consent was provided to all study participants, and the study was conducted with the approval of the ethics committee of the Nagoya University Graduate School of Medicine. The stool specimens were directly inoculated onto MacConkey agar plates (Eiken Chemical Co., Ltd, Tokyo, Japan) supplemented with 1 μg/ml of cefotaxime (CTX-MacConkey). The three to four colonies growing on the CTX-MacConkey plates were picked up and identified in terms of bacterial species by using the API-20E system (SYSMEX bioMérieux, Tokyo, Japan). These isolates were further subjected to pulsed field gel electrophoresis (PFGE) analysis. Plugs containing whole genomic DNA were digested with *Xba*I (Takara Bio., Inc., Tokyo, Japan), and electrophoresis was performed using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA) with pulses ranging from 2.2 to 54.2 sec at a voltage of 6 V/cm at 14°C for 19 h. A dendrogram showing genetic relatedness among the isolates was constructed with Fingerprinting II software (Bio-Rad Laboratories). In the isolates from each volunteer, when ≥85% genetic similarity was observed, these were classified as a clone with a common genetic background, and one representative isolate was further studied. When <85% genetic similarity was observed, these were considered a different clone and separately treated in this study.

ESBL screening and antimicrobial susceptibility test

ESBL screening was performed on the basis of the double-disk synergy test by using three different commercially available discs: ceftazidime (30 μg), cefotaxime (30 μg), and amoxicillin/clavulanic acid (20 μg/10 μg; all three from Eiken).[22] An antimicrobial susceptibility test was performed using the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI),[4] and the interpretation of minimal inhibitory concentration (MIC) results was in accordance with CLSI criteria in document M7-A9. The antimicrobial agents were obtained from the following sources: cefotaxime, fosfomycin, gentamicin, minocycline, sulfamethoxazole, trimethoprim, rifampin, and ciprofloxacin from Wako Pure Chemical Co., Inc. (Osaka, Japan); amikacin and chloramphenicol from Sigma-Aldrich JAPAN (Tokyo, Japan); and florfenicol from LKT Laboratories, Inc. (St. Paul, MN). *E. coli* ATCC 25922 was used as the control strain.

Detection of antimicrobial resistance genes

The presence of CTX-M-type β-lactamase genes was determined by polymerase chain reaction (PCR) using the CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, and CTX-M-9 group-specific primers.[5] The TEM- and SHV-type β-lactamase genes were detected by PCR, and their genotypes were further determined by sequencing analysis.[28] The presence of acquired fosfomycin resistance genes such as *fosA*, *fosA3* and *fosC2* was determined by PCR using specific primer sets (Table 1). In addition, for *fosA3*-harboring isolates, the genotypes of CTX-M-type β-lactamases were characterized by PCR and nucleotide sequencing analysis.[15,22] The *floR* gene was amplified using specific primer sets (Table 1).

Multilocus sequence typing and serotyping

Multilocus sequence typing (MLST) of the *E. coli* isolates harboring the *fosA3* gene was performed by analyzing seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) according to the protocol of the *E. coli* website (http://mlst.ucc.ie/mlst/dbs/Ecoli).

Serotyping was performed using *E. coli* antisera "SEIKEN" Set 1 (Denka Seiken, Tokyo, Japan) for the O-antigen and Set 2 (Denka Seiken) for the H-antigen, according to the manufacturer's instructions.

Transfer of plasmid and replicon typing

The conjugation experiments were performed with rifampin-resistant *E. coli* CSH-2 (*metB* F^-, nalidixic acid and rifampin resistant) as the recipient strain using the broth mating method. Transconjugants were selected on Luria–Bertani (LB) agar plates supplemented with fosfomycin (40 μg/ml), glucose-6-phosphate (G6P) (Wako; 25 μg/ml), and rifampin (100 μg/ml). Transformation by electroporation was performed using *E. coli* DH10B as the host strain, and the transformants were selected on LB agar plates containing fosfomycin (40 μg/ml) and G6P (25 μg/ml).

Replicon types of plasmids were analyzed using the PCR-based replicon-typing scheme described by Carattoli *et al.*[3] In addition, the plasmid multilocus sequence typing (pMLST) of IncF, IncN, and IncI1 plasmids were carried out as previously described.[7,8,24] The alleles or sequence types (STs) were assigned by submitting the amplicon sequence to the pMLST website (www.pubmlst.org/plasmid).

Determination of genetic environment mediating fosA3

To determine the genetic environment of *fosA3*, plasmids carrying the *fosA3* gene were extracted from the transconjugants and used as a DNA template for PCR mapping experiments. All the primers were designed based on the sequences of GenBank Accession nos. JQ343849, JQ343850, and JQ343851, which encompassed the surrounding region of *fosA3* of clinically isolated *E. coli* strains from Korea[14] (Table 1).

Nucleotide sequence accession numbers

The nucleotide sequences of the genetic environments of *fosA3* presented in this study have been deposited in GenBank under Accession nos. AB778291 and AB778503.

Results

Bacterial isolates

Of the 4,314 stool specimens, ESBL producers were recovered from 197 stool samples, which included more than one sample recovered at a different time point from a participant. PFGE was performed to determine the genetic relationships of the isolates (three to four colonies grown on CTX-MacConkey plates) collected from one participant. As a result, 145 nonduplicate ESBL producers were identified, of which 138 *E. coli* isolates harbored $bla_{\text{CTX-M}}$ genes (30 $bla_{\text{CTX-M-1 group}}$, 19 $bla_{\text{CTX-M-2 group}}$, 7 $bla_{\text{CTX-M-8 group}}$, 81 $bla_{\text{CTX-M-9 group}}$, 1 $bla_{\text{CTX-M-1 group}}$ and $bla_{\text{CTX-M-9 group}}$). The remaining seven *E. coli* isolates harbored the $bla_{\text{SHV-5}}$ genes. Finally, 138 nonduplicate CTX-M-producing *E. coli* isolates were subjected to further analyses.

Assay of fosfomycin susceptibility

Of the 138 CTX-M-producing *E. coli* isolates tested, 129 isolates (93.5%) were classified as susceptible to fosfomycin

TABLE 1. PRIMERS USED IN THIS STUDY

Primer name	*Position*	*Sequence (5′ to 3′)*	*Amplicon size (bp)*
For detecting resistance genes			
FosA3-F	—	GGCATTTTATCAGCAGT	350
FosA3-R	—	AGACCATCCCCTTGTAG	
FosC2-F	—	CGAGCCAAGATTACTGT	196
FosC2-R	—	AACGATTCCAAACGACT	
FosA-F	—	ATCTGTGGGTCTGCCTGTCGT	271
FosA-R	—	ATGCCCGCATAGGGCTTCT	
FloR-F	—	TGATCCAACTCACGTTGAGC	900
FloR-R	—	GAACGCAGAAGTAGAACGCG	
For PCR mapping			
IS26&IS903-F	IS*26*	GCCGTACGCTGGTACTGC	1,936
IS26&IS903-R	IS*903*	TTTTGAACTTTTGCTTTG	
IS26&CTX-M-14-F	IS*26*	TTATCTCTCCTCCCGTCGTA	971
IS26&CTX-M-14-R	$bla_{CTX\text{-}M\text{-}14}$	TGCTTTTGCGTTTCACTCAG	
CTX-M-14&IS26-F	$bla_{CTX\text{-}M\text{-}14}$	TGAACCTACGCTGAATACC	2,851
CTX-M-14&IS26-R	IS*26*	GCAAACTGAAACGGATAAT	
FosA3&IS26-F	*fosA3* upstream	GTCGGTGCTGATTTGGTCTT	1,521
FosA3&IS26-R	*orf2*	ATTGCCCTGCTTCTGGTTGC	
ORF&IS26-F	*orf2*	GACGGTGACGATGATGTT	914
ORF&IS26-R	IS*26*	CGAGCTATTCTGTGGGCC	
IS26&ISEcp1-R[a]	$bla_{CTX\text{-}M\text{-}55}$ upstream	GAGACGAAATAACAACAA	1,059
IS26&CTX-M-55-F	IS*26*	TCCTCCCGTCGTAACAGC	1,465
IS26&CTX-M-55-R	$bla_{CTX\text{-}M\text{-}55}$	GGTGGTATTGCCTTTCAT	
ISEcp1&CTX-M-55-F	IS*Ecp1*	CAATGTGTGAGAAGCAGT	902
ISEcp1&CTX-M-55-R	$bla_{CTX\text{-}M\text{-}55}$	CCCAGGAAGCAGGCAGTC	
CTX-M-55&IS26-F	$bla_{CTX\text{-}M\text{-}55}$	GAAAAGTGAAAGCGAACC	2,527
CTX-M-55&IS26-R	IS*26*	CGTGAAGAAGTGGCAGAT	
CTX-M-55-down-F	$bla_{CTX\text{-}M\text{-}55}$ downstream	GAAACGGAATGGGGAAAC	1,745
CTX-M-55-down-R	IS*26*	GCAAACTGAAACGGATAA	
FosA-up-F	bla_{TEM}	CGTCGTTTGGTATGGCTTCA	1,839
FosA-up-R	*fosA3*	TTCACTGCGGTTATCTTTCC	
IS26&FosA3-F	IS*26*	GCGGTAAATCGTGGAGTG	743
IS26&FosA3-R	*fosA3*	TGAAGGCGTAGTGGGTAT	
FosA3-down-F	*fosA3*	AAAGATAACCGCAGTGAA	2,282
FosA3-down-R	IS*26*	CGTGAAGAAGTGGCAGAT	
ORF3[b]	*orf3*	GACTCCCTGAATAAAATGAC	—

[a]Used in a pair with IS26&IS903-F.
[b]Used for sequencing analysis.
PCR, polymerase chain reaction.

(MIC ≤64 μg/ml); whereas 1 (0.7%) showed intermediate resistance (MIC, 128 μg/ml), and 8 (5.8%) showed resistance to fosfomycin (MIC ≥256 μg/ml) under the presence of G6P that induces production of fosfomycin influx transporters in bacterial inner membrane. The MIC_{50} and MIC_{90} were 1 and 4 μg/ml, respectively, which were far below the clinical breakpoints of fosfomycin in the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

Detection of acquired fosfomycin resistance genes

The mechanism underlying fosfomycin resistance were evaluated in eight isolates showing resistance to fosfomycin (MIC ≥256 μg/ml). It was reported that fosfomycin resistance in clinically isolated CTX-M-producing *E. coli* strains is attributable to the presence of horizontally acquired fosfomycin resistance genes such as *fosA*, *fosA3*, and *fosC2*.[26] Our analysis showed that five of the eight isolates carried *fosA3*, whereas no isolates harbored *fosA* and *fosC2* (Table 2).

