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

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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* sequence type 208 (ST208) and ST455 isolates harboring the *armA* gene and ST512 harboring the *armA* and bla_{OXA-72} genes. These findings show that *A. baumannii* isolates harboring *armA* are disseminated throughout Japan, and this is the first report to show that *A. baumannii* strains harboring bla_{OXA-72} and *armA* are emerging in hospitals in Japan.

Multidrug-resistant *Acinetobacter baumannii* has become a threatening nosocomial pathogen worldwide (1). Most strains of this species 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_{OXA-72}, one of the $bla_{OXA-40-like}$ genes, was first identified in an *A. baumannii* strain isolated in 2004 in Thailand (GenBank accession no. AY739646). Since then, *Acinetobacter* spp. harboring bla_{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 (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 it was later detected in several Gram-negative bacterial spp., including *A. baumannii*, in Africa, Asia, Europe, and North America (18).

From July to December 2012, the BML Biomedical Laboratories R&D Center (Kawagoe, Saitama, Japan) acquired 16,343 isolates of *Acinetobacter* spp. from 3,015 medical settings located in 47 prefectures throughout Japan. These included 49 isolates of multidrug-resistant *A. baumannii* obtained from 49 patients in 12 hospitals located in 7 prefectures in Japan (see Fig. S1 in the supplemental material). Of these 49 isolates, 41 were from respiratory tract, 7 from urinary tract, and 1 from blood samples. The multidrug-resistant *A. baumannii* strains were defined as having MICs of ≥16 μg/ml to imipenem/meropenem, ≥32 μg/ml to amikacin, and ≥8 μg/ml to levofloxacin/gatifloxacin or ≥4 μg/ml to ciprofloxacin, according to the criteria of the Japanese Nosocomial Infection Surveillance System (JANIS) of the Japanese Ministry of Health, Labor and Welfare (MHLW). The species were determined by the Vitek system (bioMérieux SA, Marcy l'Etoile, France) and by the sequences of the 16S rRNA, *gyrB*, and $bla_{OXA-51-like}$ genes.

The MICs were determined using the microdilution method, as described in the guidelines of the Clinical and Laboratory Standards Institute (19).

Whole genomes of the 49 multidrug-resistant isolates were extracted by DNeasy blood and tissue kits (Qiagen, Tokyo, Japan) and sequenced by MiSeq (Illumina, San Diego, CA). More-than-10-fold coverage was archived for each isolate. To identify single-nucleotide polymorphisms (SNPs) in these genomes, the sequence of *A. baumannii* strain MDR-TJ (GenBank accession no. CP003500) (20) was used as a control, with all reads of each isolate aligned against the MDR-TJ sequence using CLC Genomics Workbench version 5.5 (CLC bio, Tokyo, Japan). SNP concatenated sequences were aligned using MAFFT (http://mafft.cbrc.jp/alignment/server/). A maximum likelihood phylogenetic tree was constructed from the SNP alignment using PhyML 3.0 (21). The probability of node branching was evaluated with 100 bootstrappings. The multilocus sequence types (MLST) were deduced as described in 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). The sequences of 916 drug resistance genes, including β-lactamase-encoding genes (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 previously (25).

The 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 of ≥16 μg/ml. Five isolates showed higher MICs to imipenem and meropenem of 64 and 128 μg/ml, respectively, than the other 44

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TABLE 1 MIC_{50} and MIC_{90} values and percent antimicrobial resistance of *A. baumannii* clinical isolates ($n = 49$)

Antimicrobial agent	Breakpoint for resistance (μg/ml)[a]	% resistance	MIC data (μg/ml)		
			Range	MIC_{50}	MIC_{90}
Amikacin	≥64	100	>1,024	>1,024	>1,024
Arbekacin			1,024 to >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 to >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] Breakpoints for antimicrobial resistance were determined according to guidelines of the Clinical and Laboratory Standards Institute (document M07-A9 [19]).
[b] The MICs to tigecycline were 4 μg/ml for 6 isolates, 2 μg/ml for 7 isolates, 1 μg/ml for 18 isolates, 0.5 μg/ml for 12 isolates, and <0.25 μg/ml for 6 isolates.

isolates. All 49 isolates were resistant to amikacin, arbekacin, and gentamicin, with MICs of ≥512 μg/ml, and to ciprofloxacin, with MICs of 32 to 1,024 μg/ml. Of the 49 isolates, 45 were susceptible to colistin.

