_{\text{OXA-82}}$ genes and 4 had $bla_{\text{OXA-202}}$ (Table 2). The five isolates belonging to the ST512 clade had both $bla_{\text{OXA-66}}$ and $bla_{\text{OXA-72}}$ genes (Table 2). The $bla_{\text{OXA-66}}$, $bla_{\text{OXA-82}}$ and $bla_{\text{OXA-202}}$ are $bla_{\text{OXA-51-like}}$ variants. Among these bla_{OXA} genes, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-82}}$ and $bla_{\text{OXA-202}}$ were flanked by IS*Aba1*, whereas $bla_{\text{OXA-66}}$ and $bla_{\text{OXA-72}}$ were not. Sixteen isolates had $bla_{\text{TEM-1}}$, and all 49 had the

AmpC encoding gene, bla_{ADC-30} flanked by IS*AbaI*. None of the isolates had any other β-lactamase encoding genes registered at http://www.lahey.org/studies/. It has been reported that *A. baumannii* isolates producing OXA-66, -82 or -202 belong to European Clone II (28). There were no differences in drug-susceptibility profiles among the isolates belonging to the three clades, except for their carbapenem susceptibilities. Five isolates belonging to the ST512 clade were more resistant to imipenem and meropenem, with MICs of 64 μg/ml and 128 μg/ml, respectively. These isolates harbored bla_{OXA-72}.

In the 5 isolates harboring bla_{OXA-72}, this gene was located on pAB-NCGM253 (Accession no. AB823544). The genetic organization of pAB-NCGM253 was similar to that of pABVA01 harboring bla_{OXA-24} (29) and p2ABAYE harboring no β-lactamase gene (30), which were obtained from clinical isolates in Italy and France, respectively. The entire sequences of pAB-NCGM253 (8,970 bp) had more than 96% identity with those of pABVA01, and the most part of pAB-NCGM253 (86.0%: nt 1 to nt 5,194 and nt 6,098 to nt 8,619) had more than 99% identity with those of p2ABAYE. The bla_{OXA-72} was flanked by XerC/XerD recombination sites which were identical to those of pABVA01 (29), indicating mobilization by the site-specific recombination mechanism. The sequence analysis using MiSeq revealed that all the 5 isolates had the same sequence of pAB-NCGM253. The other than the 5 isolates harboring bla_{OXA-72} had no plasmid. The genetic environments surrounding

117 *bla*$_{OXA-23}$ [from nt 3,159,090 to nt 3,162,816 in the entire genome sequence (Accession no.
118 AP013357)] were IS*AbaI*-*bla*$_{OXA-23}$-IS*AbaI*, which was identical to *A. baumannii* transposon
119 Tn*2006* (Accession no. GQ861439) (26). All isolates had *armA*, *aac(6')-Ib* and *aadA1*, but
120 none had the genes encoding the other 16S rRNA methylases, 6'-*N*-aminoglycoside
121 acetyltransferases and aminoglycoside adenylyltransferases. The genetic environments
122 surrounding *armA* [from nt 1,398,519 to nt 1,416,800 in the entire genome sequence
123 (AP013357)] were identical to those of *A. baumannii* MDR-TJ isolated in China (31) and
124 TYTH-1 isolated in Taiwan (32). The sequences from nt 1,405,067 to nt 1,409,153 in the
125 entire genome sequence (AP013357) were identical to *A. baumannii* transposon Tn*1548*
126 (Accession no. EU014811) (33), which included the IS*CR1* insertion sequence. *tnpU*, a
127 putative transposase, was located upstream of *armA*, which was followed downstream by
128 another putative transposase gene, *tnpD*. A class I integron, including
129 *intI1-aac(6)-Ib-catB8-aadA1-qacEdelta1-sulI*, located upstream of the IS*CR1* insertion
130 sequence.
131 A complete genome sequence of *A. baumannii* NCGM237 determined using a
132 combination of PacBio and MiSeq revealed that the *armA* and *bla*$_{OXA-23}$ were located on the
133 chromosome (Accession no. AP013357). The genome consisted of a single circle
134 chromosome of 4,021,920 bp, with an average G+C content of 39.1%. The details of *A.*