Conjugation and transformation experiments

Conjugation experiments were performed to confirm the transmissibility of the fosfomycin resistance in eight resistant isolates. The results showed that the fosfomycin resistance of five *fosA3*-harboring isolates was successfully transferred to the recipient cells along with the cefotaxime resistance phenotype (Table 2). Transformation by electroporation was performed to transfer the fosfomycin resistance gene of the remaining three isolates; however, these attempts were unsuccessful. In the five *fosA3*-harboring transconjugants, the $bla_{CTX\text{-}M}$ genes (*i.e.*, three $bla_{CTX\text{-}M\text{-}14}$, one $bla_{CTX\text{-}M\text{-}3}$, and one $bla_{CTX\text{-}M\text{-}55}$), which were the same as those found in the parental clinical isolates, were also detected. The chloramphenicol and florfenicol resistance phenotype was observed in only one transconjugant, thus confirming the presence of the *floR* gene.

Genetic background of five fosA3*-harboring* E. coli *isolates*

The phenotypic and genotypic characteristics such as serotypes, MLST types, and plasmid replicon types of the five

TABLE 2. CHARACTERISTICS OF *FOSA3*-POSITIVE *E. COLI* ISOLATES AND THEIR TRANSCONJUGANTS

Strain no.	*ESBL type (parental)*	*Serotype (parental)*	*ST*	*MIC (μg/ml) of*		*Resistance (parental)*	*Inc type (parental)*	*ESBL type (conjugant)*	*Inc type (conjugant)*	*pMLST*	*MIC (μg/ml) of*	
				FOM	*CTX*						*FOM*	*CTX*
48	CTX-M-14	O128:H-	3502	> 256	128		N	CTX-M-14	N	ND	> 256	16
84	CTX-M-14	O29:HUT	224	> 256	> 256	MINO, CPFX	I1, FII, FIB	CTX-M-14	I1	ST71	> 256	256
558	CTX-M-55	OUT:H-	155	> 256	256	CP, FLO, MINO, ST, CPFX	FII, FIB	CTX-M-55	FII	FII:33	> 256	256
559	CTX-M-14	OUT:HUT	3503	> 256	128	CP, FLO, MINO, ST, CPFX	I1, FII, FIB	CTX-M-14	I1	ST97	> 256	16
608	CTX-M-3	O25:H-	3504	> 256	128	GM, MINO, ST, CPFX	FII, FIA, FIB	CTX-M-3	FII	FII:2	> 256	256

The *korA* gene of the IncN plasmid was not amplified. Resistance phenotype transferred to recipient cells by conjugation is underlined.
OUT, O-antigen untypable; HUT, H-antigen untypable; MINO, minocycline; CPFX, ciprofloxacin; CP, chloramphenicol; FLO, florfenicol; ST, trimethoprim/sulfamethoxazole; GM, gentamicin; ESBL, extended-spectrum β-lactamase; MIC, minimal inhibitory concentration; pMLST, plasmid multilocus sequence typing; ND, not determined.

fosA3-carrying plasmids were determined (Table 2). The serotypes of the five isolates varied, and they were also assigned to different STs: ST155, ST224, ST3502, ST3503, and ST3504. STs 155 and 224 were previously reported,[2,16,18] whereas the latter three are newly described for the first time in this article. The replicon types of the *fosA3*-carrying plasmids were IncI1 ($n=2$), IncN ($n=1$), and IncFII ($n=2$). The pMLST types of IncI1 plasmids were ST71 and ST97, and those of IncFII plasmids were FII:2 and FII:33. However, we could not determine the pMLST type of the IncN, because the *korA* gene could not be amplified.

Genetic environments of fosA3

The genetic environment of the *fosA3* genes located on the three plasmids belonging to different incompatibility groups (*i.e.*, IncN, IncI1, and IncFII) was determined (Fig. 1). In the IncN plasmid of *E. coli* no. 48, truncated IS*903*, $bla_{CTX\text{-}M\text{-}14}$, and truncated IS*Ecp1* were found upstream of *fosA3*. This structure was 99% identical to that found in the plasmid of ECO021TF (Accession no. JQ343849), which harbored the *fosA3* gene. On the other hand, the downstream sequences of *fosA3* showed 78% genetic identity with a part of the chromosomal nucleotide sequence of *Klebsiella pneumoniae* 342. These regions were flanked by two IS*26* elements that were oriented in opposite directions. The surrounding region of *fosA3* in the IncI1 plasmid of *E. coli* no. 559 was identical to that in the IncN plasmid of *E. coli* no. 48. In the IncFII plasmid of *E. coli* no. 558, a structure comprising a truncated $bla_{TEM\text{-}1}$, *orf477*, $bla_{CTX\text{-}M\text{-}55}$, and truncated IS*Ecp1* was found upstream of *fosA3*. This structure was flanked by two IS*26* elements, and was 100% identical to that in the plasmid of ECO141TF (Accession no. JQ343851), which harbored the *fosA3* gene. Downstream of *fosA3*, an IS*26* element and a region that had 79% nucleotide identity with a part of the chromosomal sequence of *K. pneumoniae* 342 were observed. These genetic environments surrounding the *fosA3* were 98% −100% in sequence identity compared with those reported from clinical and veterinary settings.[11,14,26]

Discussion

This study demonstrated the presence of fosfomyin-resistant CTX-M-producing *E. coli* from the gut of healthy individuals who were not exposed to any antimicrobial agents, including fosfomycin and third-generation cephalosporins in this investigation period (January–August 2010). The rate of fosfomycin resistance was 5.8%, which is similar to that reported in clinical isolates from Japan (3.6%),[26] Spain (9.1%),[19] and Korea (4.2%).[14] No significant difference in the rates of fosfomycin resistance between CTX-M-producing *E. coli* from healthy individuals and those from clinical specimens were observed. Recently, similar *E. coli* strains were detected in the gut of domestic animals in China,[12] indicating that fosfomycin resistance in CTX-M-producing *E. coli* may have occurred early on in a variety of settings.

The most prevalent mechanism of fosfomycin resistance in CTX-M-producing *E. coli* is the horizontally acquired *fosA3*.[11,14,26] As expected, the carriage of *fosA3* was identified at a high rate (62.5%, 5 out of 8) among fosfomycin-resistant CTX-M-producing *E. coli* isolates, and the *fosA3* genes were commonly linked to the $bla_{CTX\text{-}M}$ genes, although the genotypes of CTX-M β-lactamases were diverse (*i.e.*, three $bla_{CTX\text{-}M\text{-}14}$, one $bla_{CTX\text{-}M\text{-}3}$, and one $bla_{CTX\text{-}M\text{-}55}$). These results indicate that

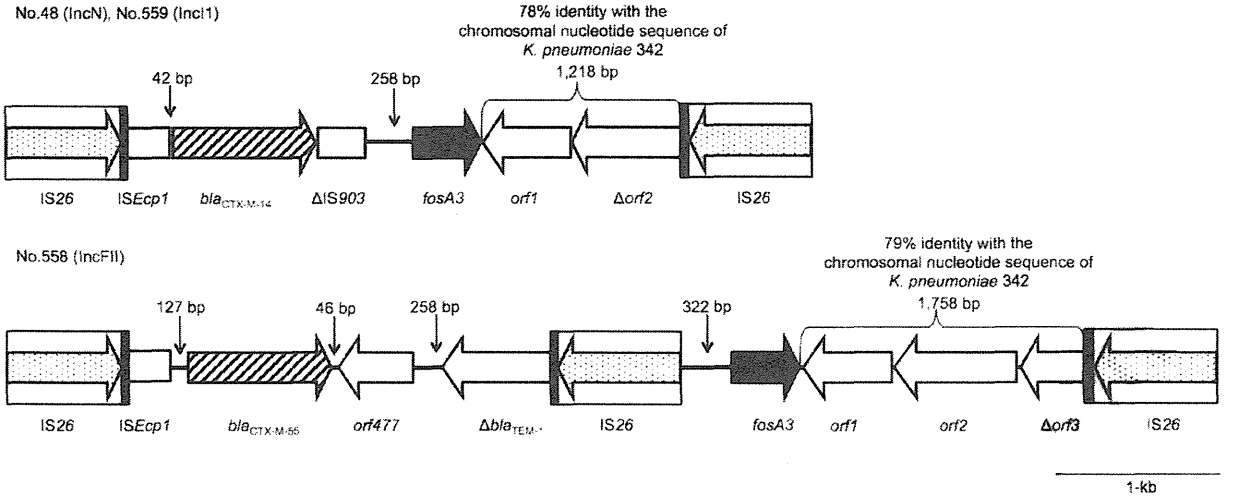

FIG. 1. Schematic representation of the genetic environments carrying the *fosA3* gene.

fosA3-harboring *E. coli* isolates associated with cefotaxime resistance that was related to CTX-M β-lactamase production naturally dwell in the intestinal flora of healthy individuals as well as in clinical and veterinary settings.[13,14,26] As previously reported, the bla_{CTX-M} gene could have widely spread across the members of family *Enterobacteriaceae* in various settings[17,21]; *fosA3* may have, thus, disseminated across these CTX-M-type ESBL producers. However, reports of *fosA3*-harboring CTX-M-type ESBL producers have so far been limited to East Asian countries such as Japan,[26] China,[11] and Korea[14]; these organisms have not yet been reported from other countries in Europe or the United States of America. A worldwide surveillance program is, thus, essential to precisely elucidate the prevalence of acquired fosfomycin-resistance genetic determinants such as that for FosA3.

The resistance determinants of the three *fosA3*-negative fosfomycin-resistant isolates could not be fully characterized in the present study. Fosfomycin resistance is largely influenced by mutations in specific chromosomal loci such as *glpT* and *murA*[23]; these determinants may well be, therefore, partly involved with the resistance phenotype of the three isolates.

We determined the phenotypic and genotypic characteristics of serotypes, MLST types, and plasmid replicon types of five *fosA3*-harboring isolates (Table 2). The serotypes of the five isolates varied and were assigned to different STs. The STs of the *fosA3*-harboring isolates differed from those found in Korea.[14] In addition, the replicon types of the plasmids carrying *fosA3* were also diverse. The *fosA3*-carrying plasmids with the replicon of FII:2 and FII:33 have been earlier identified in fosfomycin-resistant *E. coli* isolates from humans, food animals, and pets.[11–14] These results suggested that *fosA3* was incorporated into plasmids of varying incompatibility groups, namely FII:2 or FII:33, and may have been disseminated across genetically different *E. coli* clones.

