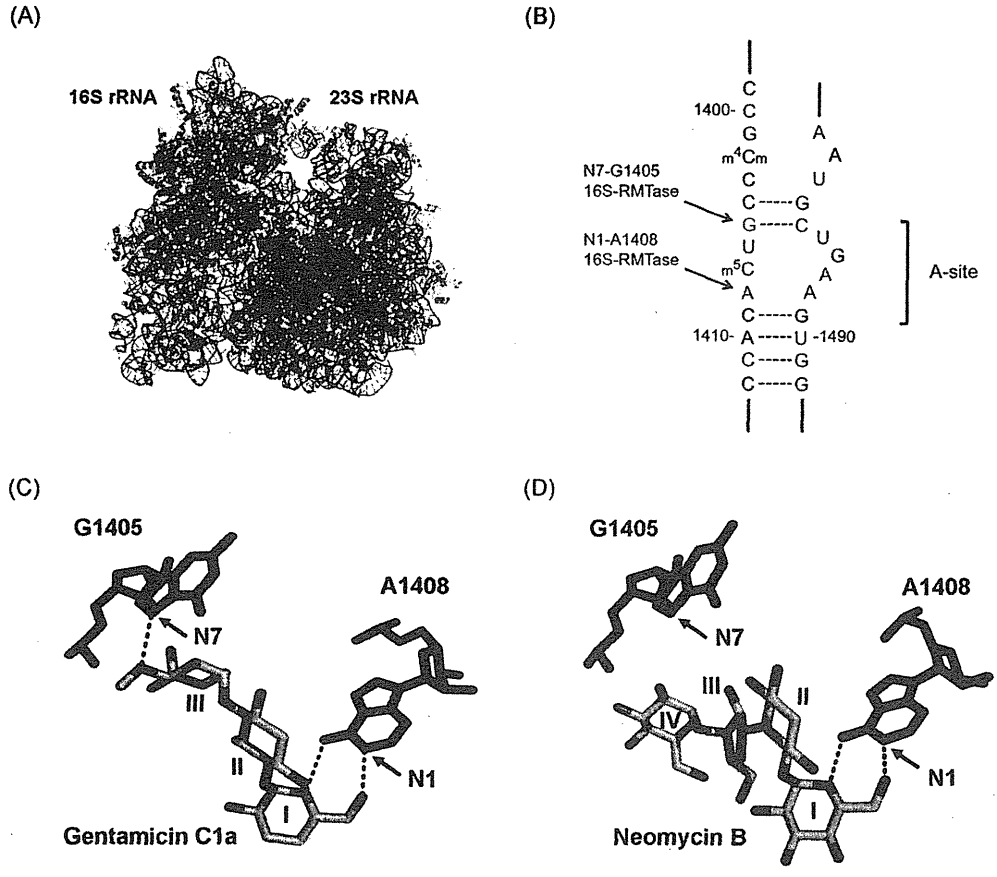

Fig. 2. (A) Structure of *E. coli* 70S ribosome [PDB code, 2QB9 and 2QBA] consisting of 16S rRNA (orange), 23S rRNA (gray), and ribosomal proteins (green and cyan). The position of gentamicin bound to 16S rRNA is shown with a square. The figure was rendered with the PyMol program. (B) A-site decoding region in 16S rRNA. (C and D) The structure of complexes between aminoglycosides (Gentamicin C1a and Neomycin B) and nucleotides in 16S rRNA (G1405 and A1408). Dashed line indicates probable hydrogen bonds. The figures were rendered with PDB data (2QB9 and 2QAL) and the PyMol program.

inherently show a high level of resistance to aminoglycosides including those which are self-produced. In the 2000s, by virtue of significant progress in the field of genetic analytical techniques, the gene clusters involved with aminoglycoside synthesis in *actinomycetes* have been aggressively analyzed (Kharel et al., 2004; Unwin et al., 2004). At the same time, these genetic analyses revealed that 16S-RMTase genes are mostly placed within aminoglycoside biosynthesis gene clusters of *actinomycetes* (Kharel et al., 2004; Unwin et al., 2004). For example, a 16S-RMTase gene, *kmr*, was found within the kanamycin biosynthesis gene cluster of *Streptomyces kanamyceticus* strain DSM40500 (GenBank Accession No. AJ628422), and the other 16S-RMTase gene, *kamA* (also called *fmrT*), was within the istamycin biosynthesis gene cluster of the *Streptomyces tenjimariensis* strain ATCC31603. Coexistence of the genes for aminoglycoside production and aminoglycoside resistance may give *actinomycetes* some ecological advantage to survive in an environment where various microbes are competing with one another.

2.2. Types of intrinsic 16S-RMTases

The 16S-RMTases implicated in aminoglycoside resistance found in aminoglycoside-producing *actinomycetes* are basically classified into two subgroups, N7-G1405 16S-RMTases and N1-A1408 16S-RMTases, depending on the nucleotide position to be modified at the A-site of 16S rRNA (Fig. 2B) (Beauclerk and Cundliffe, 1987). The N7-G1405 16S-RMTases like Kmr, FmrO, and Sgm modify N-7 position of G1405 nucleotide within 16S rRNA and confer resistance exclusively to 4,6-disubstituted DOS such as amikacin and gentamicin, but not to 4,5-disubstituted DOS, apramycin, and streptomycin (Demydchuk et al., 1998; Kojic et al., 1992; Ohta and Hasegawa, 1993b). On the one hand, a N1-A1408 16S-RMTase like KamA (FmrT) modifies N-1 position of A1408 nucleotide within 16S rRNA and confers resistance to structurally diverse aminoglycosides, 4,6- and 4,5-disubstituted DOS, and apramycin (Ohta and Hasegawa, 1993a). The difference in the modified nucleotide positions within A-site results in diversity of the aminoglycoside resistance profiles, which can be partially predicted by considering the formation of hydrogen bond networks between each sugar ring of aminoglycosides and the nitrogenous bases of the nucleotides in bacterial 30S ribosome composed of 16S rRNA and ribosomal proteins (Fig. 2C and D) (Borovinskaya et al., 2007; Yoshizawa et al., 1998). The ring III of 4,6-disubstituted DOS like gentamicin C1a forms a hydrogen bond to the N7-G1405 position, while the spatial location of the rings III and IV of 4,5-disubstituted DOS like neomycin B is far from the N7-G1405 position. Therefore, the modification by N7-G1405 16S-RMTase may well have an effect upon the interaction with 4,6-disubstituted DOS, not upon 4,5-disubstituted DOS. Introduction of a methyl group to the N7 position of G1405 by 16S-RMTase will disturb the formation of hydrogen bond between the nucleotide and aminoglycoside, and in turn reduce the binding affinity of aminoglycosides to the A-site. Regardless of the structural diversity, the ring I of aminoglycosides commonly contacts the N1-A1408 position; accordingly, N1-A1408 16S-RMTases can confer resistance to a variety of aminoglycosides including 4,6- and 4,5-disubstituted DOS, and apramycin.