IP-MLST showed that all isolates belonged to sequence type 2 (ST2). According to the MLST scheme from www.pasteur.fr, all isolates belonging to ST2 belong to the worldwide clonal lineage II (European clone II) (26), indicating that *A. baumannii* European clone II isolates have been spreading in Japan. PubMLST showed that 23, 21, and 5 isolates belonged to ST455, ST208 (clonal complex 92 [CC92]), and ST512 (CC92), respectively. CC92 is the most widely disseminated clonal 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). The PFGE pattern analysis showed 2 clusters (clusters I and II) (see Fig. S2 in the supplemental material). 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_{OXA-23} and bla_{OXA-66} genes (Table 2). Of the 21 isolates belonging to the ST208 clade, 17 had bla_{OXA-82} genes and 4 had $bla_{OXA-202}$ genes (Table 2). The five isolates belonging to the ST512 clade had both bla_{OXA-66} and bla_{OXA-72} genes (Table 2). The bla_{OXA-66}, bla_{OXA-82}, and $bla_{OXA-202}$ genes are $bla_{OXA-51-like}$ variants. Among these bla_{OXA} genes, bla_{OXA-23}, bla_{OXA-82}, and $bla_{OXA-202}$ were flanked by IS*Aba1*, whereas bla_{OXA-66} and bla_{OXA-72} were not. Sixteen isolates had bla_{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, OXA-82, or OXA-202 belong to European clone II (28). There were no differences in the drug susceptibility profiles of the isolates belonging to the three clades, except for their susceptibility to carbapenems. 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 (GenBank 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 >96% identity with those of pABVA01, and most of pAB-NCGM253 (86.0%; nucleotide [nt] 1 to nt 5194 and nt 6098 to nt 8619) had >99% identity with those of p2ABAYE. The bla_{OXA-72} gene 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 5 isolates had the same sequence of pAB-NCGM253. Isolates other than these 5 that harbored bla_{OXA-72} had no plasmid. The genetic environments surrounding bla_{OXA-23} (from nt 3159090 to nt 3162816 in the entire genome sequence [GenBank accession no. AP013357]) were IS*AbaI*-bla_{OXA-23}-IS*AbaI*, which was identical to *A. baumannii* transposon Tn*2006* (GenBank accession no. GQ861439) (26). All isolates had *armA*, *aac(6′)-Ib*, and *aadA1*, but none had the genes encoding the other 16S rRNA methylases, 6′-*N*-aminoglycoside acetyltransferases and aminoglycoside adenylyltransferases. The genetic environments surrounding *armA* (from nt

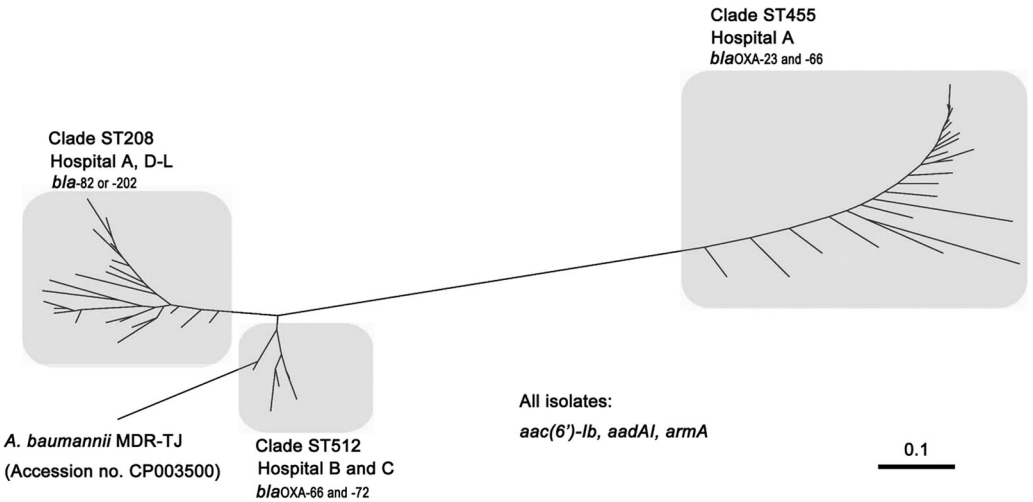

FIG 1 Molecular phylogeny of the 49 *A. baumannii* strains. Molecular phylogenetic analysis based on SNP concatenation revealed that the 49 isolates were clustered into 3 clades, with the ST208 clade composed of 2 subclades. The genes harbored by each ST are listed with the clades, and all isolates had *aac(6′)-Ib*, *aadAI*, and *armA*.

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

MLST	No. of isolates in ST	β-Lactamase-encoding genes[a]	Aminoglycoside resistance genes[a]
ST208	21	bla_{OXA-82} (17/21), $bla_{OXA-202}$ (4/21), 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)[b]	1	bla_{OXA-66}, bla_{ADC-30}	*armA*, *aac(6′)-Ib*, *aadA1*, *aph(3′)-Ib*

[a] Shown in parentheses are the numbers of isolates with the respective genes out of the total number of isolates for that ST, if not all isolates in that ST had those particular genes.
[b] MDT-TJ belonging to ST369 strain was cited to compare drug resistance genes (20).