As shown by conjugation experiments, *fosA3* could be often co-transferred along with the other antibiotic resistance genes. Currently, the second most common antibiotic resistance gene with genetic linkage to *fosA3* is the aminoglycoside-resistant 16S rRNA methyltransferase gene *rmtB*.[9,13] In this study, the chloramphenicol and florfenicol resistance phenotype, which was conferred by *floR* (chloramphenicol and florfenicol efflux transporter), along with *fosA3*, was transferred in only one of the five *E. coli* isolates. Hereafter, *fosA3* may accumulate in association with a variety of antimicrobial resistance genes in plasmids belonging to different incompatibility groups.

Contrary to the diversity of plasmids carrying *fosA3* and in STs of the parent *E. coli* isolates, the genetic environment of *fosA3* showed common patterns regardless of origin, one of which is that it is flanked by IS*26* elements.[11,13,14] This observation suggests a possibility that *fosA3* is commonly associated with mobile genetic units that are driven by the IS*26* elements distributed among plasmids with varying replicon types and have spread across various settings such as hospitals, communities, and livestock. The IS*26* element would thus be the main vehicle accelerating the spread of small genetic units mediating *fosA3*, because it usually locates upstream and/or downstream of *fosA3*. In fact, IS*26* is genetically combined with a variety of antibiotic resistance genes and this governs its transfer.[20,25,27]

In conclusion, we report the first identification of *E. coli* isolates harboring both *fosA3* and bla_{CTX-M} from healthy individuals. The *fosA3* gene could have already spread across CTX-M-producing *E. coli* in various settings such as the clinics, as well as the veterinary and community areas, and could eventually have contributed to their fosfomycin resistance. Although fosfomycin has been recently reconsidered a potent therapeutic option for infectious diseases such as community-acquired UTIs caused by CTX-M-producing *E. coli*,[6] these bacteria have already been armed with new mechanisms such as *fosA3* to hamper fosfomycin. Indeed, the prevalence of fosfomycin resistance among CTX-M-producing *E. coli* is still less than 10% in most settings at present, but it is of great concerns that *fosA3* would further spread with the increase of fosfomycin consumption in both clinical and veterinary settings, thereby decreasing its efficacy in therapeutics.

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Original Article

Prevalence of MRSA colonization in Japanese neonatal care unit patients in 2011

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Abstract ***Background***: The neonatal intensive care unit (NICU) is a high-risk setting for transmission of methicillin-resistant *Staphylococcus aureus* (MRSA). The recent prevalence of colonization with MRSA in patients and its control measures are unknown in Japanese NICU. We investigated the prevalence of MRSA colonization in patients and measures to control and prevent health-care-associated transmission in Japanese NICU in 2011.

Methods: A nationwide survey was performed in facilities certified as training hospitals for neonatologists. Data in NICU and growing care units (GCU) were collected and analyzed regarding surveillance cultures for MRSA and the proportion of MRSA-colonized patients in September 2011. Trends in the proportion of MRSA-colonized patients and the measures to control and prevent health-care-associated MRSA transmission were investigated in the surveyed NICU in 2000, 2003, and 2011.

Results: A total of 168 NICU and 158 GCU were analyzed. The proportions of NICU and GCU that conducted regular surveillance cultures for MRSA were 81% and 66%, respectively. MRSA colonization was not found in 53% of NICU and in 45% of GCU. The percentage of NICU reported to be free of MRSA colonization increased over time. Use of alcohol-based hand rub and gloves by clinical staff and cohorting for identified MRSA-positive patients became more common in 2011 than in 2000 or 2003.

Conclusions: Approximately half of Japanese NICU did not observe any patients with MRSA colonization in September 2011. Control and prevention measures have changed to use of alcohol-based hand rub and gloves in the last decade.

Key words colonization, control and preventive measure, methicillin-resistant *Staphylococcus aureus*, neonatal intensive care unit, surveillance culture.

Sepsis or infection remains a cause of death, morbidity, or poor neurodevelopmental outcome in newborn infants, particularly in those born before term or sick.[1–3] A recent multi-centre study showed that methicillin-resistant *Staphylococcus aureus* (MRSA) was the most common pathogen in the morbidity and mortality of neonatal sepsis in Japanese neonatal intensive care units (NICU).[3] Because MRSA is mainly transmitted via the hands of health-care personnel,[4–6] it is crucial to control and prevent health-care-associated (HA) MRSA transmission in NICU and growing care units (GCU).

Nationwide data regarding the prevalence of MRSA colonization are important because they may lead to opportunities to re-evaluate control and preventive measures. Recent data on prevalence of MRSA colonization in Japanese NICU or GCU, and recent control and preventive measures for HA transmission, however, have not been reported.

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The Committee for Infection Prevention and Vaccine Promotion of the Japan Society of Premature and Newborn Medicine therefore organized a survey to compare the prevalence of MRSA colonization in September 2011 with that in 2000 and 2003.[7,8] Furthermore, changes in measures to control and prevent HA transmission were also investigated.

Methods

Study design

A survey using a questionnaire was carried out regarding surveillance cultures for MRSA, the proportion of patients with MRSA colonization, and control and preventive measures for HA transmission in NICU (units for level III intensive care) and GCU (units for level II transitional care) in September 2011. We mailed this questionnaire to chief doctors of pediatrics or neonatology in 431 facilities certified by the Japan Society of Perinatal and Neonatal Medicine as training hospitals for neonatologists.[9] This study was approved by the board of directors of the Japan Society of Premature and Newborn Medicine. Data were collected by mail or fax and analyzed.

Collected data

The answers to the following questions were collected in the survey set: (i) "Do you have an NICU or GCU in your hospital?"; (ii) "Is regular or irregular surveillance culture for MRSA monitoring carried out in your NICU or GCU? (regular surveillance, surveillance culture performed once every week or every 2 weeks; irregular surveillance, surveillance culture is performed but not regularly)" "If the answer is yes, what specimen is taken for the surveillance cultures for MRSA in your NICU?"; (iii) "What is the percentage of MRSA-colonized patients in your NICU or GCU in the latest surveillance monitoring?"; (iv) "What measures are taken for the control and prevention of HA-MRSA transmission?"; and (v) "What do you use for hand hygiene of clinical staff?". For question (iv), available options were: glove wearing by clinical staff when caring for patients; cohorting for MRSA-colonized patients; use of mupirocin for MRSA-colonized patients; use of mupirocin for clinical staff with MRSA colonization; a bath with disinfectant for MRSA-colonized patients; disinfection of umbilical cord by povidone iodine; and others; and for question (v), available options were: soap; povidone iodine; benzalkonium chloride; alcohol-based hand rub; and others.

The surveyed hospitals were stratified by main or subtraining hospital based on the criteria of the Japan Society of Perinatal and Neonatal Medicine.[9] The proportion of hospitals without MRSA colonization in NICU patients was compared between the main and sub-training hospitals.

The same surveys on the prevalence of MRSA colonization in NICU patients and measures to control and prevent HA-MRSA transmission had been carried out previously in 2000 and 2003 in Japan.[7,8] Trends were investigated in the surveyed NICU in 2000, 2003, and 2011.

Statistical analysis

Statistical analysis for comparison of the proportion of NICU without MRSA-colonized patients between the main or subtraining hospitals was performed with the chi-squared test using Excel Statistics (Statcel 3; Social Survey Research Information, Tokyo, Japan). Differences were deemed statistically significant for $P < 0.05$.

Results

Responses were received for 168 of the 431 mailed surveys (39%), 10 of which were from institutions that did not have a GCU. The proportions of NICU and GCU that performed regular surveillance cultures for MRSA were 81% and 66%, respectively (Fig. 1). Either regular or irregular surveillance cultures for MRSA were performed in 89% of NICU and in 79% of GCU. Surveillance cultures were taken from multiple sites in 47% of NICU. Nasal swab was most frequently used as a specimen for MRSA surveillance culture (68%; Table 1).

On the latest surveillance monitoring, no patients with MRSA colonization were found in 53% of 150 NICU and in 45% of 125 GCU that responded, respectively (53% of 136 NICU and 46% of 125 GCU that conduct regular surveillance cultures). One hundred and fifty surveyed hospitals with an NICU in 2011 were stratified into 59 main and 91 subtraining hospitals. The proportion of main training hospitals without MRSA-colonized patients was significantly lower than that of subtraining hospitals ($P < 0.05$, Table 2).

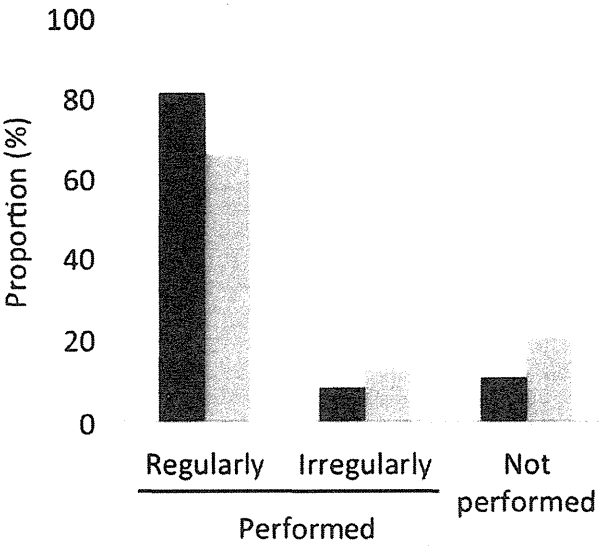

Fig. 1 Proportion of (■) neonatal intensive care units ($n = 168$) and (□) growing care units ($n = 158$) that conducted surveillance cultures for methicillin-resistant *Staphylococcus aureus* in September 2011.

The percentage of NICU reported to be free of MRSA colonization in patients increased over time. The percentage of NICU with MRSA colonization in >25% of patients continuously decreased in this decade (Fig. 2).

The proportion of NICU in which gloves were used by clinical staff when caring for patients and in which cohorting for

Table 1 MRSA surveillance culture specimen in NICU ($n = 150$)

Specimen	% hospitals
Nasal swab	68
Pharynx swab	33
Tracheal aspirate fluid	23
Feces	17
Skin swab	13
Navel swab	5

Surveillance cultures from multiple sites were performed in 47% of NICU. MRSA, methicillin-resistant *Staphylococcus aureus*; NICU, neonatal intensive care units.

Table 2 Hospitals with no MRSA colonization in NICU patients

	Main training hospitals $n = 59$	Subtraining hospitals $n = 91$
No patients with MRSA colonization	22 (37)*	55 (60)

*$P < 0.05$. MRSA, methicillin-resistant *Staphylococcus aureus*; NICU, neonatal intensive care unit.