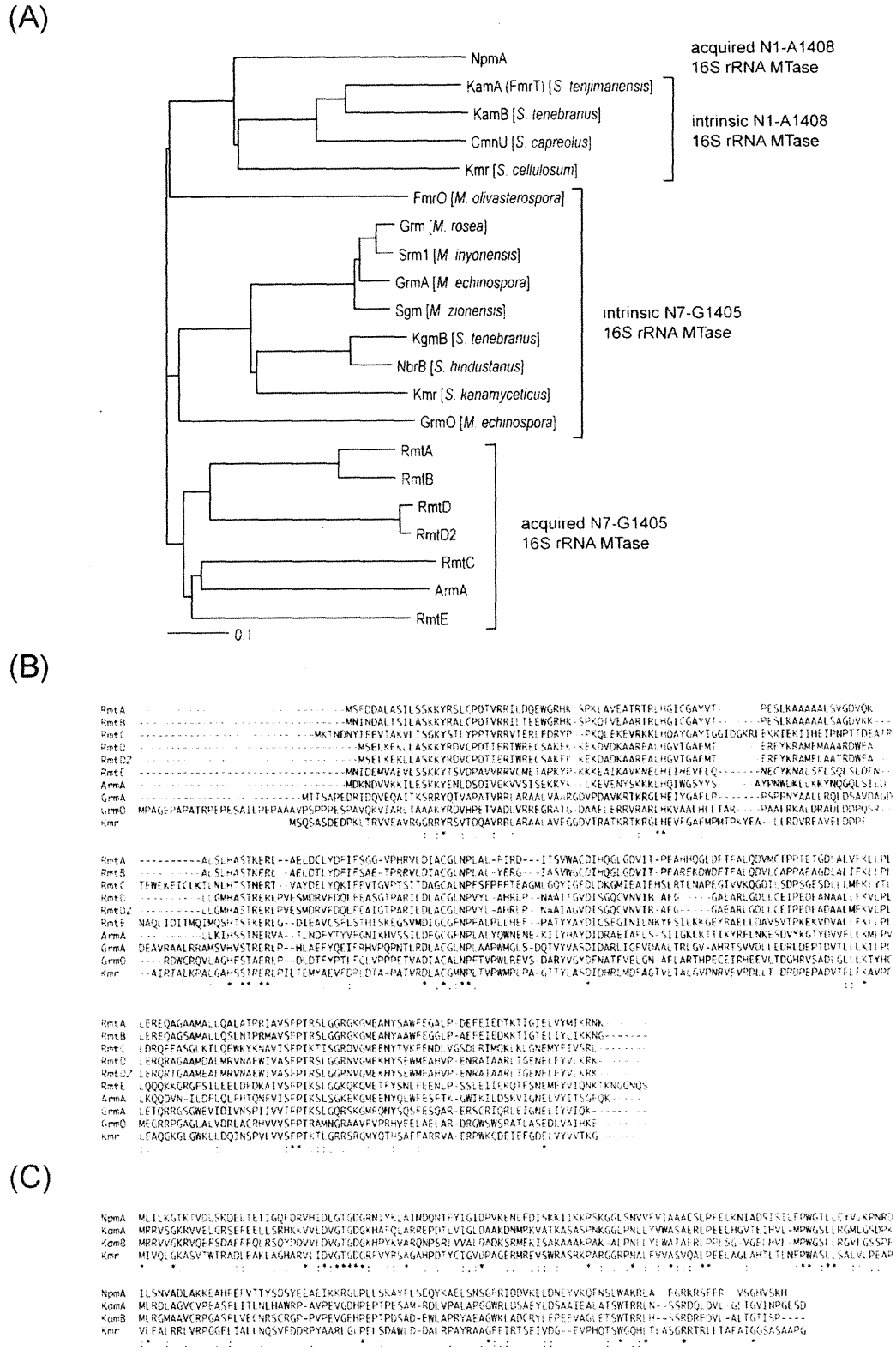

Fig. 3. *Tree view exhibiting the phylogenic similarities among N7-G1405 16S-RMTases and N1-A1408 16S-RMTases (A). Tree was constructed using the ClustalW version 1.83 program (http://clustalw.ddbj.nig.ac.jp/top-j.html) provided by DNA Data Bank of JAPAN (DDBJ). Proteins (NCBI Accession No.): FmrO (BAA02451); Grm (AAA25338); Srm1 (AAV28394); GrmA (AAR98546); Sgm (3LCU_A); KgmB (AAB20100); NbrB (AAB95477); Kmr of Streptomyces kanamyceticus (BAD20767); GrmO (AAR98541); RmtA (BAD12551); RmtB (BAC81971); RmtC (BAE48305); RmtD (ABJ53409); RmtD2 (ADW66545); RmtE (ADA63498); NpmA (BAF80809); Kmr of Sorangium cellulosum (ACB88605); KamA[FmrT] (D13170); KamB (3MQ2_A); CmnU (ABR67761). The "0.1" scale represents a genetic unit reflecting 10% of the amino acid substitutions calculated with the ClustalW program. Multiple sequence alignments of 7 exogenous and 3 intrinsic N7-G1405 16S-RMTases (B), and NpmA and 3 intrinsic N1-A1408 16S-RMTases (C). Kmr proteins shown in (B) and (C) are different enzymes derived from S. kanamyceticus and S. cellulosum, respectively. The multiple sequence alignments were illustrated by the ClustalW supported by DDBJ (http://www.ddbj.nig.ac.jp/index-j.html). (*) Indicates positions which have a single, fully conserved residue. (:) Indicates that one of the following "strong" groups is fully conserved. (STA, NHQK, NDEQ, QHRK, MLIV, MILF, HY, FYW, NEQK). (.) Indicates that one of the following "weaker" groups is fully conserved. (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY).*

Table 1
Chronological order of the first isolation of exogenously acquired 16S-RMTase-producing Gram-negative bacteria.

Year of isolation (deposition to the data base)	16S-RMTase	Bacterial species	Country	Specimen or animal	Mobile gene element	Coexisting resistance genes	Reference and Accession No.
1996 (2002)	*armA*[a]	*C. freundii*	Poland	Clinical isolate	IS*CR1*	$bla_{CTX-M-3}$, bla_{TEM-1}, *aacC2*, *aadA2*, *dfrA12*, *sul1*	Golebiewski et al. (2007), AF550415
1997 (2002)	*rmtA*	*P. aerugonosa*	Japan	Sputum	κγ		Yokoyama et al. (2003), AB083212
2002 (2003)	*rmtB*	*S. marcescens*	Japan	Sputum	Tn*3*	bla_{TEM-1}, *aadA2*	Doi et al. (2004), AB103506
2003 (2004)	*rmtC*	*P. mirabilis*	Japan	Throat swab	IS*Ecp1*	*aphA1*	Wachino et al. (2006b), AB194779
2003 (2006)	*npmA*	*E. coli*	Japan	Urine	IS*26*		Wachino et al. (2007), AB261016
2005 (2006)	*rmtD*	*P. aeruginosa*	Brazil	Urine		bla_{SPM-1}	Doi et al. (2007b), DQ914960
Unknown (2010)	*rmtE*	*E. coli*	USA	Cattle		*aph(3″)-Ia*, *aphA7*, *strA*, *strB*	Davis et al. (2010), GU201947
2007 (2009)	*rmtD2*	*Enterobacter aerogenes*	Argentina	Not reported	IS*CR14*	*cat*, *dfrA12*, *aadA2*, *sul1*	Tijet et al. (2011), AM849110

[a] Named by Galimand et al. (2003).

3. Acquired 16S-RMTases of pathogenic Gram-negative bacteria

3.1. Identification history of acquired 16S-RMTases

A *P. aeruginosa* strain AR2 was isolated from sputum in 1997 in Japan, and this isolate showed an extraordinarily high level of resistance to various aminoglycosides. A plasmid-mediated exogenous 16S-RMTase, RmtA, was first identified from this isolate (Yokoyama et al., 2003) (Table 1). In 2002, the whole nucleotide sequence of pCTX-M3 plasmid, encoding CTX-M-3 β-lactamase gene, of *Citrobacter freundii* from Poland was deposited in GenBank (Accession No. AF550415). The data revealed that the pCTX-M3 plasmid carried a probable 16S-RMTase gene, but it remained uncharacterized for its involvement in aminoglycoside resistance at that time. In 2003, Galimand et al. reported the participation of *armA* in aminoglycoside resistance, cloned from a *K. pneumoniae* clinical isolate (BM4536) showing a high level of resistance to clinically crucial aminoglycosides. The *armA* was found to be identical to the probable MTase gene detected previously in the pCTX-M3 plasmid. The G+C content of *armA* was 30%, while that of *rmtA* was 55%, resulting in only 30% identity in the alignment of amino acid residues between them. ArmA and RmtA were the index cases, which revealed the emergence of exogenously acquired 16S-RMTases conferring a wide range and high level of aminoglycoside resistance to pathogenic Gram-negative bacteria. Three years later, the enzymatic function of ArmA was characterized in detail by Liou et al., and ArmA was found to be categorized as a member of N7-G1405 16S-RMTases (Liou et al., 2006). RmtA was expected to belong to the same 16S-RMTase group on the basis of amino acid sequence similarity.