135 *baumannii* NCGM237 genome and its comparative analysis will be reported elsewhere. The
136 *armA* and bla_{OXA-23} will be located on the chromosome in the other strains belonging to
137 ST455, because the whole genome sequences using MiSeq revealed that the genomic
138 environments surrounding the *armA* and bla_{OXA-23} (18.3 Kbp and 17.9 Kbp, respectively)
139 were identical to each other and NCGM237 and PFGE analysis showed that any plasmid was
140 not found in all the isolates belonging to ST455.
141 All 49 isolates tested had point mutations in the quinolone-resistance-determining
142 regions of *gyrA* and *parC*, with amino acid substitutions of S83L in GyrA and S80L in ParC.
143 The amino acid substitutions in GyrA and ParC were reported to be associated with the
144 ciprofloxacin resistance in *A. baumannii* (24).
145 This is the first report of *A. baumannii* ST455 and ST512 isolates in Japan. *A.*
146 *baumannii* ST455 isolates were originally identified as a causative agent of nosocomial
147 infections in Taiwan (Cheng-Hsun Chiu: Chang Gung Memorial Hospital, Chang Gung
148 University College of Medicine; personal communication), and registered in 2012 in the *A.*
149 *baumannii* MLST Database website (http://pubmlst.org/abaumannii/). ST455 isolates have
150 not been reported elsewhere. The *A. baumannii* ST512 isolates were relatively close to *A.*
151 *baumannii* MDR-TJ (Fig. 1), which had been isolated in China (20). Both the ST512 isolates
152 and MDR-TJ had drug-resistance genes, including bla_{OXA-66}, *armA*, *aac(6')-Ib* and *aadA1*,

153 with the only exception of *bla*$_{OXA-72}$ (31), i.e. the ST512 isolates, but not MDR-TJ, had
154 *bla*$_{OXA-72}$ (Table 2). ST208 isolates was found in various regions in Japan (Fig. 1 and Fig. S2),
155 being first identified in 2012 in the Kanto and Kyusyu areas (34), although it was not reported
156 whether these isolates were resistant to aminoglycosides and possessed the *armA* gene.
157 To our knowledge, this is the first report showing that highly carbapenem-resistant *A.*
158 *baumannii* strains harboring *bla*$_{OXA-72}$ were emerging in Japan. OXA-72 was primarily
159 responsible for carbapenem resistance in *A. baumannii* clinical isolates from a Taiwan
160 hospital (15), although OXA-23 is much more prevalent worldwide (3). The expression of
161 OXA-72 in *E. coli* resulted in 6.0-, 2.7- and 3.9-fold increases in MICs to imipenem,
162 meropenem and doripenem, respectively, compared with control (8). It is necessary to monitor
163 highly carbapanem-resistant *A baumannii* producing OXA-72 in Japan, because outbreaks due
164 to metallo-β-lactamase producers with high MICs to carbapenems have caused serious health
165 problems throughout Japan (35). Some *A. baumannii* isolates producing CHDLs show lower
166 MICs to carbapenems (36), but a part of this population may have been undetected in this
167 study.
168 The present study strongly suggests that *A. baumannii* isolates producing a 16S
169 rRNA methylase, ArmA, have emerged and disseminated in medical settings throughout
170 Japan. Bacteria producing 16S rRNA methylases are resistant to clinically important