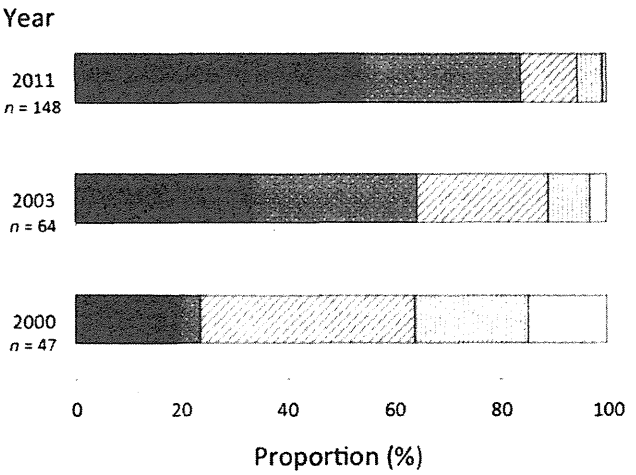

Fig. 2 Trends in the proportion of methicillin-resistant *Staphylococcus aureus*-colonized patients in the surveyed neonatal intensive care units in 2000, 2003, and 2011. (■) 0%; (▩) 1–24%; (▨) 25–49%; (▤) 50–74%; (□) 75–99%; (▣) 100%.

MRSA-colonized patients was performed, increased since 2000. The use of mupirocin to eradicate MRSA for colonized patients, the use of mupirocin to eradicate MRSA for clinical staff carrying MRSA, use of a bath with disinfectant for MRSA-colonized patients, and disinfection of the umbilical cord by povidone iodine decreased in this decade (Fig. 3). Disinfectant agents for hand hygiene of clinical staff changed from povidone iodine to

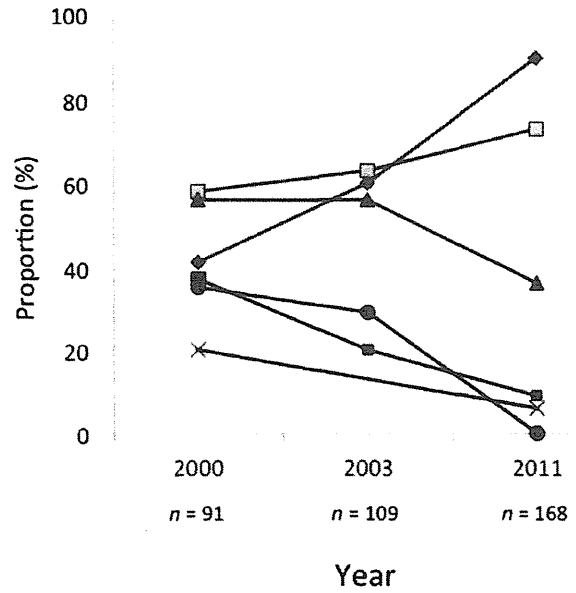

Fig. 3 Changes in measures to control and prevent methicillin-resistant *Staphylococcus aureus* transmission among the surveyed neonatal intensive care units in 2000, 2003, and 2011. (-◆-) Gloves; (-□-) cohorting; (-▲-) mupirocin for patients; (-■-) mupirocin for staff; (-✕-) bath with disinfectant; and (-●-) povidone iodine for umbilical cord.

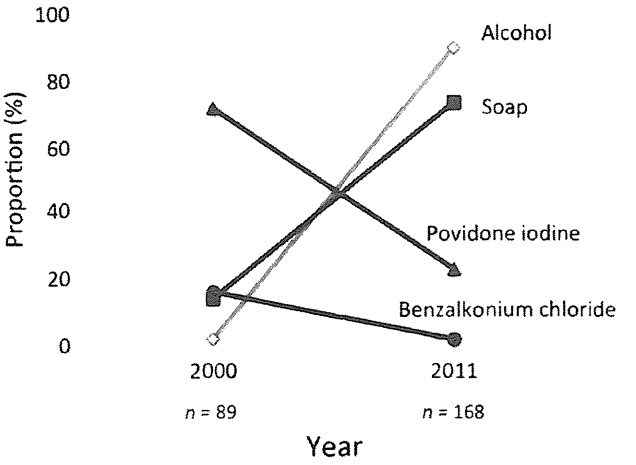

Fig. 4 Changes in disinfectants used for hand hygiene of clinical staff. Disinfectant changed from povidone iodine to alcohol and soap in the last decade.

alcohol-based hand rub and soap in the last decade (Fig. 4). In >85% of NICU in 2011, working clinical staff used an alcohol-based hand rub and wore gloves to control and prevent HA-MRSA transmission (Figs 3,4).

Discussion

We investigated the recent prevalence of MRSA colonization in patients in Japanese NICU and GCU. We found a decrease in the proportion of MRSA-colonized patients in this decade, and approximately half of Japanese NICU and GCU did not observe any patients with MRSA colonization in 2011. We also found changes in control and preventive measures for MRSA colonization in Japanese NICU in the last decade. In most of the NICU in 2011, working clinical staff used an alcohol-based hand rub and wore gloves to control and prevent HA-MRSA transmission.

In the early 2000s, MRSA spread into most NICU in Japan. This was a serious problem because 87% of major Japanese NICU had some patients with MRSA infection in 2000,[7] and the mortality rate due to HA-MRSA infection was 15% in very low-birthweight infants.[5] Furthermore, neonatal toxic shock syndrome-like exanthematous disease caused by toxic shock syndrome toxin-1 from MRSA appeared in Japanese NICU.[10] The present study, however, found that the percentage of MRSA-free NICU increased in this decade. This shows that Japanese NICU and GCU have succeeded in controlling MRSA.

The following measures were recommended from USA and Japan in the early 2000s:[5,11] use of gloves by clinical staff when caring for patients; cohorting for MRSA-colonized patients; and hand hygiene measures using alcohol and soap. They may be more effective for controlling the spread of MRSA and eliminating MRSA in NICU. Hand hygiene protocols and agents dramatically changed since the publication of recommendations from the US Centers for Disease Control and Prevention.[11] We think that these recommendations have contributed to these results.

Although the number of MRSA-colonized patients has decreased in Japanese neonatal care units, it is still concerning

that the proportion of NICU without MRSA colonization in patients in main training hospitals was significantly lower than that in subtraining hospitals (Table 2). The reason for this finding may be because of differences in the number and disease severity of admitted newborn patients. Main training hospitals accept not only many premature and severe sick newborns, but also newborn patients with surgical operations and sick newborns who were born in other hospitals and clinics. Therefore, many medical procedures are performed, including tracheal intubation and insertion of a nasogastric tube or peripherally inserted central catheter. We speculate that these many medical procedures may lead to increased risk for MRSA colonization. Furthermore, many clinical staff are on duty because of the high number of admissions of newborn patients. Bed occupancy and overcrowding are risk factors in the incidence of MRSA transmission.[12] To eliminate the spread of MRSA, we suggest that main training hospitals should have more space and higher clinical staff : patient ratios in their units. A clinical study from a Japanese NICU has also shown that introduction of another precaution reduced the incidence of HA-MRSA transmission: that is, pre-emptive precautions for higher-risk newborn patients (pre-emptive contact precaution: contact precautions were applied to all transferred outborn newborn patients while awaiting the results of active surveillance cultures on admission).[13]

This study has some limitations. First, because the response rate of the survey in 2011 was 40%, the surveyed group might have been biased. Main training hospitals, however, included 128 of 431 facilities (30%) to which we mailed this survey, and 61 (36%) of 168 facilities with NICU responded to this survey. We think that the present results are representative of the current Japanese situation because approximately 40% of very low-birthweight infants who were born in Japan in 2011 was covered in this survey (all responded facilities took care of approximately 3000 very low-birthweight infants). Second, the surveyed NICU in 2000, 2003, or 2011 were not the same; but, because we were able to observe trends in the proportion of MRSA-colonized patients and changes in measures to control and prevent HA-MRSA transmission, this study report should be valuable. Third, we do not know whether all facilities who responded used the same methodology to identify MRSA, given that each method has a different sensitivity and specificity. Finally, the current main control and preventive measures for HA-MRSA transmission should be the contact precaution; that is, that all clinical staff are required to wear a disposable vinyl gown and plastic gloves for all actions that may involve contact with MRSA-colonized patients or potentially contaminated areas in the patient environment.[13] These precautions would have been associated with a downward trend in rates of MRSA colonization in patients since they have been utilized. We recommend that these precautions should be continued until more conclusive investigations are conducted.

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Antimicrobial Agents and Chemotherapy

Novel 6′-*N*-Aminoglycoside Acetyltransferase AAC(6 ′)-Iaj from a Clinical Isolate of Pseudomonas aeruginosa

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Novel 6′-*N*-Aminoglycoside Acetyltransferase AAC(6′)-Iaj from a Clinical Isolate of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **NCGM1588 has a novel chromosomal class 1 integron, In151, which includes the *aac(6′)-Iaj* gene. The encoded protein, AAC(6′)-Iaj, was found to consist of 184 amino acids, with 70% identity to AAC(6′)-Ia. *Escherichia coli* transformed with a plasmid containing the *aac(6′)-Iaj* gene acquired resistance to all aminoglycosides tested except gentamicin. Of note, *aac(6′)-Iaj* contributed to the resistance to arbekacin. Thin-layer chromatography revealed that AAC(6′)-Iaj acetylated all aminoglycosides tested except gentamicin. These findings indicated that AAC(6′)-Iaj is a functional acetyltransferase that modifies the amino groups at the 6′ positions of aminoglycosides and contributes to aminoglycoside resistance of *P. aeruginosa* NCGM1588, including arbekacin.**

The major mechanism of resistance to aminoglycosides is the production of aminoglycoside-modifying enzymes (1). The aminoglycoside 6′-*N*-acetyltransferases [AAC(6′)s] are of particular interest because they can modify a number of clinically important aminoglycosides, including amikacin, gentamicin, netilmicin, and tobramycin. The AAC(6′)-I type confers resistance to amikacin through acetylation of the drug, whereas the AAC(6′)-II type acetylates gentamicin. To date, 43 genes, designated *aac(6′)-Ia* to *aacA43*, which encode AAC(6′)-I enzymes, have been cloned and characterized (1–3). Genes encoding aminoglycoside-modifying enzymes are often located on integrons (4), sequences that can integrate gene cassettes through site-specific recombination (5), in both plasmid and genomic DNA (4). Class 1 integrons participate in multidrug resistance in *Pseudomonas aeruginosa* (6–8).

Pseudomonas aeruginosa is a nosocomial pathogen that exhibits a remarkable ability to acquire resistance to several antibiotics. In Japan, the most serious problem has been the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains, which are defined as having resistance to carbapenems, amikacin, and fluoroquinolones (9, 10).