Several N7-G1405 16S-RMTases were subsequently discovered among pathogenic Gram-negative bacteria, and a total of seven N7-G1405 16S-RMTases have been reported thus far (Fig. 3A). In Japan, RmtB and RmtC were identified in clinical isolates of *Serratia marcescens* strain S-95 and a *Proteus mirabilis* strain ARS68, respectively (Doi et al., 2004; Wachino et al., 2006b). RmtD was discovered in a *P. aeruginosa* clinical strain (PA0905) isolated in Brazil (Doi et al., 2007b). An *Enterobacter aerogenes* clinical strain (Q4079) producing RmtD2, which has 9 amino acid substitutions compared to RmtD, was later reported from Argentina (Tijet et al., 2011). RmtE was identified in a commensal *Escherichia coli* strain originated from cattle, in the United States (Davis et al., 2010). The above-described 16S-RMTase genes were mostly embedded into plasmids, and could be experimentally moved to the recipient bacteria by conjugation and/or transformation.

3.2. Enzymatic function of 16S-RMTases

The seven N7-G1405 16S-RMTases, ArmA and RmtA to RmtE, could commonly confer resistance exclusively to aminoglycosides belonging to the 4,6-disubstituted DOS group such as amikacin, tobramycin, and gentamicin (Table 2). The enzymatic function of RmtB, RmtC, and ArmA was fully characterized in vitro with the purified proteins, and it was demonstrated that these 16S-RMTases apparently added the methyl group of *S*-adenosyl-L-methionine (SAM) as a cofactor to the N-7 position of G1405 within 16S rRNA of mature 30S ribosomal subunit (Liou et al., 2006; Perichon et al., 2007; Wachino et al., 2010). However, RmtB, RmtC and ArmA failed to transfer the methyl group to the naked 16S rRNA molecule without ribosomal proteins, indicating that the tertiary structure composed of the 16S rRNA and ribosomal proteins is essential for recognition or modification of the target site by the enzyme (Doi et al., 2004; Liou et al., 2006; Wachino et al., 2006a,b).

In contrast to the N7-G1405 16S-RMTase group, acquired N1-A1408 16S-RMTase is scarcely found in clinically isolated pathogenic bacteria. NpmA, which we first identified in an *E. coli* clinical strain (ARS3) in 2007, is currently a sole acquired N1-A1408 16S-RMTase (Wachino et al., 2007). NpmA could confer a broader range of aminoglycoside resistance rather than N7-G1405 16S-RMTases (Table 2). The *npmA* gene was located on a conjugative plasmid, and successfully transferred to an *E. coli* recipient strain. NpmA could catalyze transfer of the methyl group of SAM to the N-1 position of the A1408 residue in 16S rRNA. The preferred substrate for NpmA was 30S ribosome composed of 16S rRNA and ribosomal

Table 2
Aminoglycoside resistance profile provided by N7-G1405 and N1-A1408 16S-RMTases.

Aminoglycoside	N7-G1405 16S-RMTase	N1-A1408 16S-RMTase
	RmtA, RmtB, RmtC, RmtD/D2, RmtE	NpmA
4,6-Disubstituted DOS		
Gentamicin	R+	R
Amikacin	R+	R
4,5-Disubstituted DOS		
Neomycin	S	R+
Ribostamycin	S	R+
Monosubstituted DOS		
Apramycin	S	R+
No DOS ring		
Streptomycin	S	S

R+, highly resistant; R, resistant; S, susceptible; DOS, 2-deoxystreptamine.

proteins, not naked 16S rRNA, as was observed in the N7-G1405 16S-RMTases, RmtB, RmtC, and ArmA.

3.3. G+C contents of 16S-RMTase genes

The G+C contents of acquired 16S-RMTases genes as described above are between 30% and 55%, suggesting that their origin is less likely to be intrinsic 16S-RMTase genes of aminoglycoside-producing *actinomycetes* because their G+C content is generally above 60%. The origin of acquired 16S-RMTase genes remains uncertain. Identification of an intrinsic 16S-RMTase gene of low G+C content aminoglycoside-producing bacterium like *Bacillus circulans*, may provide a clue to seek for the origins of acquired 16S-RMTase genes found in Gram-negative pathogens.

4. Genetic platform surrounding 16S-RMTase genes

4.1. RmtA

The 16S-RMTase genes found so far in pathogenic bacteria are mostly located within the transferable plasmid and/or linked to the bacteria-specific DNA recombination system such as transposon (Fig. 4). The 6.2-kb genetic region including *rmtA* of *P. aeruginosa* was flanked by two copies of a kappa-gamma (κγ) element (Yamane et al., 2004), a 262-bp element possible for mobile element, that was previously identified within a composite transposon Tn*5041* carrying the genes responsible for mercury resistance in a *Pseudomonas* species. The genetic regions outward two κγ elements of *P. aeruginosa* were identical to those of Tn*5041* (Kholodii et al., 1997). Thus, the 6.2-kb genetic region including *rmtA* encompassed with two κγ elements appeared to be later inserted into the Tn*5041*.

4.2. RmtB and RmtC

Almost all *rmtB* genes are accompanied by Tn*3* transposon mediating TEM-1 β-lactamase gene (Doi et al., 2004). The downstream region of *rmtB* is genetically variable, but often associated with quinolone efflux transporter gene, *qepA* (Perichon et al., 2007; Yamane et al., 2007a). The *rmtC* gene found in *P. mirabilis* was located adjacent to the IS*Ecp1* element that is mainly involved in translocation of neighboring *rmtC* gene as well as providing promoter activity for *rmtC* expression (Wachino et al., 2006a). IS*Ecp1* is frequently associated with β-lactamase genes such as $bla_{CTX\text{-}M}$ and bla_{CMY} that are able to hydrolyze extended-spectrum cephalosporins and/or several cephamycins, respectively. IS*Ecp1* was found to be involved in the spread of $bla_{CTX\text{-}M}$ genes in *Enterobacteriaceaes* (Toleman and Walsh, 2011). The *rmtC* gene may well be widely distributed among the family *Enterobacteriaceae* in the near future via the transposition activity of IS*Ecp1*, because several NDM-1-producing isolates have already co-produced RmtC (Islam et al., in press; Poirel et al., 2011c).

4.3. RmtD, RmtD2 and RmtE

The *rmtD* gene of *P. aeruginosa* found in Brazil was followed by *orf494* (a putative transposase gene) and a 3′-conserved segment consisting of *qacEΔ1* and *sul1* of a class 1 integron (Doi et al., 2008). Upstream of *rmtD*, *orfA* (a putative tRNA ribosyltransferase gene), *ΔgroEL*, and another *orf494* were presented. The *rmtD* gene was bound with two copies of *orf494* with the same direction. The region adjacent to *rmtD* of a *K. pneumoniae* strain was identical with that of *P. aeruginosa*, but both copies of *orf494* were truncated with IS*26*. The genetic context of *rmtD2* found in Argentina was similar to that of *rmtD* from Brazil (Tijet et al., 2011). However, the 5′-end of *ΔgroEL*, upstream of *rmtD2*, was largely deleted compared to that of *rmtD*. Tijet et al. described the possibility that the surrounding region of *rmtD* flanked by *orf494* and that of *rmtD2* were separately assembled by individual genetic recombination machinery consisting of *orf494*, rather than derived from a common ancestral structure. The genetic environment of *rmtE* has not yet been elucidated.

4.4. ArmA

The genetic surrounding regions of the *armA* gene deposited in GenBank are almost identical, despite being widespread among *Enterobacteriaceae* and *Acinetobacter baumannii* isolated from a variety of sources (Bercot et al., 2008; Doi et al., 2007a; Galimand et al., 2005; Granier et al., 2011). The *armA* gene together with *tnpAcp1* (a transposase-like gene) is typically located downstream of an IS*CR1* element associated with a class 1 integron. Downstream of *armA*, *trpA* (a transposase-like gene), and macrolide efflux gene (*mel*) and a macrolide phosphotransferase gene (*mph*), were often located (Galimand et al., 2005). Galimand et al. reported *armA* existed within a composite transposon Tn*1548* flanked by two copies of IS*6*, and could be easily transposed to the other DNA target site (Galimand et al., 2005).

4.5. NpmA

The 9.1-kb genetic region carrying *npmA* was flanked by the two copies of IS*26*, and this region had no significant genetic similarity to the sequences deposited in GenBank to date (Wachino et al., 2007). The sequences in both external regions of the 9.1-kb probable large transposable element demonstrate considerable sequence similarities to the sequences of a part of various multidrug-resistant plasmids deposited in the databases.