1398519 to nt 1416800 in the entire genome sequence [GenBank accession no. AP013357]) were identical to those of *A. baumannii* MDR-TJ isolated in China (31) and TYTH-1 isolated in Taiwan (32). The sequences from nt 1405067 to nt 1409153 in the entire genome sequence (GenBank accession no. AP013357) were identical to *A. baumannii* transposon Tn*1548* (GenBank accession no. EU014811) (33), which included the IS*CR1* insertion sequence. *tnpU*, a putative transposase gene, was located upstream of *armA*, which was followed downstream by another putative transposase gene, *tnpD*. A class I integron, including *intI1-aac(6)-Ib-catB8-aadA1-qacEdeltaI-sulI*, was located upstream of the IS*CR1* insertion sequence.

A complete genome sequence of *A. baumannii* strain NCGM237, determined using a combination of PacBio and MiSeq platforms, revealed that the *armA* and bla_{OXA-23} were located on the chromosome (GenBank accession no. AP013357). The genome consists of a single circular chromosome of 4,021,920 bp, with an average G+C content of 39.1%. The details of the *A. baumannii* NCGM237 genome and its comparative analysis will be reported elsewhere. The *armA* and bla_{OXA-23} genes will be located on the chromosome in other strains belonging to ST455. The whole-genome sequences using MiSeq revealed that the genomic environments surrounding the *armA* and bla_{OXA-23} genes (18.3 kbp and 17.9 kbp, respectively) were identical to each other, and NCGM237 and PFGE analyses showed that no plasmid was found in all the isolates belonging to ST455.

All 49 isolates tested had point mutations in the quinolone resistance-determining regions of *gyrA* and *parC*, with amino acid substitutions of S83L in GyrA and S80L in ParC. The amino acid substitutions in GyrA and ParC were reported to be associated with the ciprofloxacin resistance in *A. baumannii* (24).

This is the first report of *A. baumannii* ST455 and ST512 isolates in Japan. *A. baumannii* ST455 isolates were originally identified as a causative agent of nosocomial infections in Taiwan (C.-H. Chiu, Chang Gung Memorial Hospital and Chang Gung University College of Medicine, personal communication), and were registered in 2012 in the *A. baumannii* MLST database website (http://pubmlst.org/abaumannii/). ST455 isolates have not been reported elsewhere. The *A. baumannii* ST512 isolates were relatively similar to *A. baumannii* MDR-TJ (Fig. 1), which had been isolated in China (20). Both the ST512 isolates and MDR-TJ had drug resistance genes, including bla_{OXA-66}, *armA*, *aac(6′)-Ib*, and *aadA1*; however, not all isolates had bla_{OXA-72} (31), i.e., the ST512 isolates, but not MDR-TJ, had bla_{OXA-72} (Table 2). ST208 isolates were found in various regions in Japan (Fig. 1; see also Fig. S2 in the supplemental material), being first identified in 2012 in the Kanto and Kyushu areas (34), although it was not reported whether these isolates were resistant to aminoglycosides and possessed the *armA* gene.

To our knowledge, this is the first report showing that highly carbapenem-resistant *A. baumannii* strains harboring bla_{OXA-72} are emerging in Japan. OXA-72 was primarily responsible for carbapenem resistance in *A. baumannii* clinical isolates from a Taiwan hospital (15), although OXA-23 is much more prevalent worldwide (3). The expression of OXA-72 in *Escherichia coli* resulted in 6.0-, 2.7-, and 3.9-fold increases in the MICs to imipenem, meropenem, and doripenem, respectively, compared with that of the control (8). It is necessary to monitor highly carbapenem-resistant *A. baumannii* producing OXA-72 in Japan, because outbreaks due to metallo-β-lactamase producers with high MICs to carbapenems have caused serious health problems throughout Japan (35). Some *A. baumannii* isolates producing CHDLs show lower MICs to carbapenems (36), but a part of this population may have been undetected in this study.

The present study strongly suggests that *A. baumannii* isolates producing a 16S rRNA methylase, ArmA, have emerged and disseminated in medical settings throughout Japan. Bacteria producing 16S rRNA methylases are resistant to clinically important aminoglycosides (37, 38). In Japan, clinical isolates of aminoglycoside-resistant Gram-negative bacteria producing 16S rRNA methylases were first reported in 2003 (39); since then, 38 of these strains have been reported throughout Japan (38–43). A nationwide surveillance of 16S rRNA methylase-producing Gram-negative pathogens in 2004 in Japan (38) revealed that only 26 of 87,626 isolates (0.03%) produced 16S rRNA methylases. Recently, there was an outbreak of *A. baumannii* harboring *armA* at a university hospital in Japan (43). In this study, we focused on multidrug-resistant *A. baumannii* isolates but not aminoglycoside-resistant isolates. Therefore, a subset of aminoglycoside-resistant *A. baumannii* isolates, such as carbapenem-sensitive ArmA-producers, might have been missed. It is necessary to continue surveying aminoglycoside-resistant *A. baumannii* isolates in Japan.

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