Previously, we described a nosocomial outbreak caused by an MDR *P. aeruginosa* strain, IMCJ2.S1 (present name, NCGM2.S1) in a hospital in the eastern part of Japan (10). IMCJ2.S1 was found to harbor an aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iae*, in a chromosomal integron (9). A study in Japan in 2008 revealed two MDR *P. aeruginosa* clinical isolates harboring *aac(6′)-Iaf* (11). In 2011, a clinical isolate of MDR *P. aeruginosa* negative for *aac(6′)-Iae* and *aac(6′)-Iaf* was found. The isolate contained a novel aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iaj*. Here, we report the structure of this gene and the properties of its product.

MATERIALS AND METHODS

Bacterial strains and plasmids. A clinical isolate of *P. aeruginosa*, NCGM1588, was obtained from the respiratory tract of a patient in 2011 in a hospital in Osaka, Japan. *P. aeruginosa* IMCJ2.S1, which is a representative strain of a cluster endemic to Japan, was used as a control (10, 12). *Escherichia coli* DH5α (TaKaRa Bio, Shiga, Japan) and *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and for expression of *aac(6′)-Iaj*, respectively. Plasmids pSTV28 and pQE2 were used for cloning of *aac(6′)-Iaj* and purification of recombinant AAC(6′)-Iaj, respectively (11).

Antimicrobial agents. Amikacin (AMK), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (CST), lividomycin A (LIV), piperacillin (PIP), polymyxin B (PMB), sisomicin (SIS), and tobramycin (TOB) were obtained from Sigma-Aldrich (St. Louis, MO), arbekacin (ABK), dibekacin (DIB), and kanamycin A (KAN) were purchased from Meiji Seika Pharma Co. (Tokyo, Japan), aztreonam (ATM) was obtained from Eizai (Tokyo, Japan), cefepime (FEP) was obtained from Bristol-Myers Squibb Co. (New York, NY), gentamicin (GEN) and neomycin B and C mixtures (NEO) were obtained from Nacalai Tesque (Kyoto, Japan), imipenem (IPM) was obtained from Banyu Pharmaceutical Co. (Tokyo, Japan), isepamicin (ISP) was obtained from Nichi-Iko Co. (Toyama, Japan), meropenem (MEM) and netilmicin (NET) were obtained from Sumitomo Pharmaceutical Co. (Osaka, Japan), ofloxacin (OFX) was obtained from LKT laboratories (St. Paul, MN), and piperacillin-tazobactam (TZP) was obtained from Toyama Pure Chemical Industries (Tokyo, Japan).

***In vitro* susceptibility tests.** MICs were determined using a microdilution method according to the protocols recommended by the Clinical and Laboratory Standards Institute (13).

Serotyping. The O serotypes of isolates were determined with a slide agglutination test kit (Denka Seiken Co., Tokyo, Japan) and sequence analysis of serotype-specific genes (14).

MLST. Multilocus sequence typing (MLST) was performed according to the protocols described on the *P. aeruginosa* MLST database website (http://pubmlst.org/paeruginosa/). PCR and sequencing were performed for 7 chromosomal genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*). The nucleotide sequences of these genes were compared with the sequences submitted to the MLST database to determine the allele numbers and sequence types (STs).

PCR amplification of a class 1 integron. Genomic DNA was extracted using a Wizard Genomic DNA purification kit (Promega, Madison, WI). A class 1 integron was detected by PCR using 5′-CS and 3′-CS primers as described previously (9) and genetically mapped using the primers listed

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TABLE 1 PCR primers used in this study

Primer	Sequence[a] (5′ to 3′)	Description
5′-CS	GGCATCCAAGCAGCAAG	5′-end common segment of class 1 integrons
3′-CS	AAGCAGACTTGACCTGA	3′-end common segment of class 1 integrons
intI-F	CTACCTCTCACTAGTGAGGGG	Positions 1–21 in *intI1*
intI-R	TGCGTGTAAATCATCGTCGT	Positions 196–177 in *intI1*
aac(6′)-Iaj-41F	ATCAGATCGATGCTGCAAGAATTC	Positions 41–64 in *aac(6′)-Iaj*
aac(6′)Iaj-543R	ACTTTTCCACATCCAAATATCGGG	Positions 543–520 in *aac(6′)-Iaj*
qacEdelta-F	TGAAAGGCTGGCTTTTTCTT	Positions 2–21 in *qacEΔ1*
qacEdelta-R	GCAATTATGAGCCCCATACC	Positions 268–287 in *qacEΔ1*
sul1-R	GGGTTTCCGAGAAGGTGATT	Positions 768–787 in *sul1*
sul1-F	TCACCGAGGACTCCTTCTTC	Positions 29–48 in *sul1*
*IS*6100-R	GGCTCTGTTGCAAAGATTGGC	Sequence 34–54 downstream of IS*6100*
PstI-aac-F	aactgcagGGCTTGTTATGACTGTTTTT	Sequence in the 180- to 161-bp upstream region of *aac(6′)-Iaj* with PstI site
SalI-aac-R	ggtcgacTCAATTGAGTAGACTTTTCCAC	Positions 555–534 in *aac(6′)-Iaj* with SalI
SphI-aac-F	ccgcatgcgATGGAATATTCAATTATCAAT	Positions 1–21 in *aac(6′)-Iaj* with SphI
NotI-aac-R	gggcggccgcTCAATTGAGTAGACTTTTCC	Positions 555–536 in *aac(6′)-Iaj* with NotI
23S-rRNA-F	CGAGGACAGTGTATGGTGGGCAGT	Positions 2207–2231 in 23S rRNA gene
23S-rRNA-R	CTCAACGCCTCACAACGCTTACACA	Positions 2856–2832 in 23S rRNA gene

[a] Lowercase letters represent restriction enzyme recognition sites attached on the 5′ ends of primers.

in Table 1. The Expand High-Fidelity PCR system (Roche Diagnostics GmbH, Penzberg, Germany) was used for PCR amplification. All PCR products were sequenced to identify genes and their orders in the integron.

DNA sequencing. DNA sequences were determined using an ABI PRISM3130 sequencer (Applied Biosystems, Foster City, CA). Homology searches of nucleotide and translated protein sequences were performed using BLAST. Multiple-sequence alignments, searches for open reading frames (ORFs), and dendrograms for AACs were performed using Genetyx software (Genetyx, Tokyo, Japan).

PFGE and Southern hybridization. DNA plugs were prepared as described previously (5) and digested overnight at 37°C with SpeI (TaKaRa Bio) or I-CeuI (New England BioLabs, Ipswich, MA). Pulsed-field gel electrophoresis (PFGE) analysis was performed as described previously (9). Southern hybridization was performed using an enhanced chemiluminescence direct nucleic acid-labeling and detection system according to the manufacturer's instructions (GE Healthcare, Tokyo, Japan), as described previously (11), to determine whether the novel class 1 integron identified in the *P. aeruginosa* isolates has a chromosomal location. Probes for *aac(6′)-Iaj* and 23S rRNA genes from NCGM1588 were amplified by PCR using the primer sets aac(6′)-Iaj-41F/aac(6′)Iaj-543R and 23S-rRNA-F/23S-rRNA-R, respectively (Table 1).

Cloning of *aac(6′)-Iaj* gene. The ORF of *aac(6′)-Iaj* and 180 bp of the upstream region of the gene, including the promoter, were amplified by PCR from *P. aeruginosa* NCGM1558 using the primer set PstI-aac-F and SalI-aac-R (Table 1). The PCR products were digested with PstI and SalI and ligated into the PstI and SalI sites of pSTV28. The plasmids were used to transform DH5α, and transformants were selected on LB agar containing 100 μg/ml of chloramphenicol. To determine the MICs of aminoglycosides, *E. coli* DH5α was transformed with pSTV28-*aac(6′)-Iaj*.

Purification of recombinant AAC(6′)-Iaj. The *aac(6′)-Iaj* gene from *P. aeruginosa* NCGM1588 was amplified by PCR using the primer set SphI-aac-F and NotI-aac-R (Table 1), and the product was digested with SphI and NotI and ligated into pQE2 (Invitrogen, Carlsbad, CA), which had been digested with the same restriction enzymes. The plasmid was used to transform DH5α, and the transformants were selected on LB agar containing 100 μg/ml of ampicillin. The resulting plasmid, pQE-*aac(6′)-Iaj*, was used to transform BL21-CodonPlus (DE3)-RIP (Agilent Technologies), which was used for recombinant protein purification. BL21-CodonPlus (DE3)-RIP carrying plasmid pQE2-*aac(6′)-Iaj* was grown in LB medium containing 200 μg/ml ampicillin at 37°C until the A_{600} reached 0.3. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a concentration of 0.1 mM to induce expression of AAC(6′)-Iaj, and the culture was incubated for 4 h at 37°C. The soluble fraction of six-histidine-tagged AAC(6′)-Iaj was obtained from the bacterial cells lysed by sonication in buffer A (20 mM Tris, 300 mM NaCl, and 10 mM imidazole, pH 8.0). The AAC(6′)-Iaj was purified from the soluble fraction using Ni-NTA agarose according to the manufacturer's instruction (Qiagen, Tokyo, Japan).

TLC analysis of acetylated aminoglycosides. Mixtures containing 2 mM aminoglycoside, 2 mM acetyl coenzyme A (acetyl-CoA), and 50 μg/ml AAC(6′)-Iaj in 20 μl of phosphate buffer (pH 7.4) were incubated for 16 h at 37°C. Aliquots of 3 μl of each aminoglycoside mixture were spotted onto the surface of a Silica Gel 60 thin-layer chromatography (TLC) plate containing a fluorescence indicator with an excitation wavelength of 254 nm (Merck, Darmstadt, Germany), and the results were developed with a 5% phosphate potassium solution. The aminoglycosides and their acetylated products were detected with 0.2% ninhydrin in acetone.

Nucleotide sequence accession number. The nucleotide sequence of In151 determined in this study has been deposited in the EMBL and GenBank databases and the DDBJ and assigned the accession number AB709942.

RESULTS AND DISCUSSION

Characterization of *P. aeruginosa* NCGM1588. The MICs of antibiotics for NCGM1588 were as follows: PIP, 32 μg/ml; TZP, 32 μg/ml; CAZ, 8 μg/ml; FEP, 16 μg/ml; IPM, 32 μg/ml; MEM, 16 μg/ml; ATM, 32 μg/ml; AMK, 128 μg/ml; ABK, 32 μg/ml; GEN, 8 μg/ml; CIP, 16 μg/ml; OFX, 32 μg/ml; PMB, 4 μg/ml; and CST, 4 μg/ml. NCGM1588 showed high levels of AMK resistance. In particular, it showed high levels of ABK resistance, whereas the representative epidemic strain of MDR *P. aeruginosa* IMCJ2.S1 in Japan was susceptible to ABK (9, 15). The serotype of NCGM1588 was O7, and the MLST was ST560. NCGM1588 showed different PFGE patterns from that of MDR *P. aeruginosa* IMCJ2.S1, with similarity of 46.2%.