4.6. Characteristics of plasmids mediating 16S-RMTase genes

In some instances, as described below, 16S-RMTase genes together with the other antimicrobial resistance genes, such as NDM-1 gene, have so far been accumulated on various conjugative plasmids of broad or narrow host ranges (Table 3). Several mobile genetic elements carrying 16S-RMTase genes were already embedded into various transferable plasmids belonging to diverse incompatibility groups, such as IncL/M, IncFII, and IncA/C. Rapid dissemination of multiple drug-resistance genes including 16S-RMTase genes mediated by broad-host range plasmids like IncN and IncA/C groups is of a great concern for human health since it apparently accelerates acquisition of a multidrug resistant nature in pathogenic microorganisms.

5. Epidemiology of 16S-RMTase producers

5.1. RmtB- and ArmA-producers

Pathogenic microbes that produce 16S-RMTases have already been distributed all over the world (Fig. 5). Among the 8 variants of exogenous 16S-RMTases described above, RmtB and ArmA are the predominant 16S-RMTases. The *rmtB* gene was detected mainly in the members of *Enterobacteriaceae* from East Asia, Europe, North and South America, and Oceania (Bogaerts et al., 2007; Fritsche et al., 2008; Kang et al., 2008; Poirel et al., 2010; Tian et al., 2011; Wu et al., 2009; Yamane et al., 2008b, 2007b). Of note, RmtB-producing bacteria have been isolated not only from human specimens in clinical settings, but also from livestock and pet animals (Table 4), suggesting probable transmission of the resistance determinants between human and animals (Chen et al., 2007; Deng et al., 2011b). The significant similarity in the genetic context of *rmtB* between

Table 3
Characteristics of plasmids that mediate exogenously acquired 16S-RMTase gene, together with clinically crucial antimicrobial resistance genes.

Year of isolation	16S-RMTase gene	Bacterial species	Plasmid	Imcompatibility type	Size	Coexisting resistance genes	Reference and Accession No.
2000	*armA*	*K. pneumoniae*	pIP1204	IncL/M	ca. 90-kb	$bla_{CTX-M-3}$, *ant3″9*, *dfrXII*, *sul1*	Galimand et al. (2005), AY220558
2002	*armA*	*E. coli*	pMUR050	IncN	57-kb	*ant3′9″*, *linF*, *aphA1*, *mel*, *mph2*, *sul1*	Gonzalez-Zorn et al. (2005a), NC007682
2005	*armA*	*K. pneumoniae*		IncN		bla_{VIM-1}, bla_{TEM-1}, $bla_{CTX-M-3}$,	Samuelsen et al. (2011b)
2006	*armA*	*K. pneumoniae*	pKP048	IncF	151-kb	bla_{KPC-2}, bla_{DHA-1}, *qnrB4*, *mel*, *mph2*, *sul1*	Jiang et al. (2010), FJ628167
2009	*armA*	*E. coli*	pNDM-1_Dok01	IncA/C	196-kb	bla_{NDM-1}, bla_{CMY-4}, bla_{TEM-1}, *dfrA12*, *aadA2*, *mel*, *mph2*, *sul1*	Sekizuka et al. (2011), AP012208
2009	*armA*	*E. coli*	pNDM-HK	IncL/M	89-kb	bla_{NDM-1}, Δbla_{DHA-1}, bla_{TEM-1}, *aacC2*, *mel*, *mph2*, *sul1*	Ho et al. (2011), HQ451074
Not reported	*armA*	*E. coli*	p271B	IncI1	110-kb	bla_{TEM-1}, bla_{OXA-9}, $bla_{CTX-M-15}$, *aadA1*, *mph2*, *mel*, *dfrA12*, *arr2*, *cmlA5*, *sul1*	Poirel et al (2011a)
2010	*armA*	*S. enterica* subsp. *enterica* serovar Paratyphi B		IncFII	~72-kb	$bla_{CTX-M-3}$, *qnrB2*, *aac(6′)-Ib-cr*, *dfrA1*, *aadA5*, *mel*, *mph2*, *sul1*	Du et al. (2012), JN225877
2011	*armA*	*P. stuartii*	pMR0211	IncA/C	178-kb	bla_{NDM-1}, bla_{CMY-2}, bla_{OXA-10}, *floR*, *tetA/R*, *strA/B*, *aadA*, *mel*, *mph2*, *cmlA7*, *aac(6′)*, *qnrA1*, *sul1*, *sul2*,	McGann et al. (2012), JN687470
2002	*rmtB*	*E. coli*	pHPA	IncFII		$bla_{CTX-M-12}$, bla_{TEM-1}, *qepA*, *fosC2*, *dfrA17*, *aadA5*	Yamane et al. (2007a), AB263754
Not reported	*rmtB*	*E. coli*	p271C	IncF	130-kb	bla_{TEM-1}, bla_{OXA-1}, bla_{OXA-10}, *aphA1-IAB*, *ermB*, *catB4*, *qepA*, *sul1*	Poirel et al (2011a)
2000–2005	*rmtB*	*E. coli*	pIP1206	IncFI	168-kb	bla_{TEM-1}, *qepA*, *dfrA17*, *tetA*, *catA1*, *aadA4*, *sul1*	Perichon et al. (2008), AM886293
2006	*rmtB*	*E. aerogenes*		IncFI	ca. 120-kb	*qepA*, *qnrS1*, bla_{TEM-1}, bla_{LAP-1}	Park et al. (2009)
2006–2010	*rmtB*	*E. coli*	p3D12T	IncFII		$bla_{CTX-M-65}$, *fosA3*	Hou et al. (2012), JF411007
2009	*rmtB*	*E. coli*	pXD2	IncFII	~181-kb	bla_{TEM-1}, *qnrS1*, *aac(6′)-Ib-cr*, $bla_{CTX-M-15}$	Li et al. (2012), JN315966
2007–2009	*rmtC*	*K. pneumoniae*	pNDM-KN	IncA/C	163-kb	bla_{NDM-1}, bla_{CMY-6}, *cmlA7*, *aadA1*, *ereC*, *arr2*	Carattoli et al. (2012), JN157804
Not reported	*rmtC*	*K. pneumoniae*	pNDM10469	IncA/C	138-kb	bla_{NDM-1}, bla_{CMY-6}, *aac(6′)-Ib*, *sul1*	JN861072