171 aminoglycosides (37, 38). In Japan, clinical isolates of aminoglycoside-resistant
172 Gram-negative bacteria producing 16S rRNA methylases were first reported in 2003 (39);
173 since then, 38 of these strains have been reported throughout Japan (38-43). A nationwide
174 surveillance of 16S rRNA methylase-producing Gram-negative pathogens in 2004 in Japan
175 (38) revealed that only 26 of 87,626 isolates (0.03%) produced 16S rRNA methylases.
176 Recently, there was an outbreak of *A. baumannii* harboring *armA* at a university hospital in
177 Japan (43). We focused on multidrug-resistant *A. baumannii* isolates in this study, but not
178 aminoglycoside-resistant isolates. Therefore, a part of aminoglycoside-resistant *A. baumannii*
179 isolates, such as carbapenem-sensitive ArmA-producers, could have been missed. It is
180 necessary to survey aminoglycoside-resistant *A. baumannii* isolates in Japan.
181

ACKNOWLEDGMENTS

We thank Takashi Hirano, Kazuhito Satou and Kuniko Teruya (Okinawa Institute of Advanced Sciences) for a complete genome sequence of *A. baumannii* NCGM237 using PacBio. This study was supported by grants [International Health Cooperation Research (24-S-5), and a grant from the Ministry of Health, Labor and Welfare of Japan (H24-Shinko-Ippan-010), and JSPS KAKENHI Grant Number 24790432].

TABLE 1. MIC_{50} and MIC_{90} values and percent antimicrobial resistance of *A. baumannii* clinical isolates

Antimicrobial agent	*A. baumannii* (n=49)				
	Breakpoint for resistance[a] (μg/ml)	% Resistance	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Amikacin	≥64	100	>1,024	>1,024	>1,024
Arbekacin	-	-	1,024->1,024	>1,024	>1,024
Colistin	≥4	8	≤0.25-4	2	2
Ciprofloxacin	≥4	100	32-1,024	256	512
Gentamicin	≥16	100	512->1,024	>1,024	>1,024
Imipenem	≥16	100	16-64	16	64
Meropenem	≥16	100	16-128	16	128
Tigecycline[b]	-	-	≤0.25-4	1	4

[a]Break points for antimicrobial resistance were determined according to the guidelines of the Clinical and Laboratory Standards Institute (M07-A9).

[b]MICs to tigecycline were 4 μg/ml for 6 isolates, 2 μg/ml for 7, 1 μg/ml for 18, 0.5 μg/ml for 12, and ≤0.25 μg/ml for 6.

189

TABLE 2. MLST and drug resistance genes in *A. baumannii* isolates

MLST	No. of isolates[a]	β-lactamase encoding genes	aminoglycoside-resistance genes
ST208	21	bla_{OXA-82} (17/21), $bla_{OXA-202}$(4/21)[b], bla_{ADC-30}, bla_{TEM-1}(10/21)	*armA*, *aac(6')-Ib*, *aac(3)-Ia*(16/21), *aadA1*, *aph(3')-Ib*(14/21)
ST455	23	bla_{OXA-23}, bla_{OXA-66}, bla_{ADC-30}, bla_{TEM-1}(1/23)	*armA*, *aac(6')-Ib*,*aadA1*, *aph(3')-Ib*(1/23)
ST512	5	bla_{OXA-66}, bla_{OXA-72}, bla_{ADC-30}, bla_{TEM-1}	*armA*, *aac(6')-Ib*,*aadA1*, *aph(3')-Ib*
ST369 (MDR-TJ)[c]	1	bla_{OXA-66}, bla_{ADC-30}	*armA*, *aac(6')-Ib*,*aadA1*, *aph(3')-Ib*

[a]Numbers of isolates belonged to the same sequence type.

[b]Of 21 isolates belonging to ST208, the remaining 4 had $bla_{OXA-202}$.

[c]MDT-TJ belonging to ST369 strain was cited to compare drug resistance genes (20).

190

Figure legends

FIG. 1. Molecular phylogeny of the 49 *A. baumannii* strains. Molecular phylogenetic analysis based on SNP concatenation revealed that the 49 isolates were clustered into 3 clades, with the ST208 clade composed of 2 subclades.