P. aeruginosa NCGM1588 is an emerging MDR pathogen in Japan. Therefore, it is necessary to carefully investigate whether the NCGM1588 will expand in medical settings. NCGM1588 seems to be quite different from the epidemic strain of MDR *P. aeruginosa* IMCJ2.S1, which is widespread in Japan (9, 10, 12), because of different PFGE patterns, MLSTs (ST560 versus ST235), and serotypes (O7 versus O11). IMCJ2.S1 causes mainly urinary

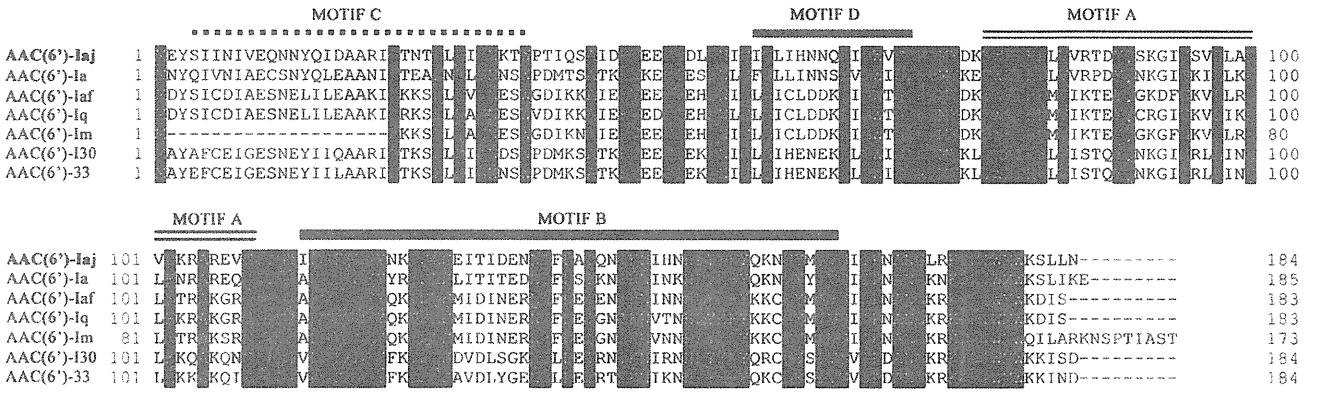

FIG 1 Alignment of the AAC(6′)-Iaj amino acid sequence with those of six members of AAC(6′)-I subfamily. Identical residues are marked with black boxes. Four motifs, C, D, A, and B, are indicated by a dotted line, a gray line, double lines, and a black line, respectively.

tract infections (9), whereas NCGM1588 caused respiratory infection. During our surveillance from 2009 to 2010, we found 16 isolates of MDR *P. aeruginosa*, including a novel MDR *P. aeruginosa* strain, NCGM1179 (16), which had identical PFGE patterns and were ST991 and serotype O18; all of these isolates were isolated from the respiratory tract (17). To date, 14 strains of *P. aeruginosa* showing ST560, including NCGM1588, have been reported—8 in Australia, 3 in China, 1 in the Netherlands, and 1 in Spain (http://pubmlst.org/paeruginosa/). This is the first report of the isolation of *P. aeruginosa* showing ST560 in Japan.

***aac(6′)-Iaj* in the class 1 integron.** To identify the drug resistance genes of NCGM1588, the variable regions of class 1 integrons were amplified with the primers 5′-CS and 3′-CS (Table 1). PCR products of 1.1 kb were generated from this strain. DNA sequence analysis revealed a variable region containing a cassette of a novel *aac(6′)* gene. Based on the standard nomenclature (18), we named this ORF *aac(6′)-Iaj*. The novel gene consisted of an ORF of 555 bp, and its sequence showed 70% identity to that of *aac(6′)-Ia* from *Corynebacterium resistens* (accession number FN825254) (19). The *aac(6′)-Iaj* gene had a G+C content of 31.2%.

We designated the gene *aac(6′)-Iaj* according to a system of nomenclature proposed by Shaw et al. (1), which is easy to understand and indicates the functional properties of the enzymes in a straightforward manner as follows: numbers in parentheses, e.g., (1), (2), (3), and (6′), etc., for the site of modification; roman numerals, e.g., I, II, IV, etc., for unique resistance profiles; and lowercase letters, e.g., a, b, c, etc., for unique protein designations (1, 20).

The structure of the class 1 integron harboring *aac(6′)-Iaj* was determined using external primers (Table 1). The sequence of the integron was not found in any database; therefore, it was named In151. In151 had a structure similar to that of In4 integron (accession number U12338) except for the gene cassette array (21). Between the 5′-CS and 3′-CS, In151 had one gene cassette that contained *aac(6′)-Iaj* and a 60-nt 59-base element, which is known as *attC*, located 11 bp downstream of *aac(6′)-Iaj* (22).

In151 could be derived from the same origin as In4 of *P. aeruginosa* plasmid R1033. In4 was found in plasmid R1033 of a *P. aeruginosa* strain isolated in 1975 (23). The In151 backbone differed from that of In4 by the presence of a partial copy of IS*6100*; i.e., In151 had the 5′-CS, 3′-CS, and a complete copy of IS*6100* located downstream of the 3′-CS.

Amino acid sequence of AAC(6′)-Iaj enzyme. AAC(6′)-Iaj consists of 184 amino acids. Multiple sequence alignments among AAC(6′) enzymes revealed that AAC(6′)-Iaj had 70% identity to AAC(6′)-Ia from *Shigella sonnei* (24), 66% to AAC(6′)-Iaf from *P. aeruginosa* (11), 65% to AAC(6′)-Iq from *Klebsiella pneumoniae* (25), 64% to AAC(6′)-Im from *Citrobacter freundii* (26), 63% to AAC(6′)-33 from *P. aeruginosa* (27), and 63% to AAC(6′)-I30 from *Salmonella enterica* (28). Based on the work of Neuwald and Landsman (29), four motifs in the amino acid sequences of the subfamily proteins belonging to AAC(6′)-Iaj were designated motifs C, D, A, and B (Fig. 1). Comparison of amino acid sequences of members of the AAC(6′)-I subfamily with that of AAC(6′)-Iaj revealed that motifs C, D, A, and B, which are found in most

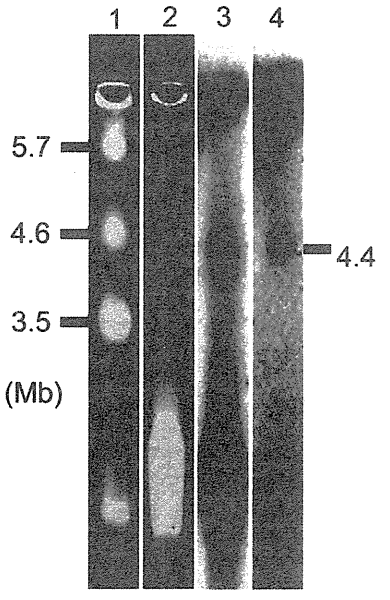

FIG 2 Localization of the *aac(6′)-Iaj* gene on I-CeuI-digested total DNA of *P. aeruginosa* strain NCGM1588 separated by PFGE. *P. aeruginosa* NCGM1588 genomic DNA digested by I-CeuI was done as previously described (11). Lane 1, molecular standard of *Schizosaccharomyces pombe* chromosomal DNA; lane 2, I-CeuI-digested total DNA of *P. aeruginosa* strain NCGM1588 with ethidium bromide; lane 3, Southern hybridization was performed with probes for 23S rRNA gene; lane 4, Southern hybridization was performed with probes for *aac(6′)-Iaj*.

TABLE 2 MICs of various aminoglycosides for *P. aeruginosa* NCGM1588 and *E. coli* strains transformed with *aac(6′)-Iaj*

Strain[a]	MIC[b] (μg/ml)									
	AMK	ABK	DIB	GEN	ISP	KAN	NEO	NET	SIS	TOB
NCGM1588	128	32	1,024	8	512	1,024	256	>1,024	1,024	128
E. coli DH5α/pSTV28	0.5	0.5	0.5	0.5	0.5	0.5	2	0.25	1	0.25
E. coli DH5α/pSTV28-*aac(6′)-Iaj*	16	4	16	0.5	4	32	8	32	4	16

[a] The MICs for *E. coli* strains were determined with Mueller-Hinton broth preparations containing chloramphenicol (30 μg/ml) and individual aminoglycosides.
[b] AMK, amikacin; ABK, arbekacin; DIB, dibekacin; GEN, gentamicin; ISP, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin.

GCN5-related *N*-acetyltransferases (GNATs) (29, 30), were conserved in AAC(6′)-Iaj (Fig. 1). A large motif at the C terminus, motif B (30), was 77.6% identical between AAC(6′)-Ia (24) and AAC(6′)-Iaj.

Localization of the *aac(6′)-Iaj* gene. PFGE analysis and Southern hybridization using NCGM1588 genomic DNA digested by I-CeuI revealed that the probes specific for the 23S rRNA and *aac(6′)-Iaj* were detected in a chromosomal fragment of about 4.4 Mb (Fig. 2). These results indicate that *aac(6′)-Iaj* was located on the chromosomal DNA. Lower DNA bands were observed in lanes 2 to 4 of Fig. 2. They were probably due to nonspecific cleavage during DNA preparation and enzyme digestion. However, we cannot exclude the possibility that NCGM1588 has multiple copies of *aac(6′)-Iaj*.

Drug resistance mediated by AAC(6′)-Iaj enzyme. *P. aeruginosa* NCGM1588 was resistant to all aminoglycosides tested except GEN (Table 2). A vector control of *E. coli* DH5α/pSTV28 was susceptible to all aminoglycosides tested, whereas *E. coli* DH5α/pSTV28-*aac(6′)-Iaj* was resistant to all aminoglycosides, including ABK, except GEN, with 4- to 128-fold-higher MIC values than those of the vector control (Table 2). The MIC of GEN in *E. coli* DH5α/pSTV28-*aac(6′)-Iaj* was the same as that in the vector control.