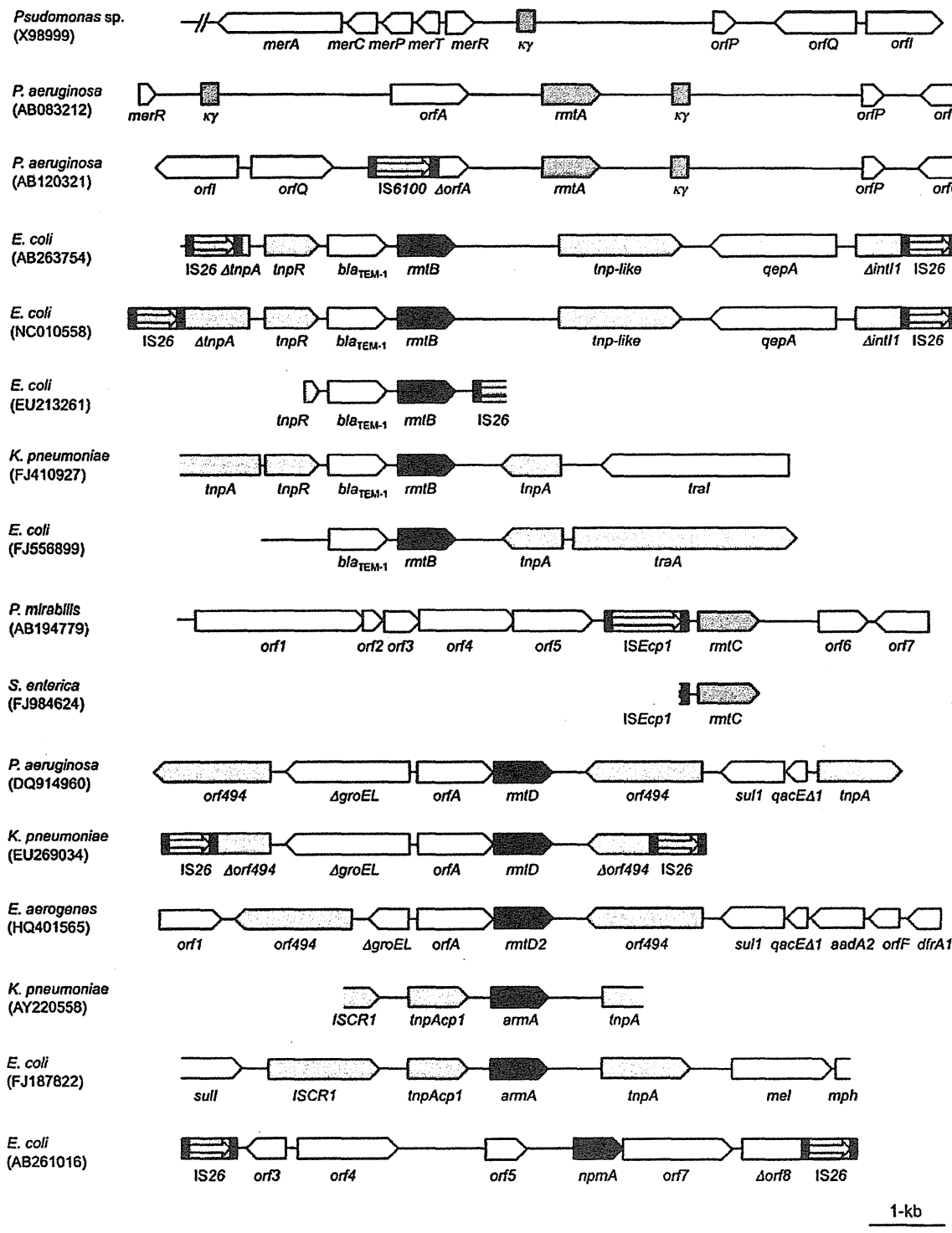

Fig. 4. Genetic context of aminoglycoside resistance 16S-RMTase genes. Antimicrobial resistance genes except for 16S-RMTase genes are shown with yellow. Genes which seem to be involved in DNA recombination are shown in greenish yellow.

human and animal sources would support the above possibility. ArmA, as well as RmtB, has been widely spread among the family *Enterobacteriaceae* including *Shigella flexneri*, and additionally in *Acinetobacter* species (Brigante et al., 2012; Fritsche et al., 2008; Galimand et al., 2005; Karah et al., 2011; Samuelsen et al., 2011a; Yamane et al., 2007b). ArmA was also detected in *Salmonella enterica* from chicken meat sampled in a supermarket in a French island in the Indian Ocean, suggesting it possibly spread the resistance determinant via food chain (Granier et al., 2011). Recently, ArmA-producing *Salmonella* isolates have been increasingly reported from Algeria and China (Naas et al., 2011; Du et al., 2012) (Table 5). The identification of RmtC had been limited in *P. mirabilis* from Japan and Australia before 2009 (Wachino et al., 2006b; Zong et al., 2008), but it has been recently detected in *S. enterica* from the United Kingdom and the United States, *K. pneumoniae* from Kenya and India, *E. coli* from the United Kingdom and Germany, and *Providencia stuartii* from India (Folster et al., 2009; Livermore et al. 2011; Hopkins et al., 2010; Poirel et al., 2011c,d).

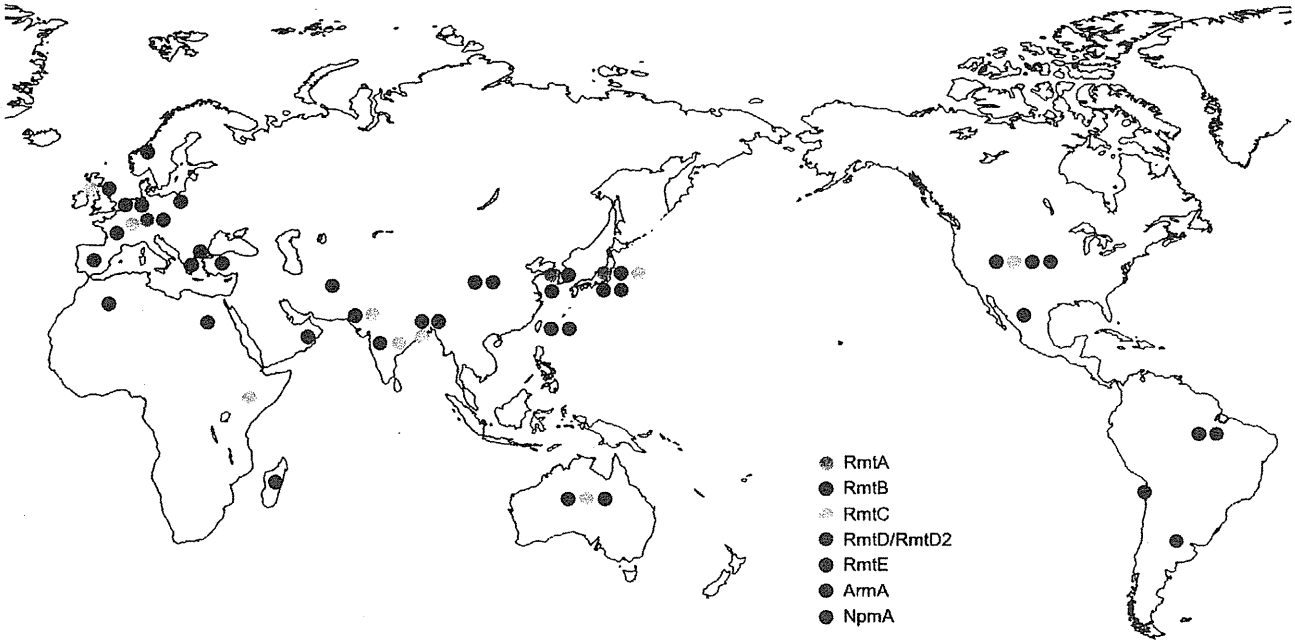

Fig. 5. Worldwide distribution of aminoglycoside resistance 16S-RMTases. Approximate locations of countries or regions where any of the acquired 16S-RMTase-producing microbes were isolated are indicated with circles in color.

Table 4
Identification of exogenously acquired 16S-RMTase-producing Gram-negative microbes recovered from livestock, pet animal or food.

Year of isolation	Country	16S-RMTase	Bacterial species	Livestock, pet animal or food	Reference
2002	Spain	ArmA	*E. coli*	Pig	Gonzalez-Zorn et al. (2005b)
2005–2006	China	RmtB	*E. coli* *E. cloacae*	Pig	Chen et al. (2007)
2005–2006	China	RmtB	*E. coli*	Pig	Liu et al. (2008)
2008	China	ArmA and rmtB	*E. coli*	Chicken	Du et al. (2009)
Not reported	USA	RmtE	*E. coli*	Cattle	Davis et al. (2010)
2008	UK	RmtC	*S. enterica* serovar Virchow	Food (frozen)	Hopkins et al. (2010)
2004–2007	Korea	ArmA	*E. coli*	Farm animals (cattle, pig, chicken)	Choi et al. (2011)
2002	China	RmtB	*E. coli*	Pig	Deng et al. (2011b)
2006–2008	China	ArmA and/or RmtB	*Enterobacteriaceae* (*E. coli*, *K. pneumoniae*, *E. cloacae*, *C. freundii*)	Pets (dog or cat)	Deng et al. (2011a)
2010	China	RmtB	*E. coli*, *M. morganii*, *L. adecarboxylata*, *E. aerogenes*, *E. cloacae*	Pig (feces or soil)	Yao et al. (2011)
2009	French island in the Indian Ocean	ArmA	*S. enterica*	Chicken meat	Granier et al. (2011)
2007	China	RmtB	*E. coli*	Chicken	Xia et al. (2011)
2010	China	ArmA	*S. enterica* Paratyphi B	Chicken	Du et al. (2012)
2009	China	RmtB	*E. coli*	Bovine	Li et al. (2012)

Table 5
Identification of 16S-RMTases in *Salmonella* or *Shigella* spp.

Year of isolation	16S-RMTase	Bacterial species	Country	Other resistance factor	Reference and Accession No.
Unknown	ArmA	*S. enterica* *S. flexneri*	Bulgaria	CTX-M-3	Galimand et al. (2005)
1999	ArmA	*S. enterica* serotype Stanley	USA		Folster et al. (2009)
2005	RmtC	*S. enterica* serotype Virchow			
2004–2008	RmtC	*S. enterica* serotype Virchow	UK		Hopkins et al. (2010)
Unknown	ArmA	*S. enterica* serotype Oranienburg	UK		DQ177329
2005	ArmA	*S. enterica* serotype Gambia	France	CTX-M-3	Moissenet et al. (2011)
2008–2009	ArmA	*S. enterica* serotype Infantis	Algeria	CTX-M-15	Naas et al. (2011)
2008–2009	ArmA	*S. enterica* serotype Typhimurium *Salmonella* 4,12:-:1,2 *S. enterica* serotype Enteritidis	Algeria	CTX-M-15, CMY-2 CTX-M-15, CMY-2 CTX-M-15	Bouzidi et al. (2011)
2009	ArmA	*S. enterica* subspecies I.4,12:i:-	France	CTX-M-3, CMY-2	Granier et al. (2011)
2010	ArmA	*S. enterica* serotype Paratyphi B	China	CTX-M-3, AAC(6′)-Ib-cr, QnrB2	Du et al. (2012)

5.2. RmtA-, RmtD- and NpmA-producers

On the other hand, the other 16S-RMTases, RmtA, RmtD, and NpmA, have been sporadically found. RmtA had been identified solely in *P. aeruginosa* from East Asian countries, Japan and South Korea (Jin et al., 2009; Yamane et al., 2007b), but quite recently it was identified in a *K. pneumoniae* strain from Switzerland (Poirel et al., 2011e). RmtD and RmtD2 were locally distributed in countries in South America like Brazil, Chile, and Argentina (Fritsche et al., 2008; Tijet et al., 2011; Yamane et al., 2008a). RmtE and NpmA were detected in *E. coli* from the United States and from Japan, respectively (Davis et al., 2010; Wachino et al., 2007).

5.3. Epidemiological data from countrywide scale surveys

Epidemiological data from the survey of 16S-RMTase-producing bacteria on a countrywide scale are little available. In Japan, twenty-six bacterial isolates (0.03%) were positive for the 16S-RMTase genes, among the 87,626 clinical isolates collected from 169 geographical disparate hospitals in 2004 (Yamane et al., 2007b). In Argentina, 7 enterobacterial strains (0.7%) were positive for *rmtD2* among 1064 isolates collected from 66 hospitals belonging to the WHONET-Argentina Resistance Surveillance Network in 2007 (Tijet et al., 2011). In South Korea, the 16S-RMTase production rate was significantly high, 11.4%, among a total of 413 non-duplicate *Enterobacteriaceae* isolates including *S. marcescens*, *E. cloacae*, and *C. freundii* collected from 11 university hospitals (Park et al., 2006). Livermore et al. exhibited SENTRY Data for 2007–2008 indicating the prevalence of 16S-RMTase producers among *Enterobacteriaceae*, 10.5% in India, 6.9% in China, 6.1% in Korea, 5% in Taiwan, and 3.1% in Hong Kong (Livermore et al., 2011).

5.4. Prevalence data on local scale investigations

In addition, a number of reports describing the prevalence of 16S-RMTase-producing organisms on a local scale have been published. The proportion of 16S-RMTase positive strains in ESBL-producing *Enterobacteriaceae* was low at medical institutes in European countries, 0.7% in a Turkish medical center and 1.3% in a French university hospital (Bercot et al., 2008, 2010). Among the *Enterobacteriaceae* collected from two Belgian hospitals, the rate of 16S-RMTase producers was 0.12% (Bogaerts et al., 2007). In a cancer hospital in Bulgaria, 20 out of 1310 (1.5%) *Enterobacteriaceae* isolates were 16S-RMTase producers (Sabtcheva et al., 2008). On the other hand, several studies published especially from East Asian study groups reported a slightly higher rate of prevalence of 16S-RMTase producers. Thirty-seven *E. coli* clinical isolates (5.4%) were positive for the 16S-RMTase genes among 680 strains collected between 2006 and 2008 in a teaching hospital in China (Yu et al., 2010). Among the 7127 *Enterobacteriaceae* clinical isolates collected in a university hospital in South Korea, 16S-RMTase genes were detected in 204 isolates (2.9%) (Kang et al., 2008). There appear to be a hospital to hospital variability in the occurrence of 16S-RMTase-producers, but on the whole, 16S-RMTases seem to be a little more prevalent in Asian countries than in Europe. Spread of 16S-RMTase genes will likely be escalated considering they can be embedded into mobile genetic apparatus and in turn associated with transferable plasmids.

6. Resistance determinants coexisting with 16S-RMTases

6.1. NDM-1, SPM-1, IMP-type and VIM-type MBLs

One great concern is the multidrug resistance development in 16S-RMTase-producing pathogenic bacteria through further accumulation of various antimicrobial resistance genes (Tables 3 and 6). Especially, the acquisition of carbapenem resistance via production of carbapenem hydrolyzing β-lactamases would be a serious concern in clinical settings, because carbapenems are still important agents for the treatment of infectious diseases caused by Gram-negative pathogens. This is certainly warranted, however, for the emergence of the member of family *Enterobacteriaceae* coproducing NDM-1 metallo-β-lactamase (MBL) and 16S-RMTases (RmtB, RmtC, and ArmA) (Livermore et al., 2011; Poirel et al., 2011a). To make matters worse, these two resistance determinants were often co-located on the same broad-range conjugative plasmid of the *Enterobacteriaceae* (Ho et al., 2011; Sekizuka et al., 2011), and this fact would imply further concern due to rapid evolution of multidrug resistance in pathogenic microorganisms.

SPM-1 is another MBL associated with a 16S-RMTase, RmtD (Doi et al., 2007b). The coproduction of SPM-1 and RmtD was identified only in *P. aeruginosa* clinical isolates in Brazil. Two articles have reported the variability in the prevalence of *P. aeruginosa* coproducing SPM-1 and RmtD in Brazil (Castanheira et al., 2008; Doi et al., 2007c). Interestingly, the SPM-1 and RmtD-coproducing *P. aeruginosa* was also identified from an urban river, suggesting its potential dissemination through the environment (Fontes et al., 2011). Although the report of SPM-1 and RmtD-coproducing *P. aeruginosa* has been limited in Brazil so far, it may be found in different geographical areas, given that SPM-1-producing *P. aeruginosa* has already spread to a European country (Salabi et al., 2010).

Co-productions of 16S-RMTase and IMP- or VIM-type MBL are not common, and such association was reported from Korea, Greece and Sweden to date (Galani et al., 2012; Gurung et al., 2010; Lee et al., 2007; Samuelsen et al., 2011b).

6.2. OXA-type and KPC-type carbapenemases

Emergence of ArmA-producing *A. baumannii* was first reported in Korea (Lee et al., 2006), but co-production of OXA-type carbapenemase in the isolate was only suggested by the fact that the isolate demonstrated nonsusceptibility to carbapenems. Coexistence of carbapenem-hydrolyzing oxacillinase, OXA-23, and a 16S-RMTase, ArmA, was later identified in multidrug-resistant *A. baumannii* (MDRA) in the United States in 2007 (Doi et al., 2007a). After that, the presence of MDRA co-producing OXA-23 and ArmA was sporadically reported from China, India, and South Korea, and a part of these MDRA additionally produce NDM-1 MBL (Karthikeyan et al., 2010; Kim et al., 2008; Zhao et al., 2011). Coproduction of KPC-2 carbapenemase, which is one of the widespread carbapenem-hydrolyzing class A β-lactamases, and a 16S-RMTase, ArmA, were seen in *Enterobacteriaceae* from Poland and China (Jiang et al., 2010; Zacharczuk et al., 2011). Moreover, *K. pneumoniae* isolates that co-produce KPC-2 and RmtB were reported from Greece (Galani et al., 2012) and China (Sheng et al., in press).

6.3. CTX-M-type, CMY-type β-lactamases and PMQR

CTX-M-type extended-spectrum β-lactamases (ESBLs), especially CTX-M-15, which is a globally prevalent CTX-M-type ESBL, are frequently combined with ArmA/RmtB in *Enterobacteriaceae* (Arpin et al., 2009; Poirel et al., 2011a). Coexistence of RmtB and another CTX-M group, CTX-M-9 group (including CTX-M-9 and CTX-M-14), was also found (Deng et al., 2011a; Yan et al., 2004). Besides, plasmid-mediated quinolone resistance (PMQR)

Table 6
Global dissemination of Gram-negative bacteria harboring exogenously acquired 16S-RMTase gene, together with clinically crucial antimicrobial resistance genes.