To examine the acetylase activity of AAC(6′)-Iaj to aminoglycosides, we performed thin-layer chromatography using the purified recombinant AAC(6′)-Iaj. LIV, an aminoglycoside compound, was used as a negative control. LIV has a hydroxyl group instead of an amino group at the 6′ position and therefore cannot be acetylated by AAC(6′). As shown in Fig. 3, all of these aminoglycosides, except GEN, were acetylated by AAC(6′)-Iaj. The acetylation rates were only 2% for GEN when estimated with the ImageJ analyzer (http://rsbweb.nih.gov/ij/index.html). The TLC data for GEN were consistent with the MICs of GEN for *E. coli* DH5α/pSTV28-*aac(6′)-Iaj* and *E. coli* DH5α/pSTV28 (Table 2). The reason for the incomplete acetylation is that commercially available gentamicin is a mixture of its derivatives; some of them have a methyl group on N-6′ and are refractory to AAC(6′)-I enzymes (1).

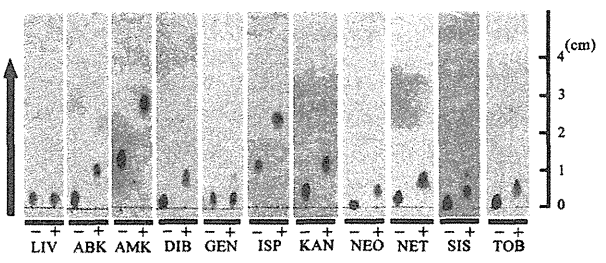

FIG 3 Analysis of acetylated aminoglycosides by TLC. AAC(6′)-Iaj and various aminoglycosides were incubated in the absence (−) or presence (+) of acetyl coenzyme A. The arrow indicates the direction of development.

AAC(6′)-Iaj-producing *P. aeruginosa* NCGM1588 was more resistant to ABK (MIC, 32 μg/ml) than AAC(6′)-Iae-producing IMCJ2.S1 (MIC, 2 μg/ml) (see Table 2 in reference 9), a representative epidemic MDR *P. aeruginosa* strain in Japan, indicating that AAC(6′)-Iaj could inactivate ABK more effectively than AAC(6′)-Iae. *E. coli* DH5α producing AAC(6′)-Iaj was relatively resistant to ABK compared to *E. coli* DH5α producing AAC(6′)-Iae (compare Table 2 in this paper and Table 3 in reference 9). As demonstrated by TCL analyses, both AAC(6′)-Iaj and AAC(6′)-Iae catalyzed inactivation of ABK (compare Fig. 3 in this paper and Fig. 6 in reference 9). The enzymatic activity of AAC(6′)-Iaj against ABK may be stronger than that of AAC(6′)-Iae, although further kinetic studies of both enzymes and chemical analysis of the products of acetylation by both enzymes will be necessary. The chemical structures of AMK and ABK are nearly identical, with only a few differences at the 2′, 3′, and 4′ positions in ring I; that is, AMK has 2′-, 3′-, and 4′-hydroxyl groups, whereas ABK has a 2′-amino group (31). The different substitutions at the 2′, 3′, and 4′ positions in ring I would be responsible for the different levels of ABK resistance between NCGM1179 and IMCJ2.S1.

ACKNOWLEDGMENTS

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NDM-8 Metallo-β-Lactamase in a Multidrug-Resistant *Escherichia coli* Strain Isolated in Nepal

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A novel metallo-β-lactamase, NDM-8, was identified in a multidrug-resistant *Escherichia coli* isolate, IOMTU11 (NCGM37), obtained from the respiratory tract of a patient in Nepal. The amino acid sequence of NDM-8 has substitutions at positions 130 (Asp to Gly) and 154 (Met to Leu) compared with NDM-1. NDM-8 showed enzymatic activities against β-lactams similar to those of NDM-1.

Metallo-β-lactamases (MBLs) produced by Gram-negative bacteria confer resistance to all β-lactams except monobactams (1). New Delhi metallo-β-lactamase-1 (NDM-1), a recently discovered MBL, was initially isolated from *Klebsiella pneumoniae* and *Escherichia coli* in 2008 in Sweden (2). Since then, NDM-1-producing members of the *Enterobacteriaceae* have been isolated in various parts of the world, including Australia, Bangladesh, Belgium, Canada, France, India, Japan, Kenya, the Netherlands, New Zealand, Pakistan, Singapore, Taiwan, and the United States (3, 4). In addition, isolates producing six NDM variants have been reported, including NDM-2-producing *Acinetobacter baumannii* strains from Egypt (5, 6), Israel (5), Germany (7), and the United Arab Emirates (8); an NDM-3-producing *E. coli* strain from Australia (accession no. JQ734687); an NDM-4-producing *E. coli* strain from India (9); an NDM-5-producing *E. coli* strain from the United Kingdom (10); an NDM-6-producing *E. coli* strain from New Zealand (11); and an NDM-7-producing *E. coli* strain from Canada (accession no. JX262694).

E. coli IOMTU11 (NCGM37) and *Pseudomonas aeruginosa* IOMTU9 (NCGM1841) were isolated from pus from a surgical site and from sputum of patients, respectively, in 2012 at Tribhuvan University Teaching Hospital in Kathmandu, Nepal. The isolates were phenotypically identified, and species identification was confirmed by 16S rRNA sequencing (12). MICs were determined using the microdilution method recommended by the Clinical and Laboratory Standards Institute (13). *E. coli* IOMTU11 was resistant to all antibiotics tested excepted fosfomycin (MIC, 4 μg/ml). The MICs of β-lactams are shown in Table 1, and those of other antibiotics were as follows: arbekacin, >1,024 μg/ml; amikacin, >1,024 μg/ml; colistin, 0.25 μg/ml; gentamicin, >1,024 μg/ml; and tigecycline, 0.5 μg/ml. MBL production was examined with an MBL Etest (Sysmex; bioMérieux Co., Marcy l'Etoile, France), with MICs of 256 μg/ml of imipenem and 2 μg/ml of imipenem-EDTA. PCR analysis for MBL genes (14, 15, 16) and 16S rRNA methylase genes (17) was performed. The isolates were positive for bla_{NDM} and *rmtB*. Sequence analysis showed that the *bla*NDM was a novel variant, and it was designated bla_{NDM-8}. Multilocus sequence typing (MLST) of IOMTU11 showed that it was ST101 (*Escherichia coli* MLST database [http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html]). *P. aeruginosa* IOMTU9 had bla_{NDM-1}, which was used as a reference gene.

The sequence of the bla_{NDM-8} gene showed mutations corresponding to two amino acid substitutions compared with bla_{NDM-1} (accession number JF798502). Analysis of the predicted amino acid sequence revealed two substitutions (D130G and M154L) compared with NDM-1, one substitution (D130G) compared with NDM-4, and one substitution (L88V) compared with NDM-5.

The bla_{NDM-8} and bla_{NDM-1} genes were cloned into the corresponding sites of pHSG398 (TaKaRa Bio, Shiga, Japan) with the primer set EcoRI-NDM-F (5′-GGGAATTCATGGAATTGCCCA ATATTATG-3′) and PstI-NDM-R (5′-AACTGCAGTCAGCGC AGCTTGTCGGCCAT-3′). *E. coli* DH5α was transformed with pHSG398-NDM-8 or pHSG398-NDM-1 to determine the MICs of β-lactams.

The open reading frames of NDM-1 and NDM-8 without signal peptide regions were cloned into the expression vector pQE2 (Qiagen, Tokyo, Japan) with the primer set SacI-NDM-F (5′-CCCC TCGAGCAGCAAATGGAAACTGGCGACCAACGGT-3′) and SalI-NDM-R (5′-CCCGAGCTCTCAGCGCAGCTTGTCGGCC ATGCGGGCC-3′). The plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA). The recombinant NDM proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose according to the manufacturer's instruction (Qiagen). His tags were removed by digestion with DAPase (Qiagen), and untagged proteins were purified by an additional passage over Ni-NTA agarose. The purities of NDM-1 and NDM-8 were over 90%, as estimated by SDS-PAGE. During the purification procedure, the presence of β-lactamase activity was monitored with nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). Initial hydrolysis rates were determined in 50 mM phosphate buffer (pH 7.0) at 25°C with a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m and k_{cat} values and the k_{cat}/K_m ratio were determined by analyzing β-lactam hydrolysis by use of the Lineweaver-Burk plot. Wavelengths and extinc-

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TABLE 1 MICs of various β-lactams for *E. coli* strain IMOTU11 and *E. coli* strains transformed with NDM-1 or NDM-8

Antibiotic	MIC (μg/ml)			
	IOMTU11	pHSG398/NDM-8	pHSG398/NDM-1	pHSG398
Ampicillin	>1,024	256	256	4
Ampicillin-sulbactam	>1,024	128	128	2
Aztreonam	>1,024	0.03	0.03	0.03
Cefepime	1,024	0.5	0.5	<0.25
Cefmetazole	ND[a]	4	2	1
Cefoselis	ND	8	4	<0.25
Cefotaxime	>1,024	8	8	<0.25
Cefoxitin	>1,024	64	64	8
Cefozopran	ND	8	8	<0.25
Cefpirome	ND	2	1	<0.25
Cefsulodin	ND	>512	>512	256
Ceftazidime	>1,024	256	256	<0.25
Ceftriaxone	ND	16	32	<0.25
Cefuroxime	ND	512	512	4
Cephradine	>1,024	512	256	16
Doripenem	ND	0.125	0.06	0.03
Imipenem	256	0.5	0.25	0.06
Meropenem	256	0.25	0.5	0.03
Moxalactam	ND	16	8	<0.25
Panipenem	ND	0.5	0.25	0.06
Penicillin G	>1,024	256	256	32
Piperacillin	>1,024	16	16	2
Piperacillin-tazobactam	>1,024	8	8	1
Ticarcillin	>1,024	>512	>512	2
Ticarcillin-clavulanic acid	512	512	512	4

[a] ND, not determined.

tion coefficients for β-lactam substrates have been reported elsewhere (18, 19, 20).

Expression of the bla_{NDM-8} and bla_{NDM-1} genes in *E. coli* DH5α conferred resistance or reduced susceptibility to all cephalosporins, moxalactam, and carbapenems (Table 1). The MICs of cefmetazole, cefoselis, cefpirome, doripenem, imipenem, panipenem, and moxalactam were one dilution higher for the *E. coli* strain expressing NDM-8 than for that expressing NDM-1. In contrast, those of ceftriaxone and meropenem were one dilution lower for the NDM-8-expressing strain than for the NDM-1-expressing strain.

As shown in Table 2, recombinant NDM-8 and NDM-1 hydrolyzed all β-lactams tested except aztreonam. The profile of enzymatic activities of NDM-8 against β-lactams was similar to that of NDM-1, although NDM-8 had slightly lower k_{cat}/K_m ratios for penicillin G, ampicillin, cephradine, cefotaxime, and meropenem than NDM-1.