Year of isolation	16S-RMTase gene	Bacterial species	Country	Specimen or animal	Coexisting resistance genes	Reference
2000	*armA*	*K. pneumoniae*	France	Urine	$bla_{CTX-M-3}$, bla_{TEM-1}, *aacC2*, *aadA2*, *dfrA12*, *sul1*	Galimand et al. (2003)
2002	*rmtB*	*E. coli*	Japan	Urine	bla_{TEM-1}, *qepA*	Yamane et al. (2007a)
2004	*armA*	*Providencia* sp.	Korea	Urinary specimen	bla_{VIM-2}, bla_{PER-1}	Lee et al. (2007)
2005	*rmtD*	*P. aeruginosa*	Brazil	Urine	bla_{SPM-1}	Doi et al. (2007b)
2006	*armA*	*E. coli*	France	Urine	$bla_{CTX-M-15}$	Arpin et al. (2009)
Not reported	*armA*	*A. baumannii*	India		bla_{NDM-1}, bla_{OXA-23}	Karthikeyan et al. (2010)
Not reported	*armA* and *rmtB*	*E. coli*	Australia	Urine	bla_{NDM-1}, $bla_{CTX-M-15}$	Poirel et al. (2010)
Not reported	*armA*	*E. cloacae*	China		bla_{KPC-2}, bla_{SHV-12}, $bla_{CTX-M-14}$, bla_{TEM-1}	Wu et al. (2010)
2008–2009	*armA*	*P. aeruginosa*	Korea		bla_{IMP-1}	Gurung et al. (2010)
2008–2010	*armA*	*A. baumannii*	Korea		*aac(6′)-Ib*, *aph(3′)-Ia*, bla_{OXA-23}	Sung et al. (2011)
Not reported	*rmtB*	*E. coli*	Belgium	Pus	bla_{NDM-1}, bla_{CMY-58}, $bla_{CTX-M-15}$, bla_{TEM-1}	Bogaerts et al. (2011)
Not reported	*armA*	*E. coli*	Hong Kong		bla_{NDM-1}, bla_{TEM-1}, *aacC2*, *sul1*, *mel*, *mph2*	Ho et al. (2011)
2009–2010	*armA*	*E. coli*	Switzerland		bla_{CMY-30}, bla_{OXA-1}, bla_{TEM-1}	Poirel et al. (2011e)
		P. mirabilis			bla_{CMY-16}, bla_{OXA-1}, bla_{OXA-10}, bla_{TEM-1}	
Not reported	*armA* or *rmtC*	Family *Enterobacteriaceae*	UK		bla_{NDM-1},	Livermore et al. (2011)
Not reported	*armA*	*K. pneumoniae*	Oman		bla_{NDM-1}, bla_{SHV-28}, $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{OXA-9}	Poirel et al. (2011b)
					bla_{NDM-1}, bla_{SHV-11}, bla_{OXA-1}	
2007–2009	*rmtC*	*K. pneumoniae*	Kenya		bla_{NDM-1}, bla_{CMY-6}, $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{OXA-9},	Poirel et al. (2011d)
Not reported	*armA*	*E. coli*	Spain		bla_{NDM-1}, bla_{TEM-1}, $bla_{CTX-M-15}$	Sole et al. (2011)
2005	*armA*	*K. pneumoniae*	Sweden	Sputum	bla_{VIM-1}, bla_{TEM-1}, bla_{SHV-11}, $bla_{CTX-M-3}$	Samuelsen et al. (2011b)
2010	*armA*	*K. pneumoniae*	Norway	Catheter urine	bla_{NDM-1}, bla_{TEM-1}, bla_{SHV-11}, $bla_{CTX-M-15}$	Samuelsen et al. (2011a)
2007–2009	*armA*	*A. baumannii*	Norway		bla_{OXA-66}	Karah et al. (2011)
Not reported	*armA*	*E. coli*	UK		bla_{NDM-1}	Mushtaq et al. (2011)
			Pakistan		bla_{NDM-1}	
	rmtC		UK		bla_{NDM-1}	
			India		bla_{NDM-1}	
			Pakistan		bla_{NDM-1}	
	armA and *rmtC*		Pakistan		bla_{NDM-1}	
Not reported	*armA*	*K. pneumoniae*	Bangladesh		bla_{NDM-1}, $bla_{CTX-M-15}$,	Islam et al. (in press)
	rmtB	*C. freundii*			bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{CMY}	
2007–2009	*rmtB*	*K. pneumoniae*	Greece		bla_{KPC-2}, bla_{OXA-10}	Galani et al. (2012)
					bla_{DHA-1}	
		P. mirabilis			bla_{VIM-1}, bla_{OXA-10}	
					bla_{OXA-10}	
2011	*armA*	*P. stuartii*	Afghanistan	Blood	bla_{NDM-1}, bla_{OXA-10}, *sul1*, *aadA*, *aac(6′)*, *qnrA1*	McGann et al. (2012)
Not reported	*armA*	*K. pneumoniae*	Spain	Pus from abdominal abscess	bla_{NDM-1}, $bla_{CTX-M-15}$, *sul1*, *aac(6′)-Ib-cr*, *qnrB*, *dfrA12*	Oteo et al. (in press)
Not reported	*armA*	*K. pneumoniae*	Oman		bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{SHV-12}, bla_{OXA-1}, bla_{TEM-1}	Dortet et al. (in press)
					bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{SHV-12}, bla_{OXA-9}, bla_{TEM-1}	
					bla_{NDM-1}, $bla_{CTX-M-15}$, $bla_{SHV-130}$, bla_{OXA-1}	
					bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{SHV-12}, bla_{OXA-1},	
					bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{SHV-2}, bla_{OXA-1},	

proteins QnrA, QnrB, and AAC(6′)-Ib-cr, and plasmid-mediated class C β-lactamases like CMY- and DHA-types were coproduced with 16S-RMTases (Jiang et al., 2010; Liu et al., 2008; Poirel et al., 2011a). The fluoroquinolone efflux transporter gene, *qepA*, was specifically linked to *rmtB* in the *Enterobacteriaceae* from sources of human, pets, and livestock-farming animals (Deng et al., 2011a,b; Liu et al., 2008; Yamane et al., 2008b), and this genetic association is generally observed as shown in Fig. 4. Acquisition of 16S-RMTase gene together with other antimicrobial resistance determinants is apparently implicated in development of multidrug-resistant properties in Gram-negative pathogens, and this would limit antimicrobial therapeutic options in both clinical and veterinary settings.

7. Screening procedure of 16S-RMTase-producing bacteria

7.1. N7-G1405 16S-RMTase producers

Development of practical screening techniques for detection of 16S-RMTase-producing pathogens will be of great assistance in their epidemiological study and rapid identification in clinical microbiology laboratories. The hallmark of N7-G1405 16S-RMTase-producing strains is their high resistance to the 4,6-disubstituted DOS group (Table 2). The high MIC values (≥128 μg/ml) of both amikacin and gentamicin are a good indicator of N7-G1405 16S-RMTase-producers (Doi and Arakawa, 2007). However, their susceptibility profiles are routinely determined only at around breakpoint concentrations in clinical microbiology laboratories when using automated susceptibility testing equipment. Therefore, it seems economical that only bacterial strains, that are determined to be resistant to both amikacin and gentamicin by routine automated susceptibility testing, be subjected to the susceptibility testing method covering a high MIC range for screening of N7-G1405 16S-RMTase-producers. The disk diffusion method is available for screening. The N7-G1405 16S-RMTase-producers can exhibit little or no inhibitory zone around both amikacin and gentamicin disks. The susceptibility to 4,5-disubstituted DOS (like neomycin) and streptomycin is less likely to be an indicator of production of N7-G1405 16S-RMTase, because N7-G1405 16S-RMTase production cannot confer evident resistance to these aminoglycosides, whereas aminoglycoside phosphotransferase and nucleotidyltransferase, that are often coproduced with 16S-RMTase, can afford resistance to these agents. One remaining issue is to what bacterial species the screening scheme described above would be applicable. Indeed, the application of the screening method to the member of family *Enterobacteriaceae* and a part of non-fermentative Gram-negative rods such as *P. aeruginosa* and *A. baumannii* has so far been demonstrated to work. However, the method would also select some member of non-fermentative Gram-negative rods, e.g., *Stenotrophomonas maltophilia*, *Chryseobacterium indologenes*, and *Achromobacter xylosoxidans* that lack production of the 16S-RMTase, since these microbes usually demonstrate inherent pan-aminoglycoside-resistance phenotype. No identification of N7-G1405 16S-RMTase gene in a member of non-fermentative Gram-negative rods with the exception of *P. aeruginosa* and *A. baumannii* has so far been reported. Accordingly, PCR seems the only confirmatory method for detecting N7-G1405 16S-RMTase genes at present. Recommended primers and cycle condition have been described in the previous literature (Doi and Arakawa, 2007; Poirel et al., 2011c).