Two amino acid substitutions at positions 88 and 130 slightly affected the enzymatic activities of NDM-8 compared to those of NDM-1 (Table 2). Among all eight NDM variants, amino acid substitutions were found at 6 positions (i.e., positions 28, 88, 95, 130, 154, and 233). It is not yet known which position(s) plays a critical role in the enzymatic activities. 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 (21, 22, 23, 24). Residues 88 and 130, however, were not lo-

TABLE 2 Kinetic parameters of NDM-8 and NDM-1[a]

β-Lactam	NDM-8			NDM-1		
	K_m (μM)[b]	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)[b]	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
Penicillin G	74 ± 10	91 ± 3	1.20	29 ± 2	79 ± 1	2.70
Ampicillin	193 ± 6	158 ± 5	0.82	122 ± 12	137 ± 5	1.10
Cephradine	52 ± 7	52 ± 4	1.00	37 ± 4	63 ± 1	1.70
Cefoxitin	34 ± 1	3 ± 0.1	0.10	25 ± 6	4 ± 0.3	0.05
Cefotaxime	30 ± 6	38 ± 3	1.30	28 ± 4	45 ± 1	1.70
Ceftazidime	63 ± 3	12 ± 0.2	0.20	74 ± 9	32 ± 2	0.45
Cefepime	153 ± 13	25 ± 1	0.17	152 ± 31	33 ± 5	0.22
Aztreonam	NH[c]	NH	NH	NH	NH	NH
Imipenem	167 ± 8	46 ± 2	0.28	194 ± 38	60 ± 7	0.31
Meropenem	127 ± 20	169 ± 12	1.30	54 ± 10	66 ± 3	1.20

[a] The proteins were initially modified by a His tag, which was removed after purification.
[b] Values are means from three independent experiments ± standard deviations.
[c] NH, no hydrolysis was detected under conditions with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

cated in these loops. These residues may indirectly affect the formation of the active site. NDM-1 may not bind to the carbapenems as tightly as IMP-1 or VIM-2, and it turns over the carbapenems at a rate similar to that of VIM-2 (2). NDM-4 possessed increased hydrolytic activity for carbapenems and several cephalosporins compared to NDM-1 (9). NDM-4 with an amino acid substitution at position 130 (Met to Leu) showed increased hydrolytic activity for carbapenems and several cephalosporins compared to NDM-1 (9). NDM-5 with substitutions at positions 88 (Val to Leu) and 154 (Met to Leu) reduced the susceptibility of *E. coli* transformants to cephalosporins and carbapenems (9). The drug susceptibilities of *E. coli* transformants with bla_{NDM}-2, bla_{NDM-3}, bla_{NDM-6}, and bla_{NDM-7} have not yet been reported. NDM must have only recently started to evolve, and therefore careful monitoring of NDM-producing pathogens is required.

bla_{NDM-8} was found in a plasmid of >100 kb (data not shown). The plasmid was sequenced by using the GS Junior system (Roche Diagnostics K.K., Tokyo, Japan). The sequence surrounding bla_{NDM-8} was *tra*-bla_{NDM-8}-*ble-trpF-tat*, and the genetic environment of bla_{NDM-8} had more than 99.9% identity at the nucleotide sequence from position 4564 to 8780 bp of *K. pneumoniae* strain GN529 (accession no. HQ416416), which was isolated in Ontario, Canada.

This is the first report describing NDM-1- and NDM-8-producing Gram-negative pathogens in Nepal.

Nucleotide sequence accession number. bla_{NDM-8} has been deposited in GenBank with the accession number AB744718.

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RESEARCH ARTICLE Open Access

Emergence of 16S rRNA methylase-producing *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates in hospitals in Vietnam

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Abstract

Background: 16S rRNA methylase-producing Gram-negative bacteria are highly resistant to all clinically important aminoglycosides. We analyzed clinical strains of 16S rRNA methylase-producing *Acinetobactor baumannii* and *Pseudomonas aeruginosa* obtained from clinical isolates in medical settings in Vietnam.

Methods: From 2008 to 2011, 101 clinical strains of *A. baumannii* and 15 of *P. aeruginosa* were isolated from patients in intensive care units (ICUs) in two medical settings in Vietnam. Antimicrobial susceptibilities were determined using the microdilution method and epidemiological analysis was performed by pulsed-field gel electrophoresis and MLST. Genes encoding the 16S rRNA methylases, OXAs and CTX-Ms were analyzed by PCR and sequence analysis.

Results: 16S rRNA methylase-producing Gram-negative pathogens were detected in two hospitals in Vietnam. Of the 101 clinical isolates of *A. baumannii* and the 15 of *P. aeruginosa* isolated from two ICUs in these hospitals, 72 (71.3%) were highly resistant to amikacin, arbekacin and gentamicin, with MICs greater than 1,024 mg/L. The 16S rRNA methylases ArmA and RmtB were produced by 61 and 9 isolates of *A. baumannii*, respectively, and RmtB was produced by 2 isolates of *P. aeruginosa*. Moreover, 52 of the *A. baumannii* isolates producing 16S rRNA methylases harbored both *bla*OXA-23-like and *bla*OXA-51-like genes. Most *A. baumannii* isolates producing 16S rRNA methylase obtained in hospital A in Hanoi were ST91 and ST231, whereas most from hospital B in Ho Chi Minh City were ST136, ST195, and ST254.
The two *P. aeruginosa* isolates harboring *rmtB* showed different patterns on PFGE, one each corresponding to ST217 and ST313.

Conclusions: Gram-negative bacteria producing the 16S rRNA methylases ArmA and RmtB are emerging in medical settings in Vietnam. *A. baumannii* isolates in northern and southern regions of Vietnam may be of different lineages.

Keywords: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, Aminoglycoside resistance, Intensive care unit, 16S rRNA methylase

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Background

Aminoglycosides widely used to treat infectious diseases caused by Gram-negative bacteria have a high affinity for the 16S rRNA of the bacterial 30S ribosome and block protein synthesis [1]. Enzymatic modification [1] and the methylation of 16S rRNA makes these bacteria highly resistant to all clinically important aminoglycosides [2]. In 2003, clinical isolates of highly aminoglycoside-resistant Gram-negative bacteria producing16S rRNA methylase were identified in France [3] and Japan [4]. Since then, 16S rRNA methylase-producing Gram-negative bacteria have been isolated in other parts of the world, including Asian countries such as Afghanistan, Bangladesh, China, Hong Kong, India, Japan, Korea, Oman and Pakistan [5]. To date, however, there have been no reports of these isolates from South-East Asian countries, including Vietnam.

Since 2003, eight plasmid-associated 16S rRNA methylase genes, *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF* and *npmA*, have been identified in clinical and veterinary isolates from various geographic areas, including East Asia, Europe and the Americas, since 2003 [5,6].

Methods

Bacterial strains

From 2008 to 2011, 50 clinical strains of *A. baumannii* and 15 of *P. aeruginosa* were isolated from patients in an ICU in hospital A in Hanoi, Vietnam; and 51 strains of *A. baumannii* were isolated from patients in an ICU in hospital B in Ho Chi Minh City, Vietnam. Of the 101 *A. baumannii* strains isolated, 98 were from patients' respiratory tracts and 3 from blood. Of the 15 *P. aeruginosa* strains, 14 were from respiratory tracts and 1 from pus. Most patients were on ventilators, and the samples were mostly aspirates from ventilation tubes. All clinical isolates used in this study were obtained during standard patient care.

Antimicrobial susceptibility and pulsed-field gel electrophoresis (PFGE)

MICs of all bacteria to amikacin (Sigma-Aldrich, St. Louis, MO), arbekacin (Meiji Seika Pharma Co., Tokyo, Japan), aztreonam (Eizai, Tokyo, Japan), ceftadizime (Sigma-Aldrich), ciprofloxacin (Daiichi Pharmaceutical Co, Tokyo, Japan), colistin (Sigma-Aldrich), gentamicin (Nacalai Tesque, Kyoto, Japan), imipenem (Banyu Pharmaceutical Co, Tokyo, Japan), meropenem (Sumitomo Pharmaceutical Co., Osaka, Japan), piperacillin (Sigma-Aldrich) and pipiracillin/tazobactam (Toyama Chemical Co., Tokyo, Japan) were determined using the microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (M07-A9). *A. baumannii* DNA was digested with the restriction enzyme *ApaI* and *P. aeruginosa* DNA was digested with *SpeI*, followed by pulsed-field gel electrophoresis (PFGE). PFGE analysis was performed as described previously [7]. Fingerprinting patterns were analyzed by the unweighted-pair-group method using Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories, Hercules, CA, USA) to create an average linkage-based dendrogram.

Multilocus sequence typing (MLST)

MLST of 16S rRNA methylase-producing pathogens was performed according to the protocols described on the *A. baumannii* (http://pubmlst.org/abaumannii/) and *P. aeruginosa* (http://pubmlst.org/paeruginosa/) MLST Database websites. Seven chromosomal genes were PCR amplified and sequenced, with their nucleotide sequences compared with the sequences submitted to the MLST database to determine allele numbers and STs.

Detection of aminoglycoside-resistant genes

PCR with 16S rRNA methylase gene specific primers [2,8,9] was performed to detect the *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmt E* and *npmA* genes. All PCR amplicons were sequenced using an ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA, USA).

Whole genomes of methylase-negative *A. baumannii* and *P. aeruginosa*, which had MICs 128 mg/L to amikacin, 32 mg/L to arbekacin and 128 mg/L to gentamicin, were extracted by DNeasy Blood & Tissue kit (QIAGEN, Tokyo, Japan) and sequenced by MiSeq (Illumina, San Diego, CA). The sequence data were used to confirm aminoglycoside-resistant genes.

Detection of OXAs and CTX-Ms encoding genes

The presence of *bla*OXA-23-like, *bla*OXA-24-like, *bla*OXA-51-like, *bla*OXA-58-like and *bla*CTX-Ms in 16S rRNA methylase-producing isolates were investigated by PCR [10,11].

Determination of the genetic environment surrounding *rmtB*

A draft genome sequence of an isolate of *A. baumannii*, NCGM36, harboring *rmtB* was determined using the GS Junior System (Roche Diagnostics K.K, Tokyo).

Ethical approval

This study was approved in 2007 by Ministry of Health, Bach Mai Hospital (Memorandum of agreement for the collaborative research project on epidemiology of nosocomial infections at the Bach Mai Hospital) and in 2011 by Cho Ray Hospital (approval number: 1644/QD-BVCR), and by the Biosafety Committee, National Center for Global Health and Medicine (approval number: 23-M-49).