7.2. N1-A1408 16S-RMTase producers

A practical screening method to detect the production of the other kind of 16S-RMTase, N1-A1408 16S-RMTase, has not been established yet, because only one isolate was reported to date. However, it is expected to be quite difficult to find N1-A1408 16S-RMTase producers depending on the result of routine antimicrobial susceptibility testing alone, because production of N1-A1408 16S-RMTase has hardly any distinctive outstanding characteristics like the highly aminoglycoside resistant nature as seen among N7-G1405 16S-RMTase producers (Table 2). The resistance profile to clinically utilized aminoglycosides conferred by N1-A1408 16S-RMTase was similar to that by co-production of multiple aminoglycoside-modifying enzymes. The possible hallmark of N1-A1408 16S-RMTase producers is its high resistance to mono-substituted DOS, apramycin, a veterinary aminoglycoside used for the treatment of bacterial infections and growth promotion in some countries. In fact, an *E. coli* clinical isolate (ARS3) producing N1-A1408 16S-RMTase, NpmA, was identified in the selection with apramycin. On the one hand, the selection with the phenotype of apramycin resistance reportedly could also enable identification of another resistance mechanism, *aac(3)-IV*, which is the most prevalent gene for apramycin resistance in *E. coli* (Jensen et al., 2006). Further collection of nonclonal N1-A1408 16S-RMTase producers and substantial additional examination will be necessary to establish the practical and feasible screening methods for N1-A1408 16S-RMTase producers.

8. Future considerations

8.1. Potential transfer of 16S-RMTase gene to Gram-positive bacteria

Acquisition of multidrug resistance in Gram-negative pathogens is becoming one of the most serious problems for human health. Among the genetic factors responsible for aminoglycoside resistance, the 16S-RMTases can confer a higher and broader spectrum aminoglycoside resistance than any other resistance determinants known so far, and they are a major factor contributing to the very high aminoglycoside resistance among members of the family *Enterobacteriaceae*, together with *P. aeruginosa* and *A. baumannii*. Identification of 16S-RMTases has been exclusively limited to Gram-negative pathogens to date, but not in Gram-positive pathogens such as genus *Staphylococcus*, *Streptococcus* and *Enterococcus*. However, it was confirmed that 16S-RMTases derived from Gram-negative pathogens have been functional and could provide a high level of aminoglycoside resistance under the native promoters in heterologous Gram-positive bacteria, *Bacillus subtilis*, and *Staphylococcus aureus* when artificially expressed under the appropriate promoters (Liou et al., 2006; Wachino et al., 2010). In Japan, arbekacin, a semisynthetic aminoglycoside, as well as vancomycin, has been used as one of the antimicrobial agents effective for MRSA infections for more than 10 years. Under such clinical circumstances, the emergence of 16S-RMTase-positive MRSA would become an actual clinical obstruction through limiting the choice of available antimicrobials in chemotherapy. We should, therefore, pay careful attention to the emergence and spread of the 16S-RMTase-producers, hereafter, not only in Gram-negative, but also in Gram-positive microbes.

8.2. Development of new potent agents against 16S-RMTase producers

Recently, a next-generation aminoglycoside (called neoglycoside), ACHN-490 (a semi-synthetic aminoglycoside derived from sisomicin), was developed as a candidate agent to cope with multidrug-resistant bacteria (Armstrong and Miller, 2010). In fact, ACHN-490 had potent activity in vitro against both Gram-negative and -positive pathogens, even amikacin- and/or

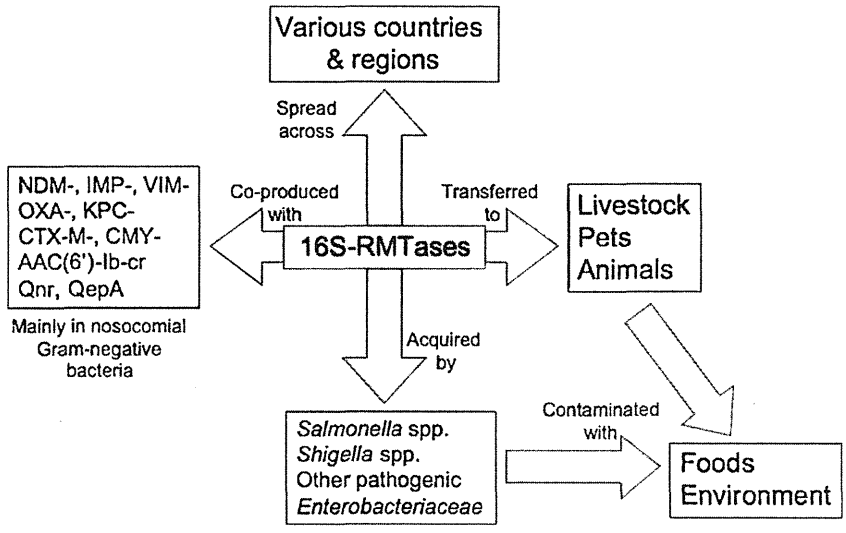

Fig. 6. 16S-RMTases posing a new threat to human health. Various Gram-negative pathogenic bacteria belonging to the family *Enterobacteriaceae* including *Salmonella* and *Shigella* spp., have acquired the 16S-RMTase genes, and they have been disseminated worldwide. These 16S-RMTase-producing microbes usually co-produce other antimicrobial resistance enzymes such as NDM-1 and CTX-M-type ESBL. Moreover, they are also recovered from livestock, pet animals and foods, as well as human. Thus, their further global spread is a great concern from the viewpoint of human health.

gentamicin-resistant strains that produce a variety of aminoglycoside-modifying enzymes (Landman et al., 2010; Tenover et al., 2011). However, ACHN-490 is reportedly less active against the 16S-RMTase producers (Livermore et al., 2011). This fact suggests that even newly developed ACHN-490 is already vulnerable to the defense mechanism provided by 16S-RMTases. Thus, exploitation of next-generation aminoglycosides or other antimicrobials not influenced by 16S-RMTase production is warranted.

One possible way to mitigate obstacles by 16S-RMTases and restore the clinical efficacy of existing aminoglycosides for the treatment of infectious diseases is to develop potent inhibitors that can specifically block 16S-RMTase activity. To realize the above objective, it is essential to understand the mode of interaction between the 16S-RMTase and its substrate including a cofactor through elucidation of three-dimensional structure provided by X-ray crystallographic analyses. To date, the structures of N7-G1405 16S-RMTases (Sgm, ArmA, RmtB) and N1-A1408 16S-RMTases (KamB, NpmA) from aminoglycoside-producing *actinomycetes* and pathogenic bacteria have been solved and deposited in the Protein Data Bank (Husain et al., 2010; Macmaster et al., 2010; Schmitt et al., 2009). Interestingly, the overall structures of 16S-RMTases from *actinomycetes* and pathogenic bacteria are quite similar, and the key amino acid residues essential for their activity are well conserved among them, despite their significant dissimilarity (approximately less than 30%) in view of the overall alignments of amino acid residues. Seven N7-G1405 16S-RMTases, ArmA and RmtA to RmtE, demonstrate a considerable similarity in the amino acid sequence level. At least 6 amino acid residues are generally conserved among the seven acquired N7-G1405 16S-RMTases, and the consensus residues are also conserved in GrmO, GrmA, and Kmr of *S. kanamyceticus* (Fig. 3B). These conserved residues were initially speculated to form the active center of the enzymes and play an important role in the enzyme reactions. Some of these amino acid residues were later found to locate in the catalytic motifs of the enzymes involved in the transfer of the methyl group to the N7 position of G1405 of bacterial 16S rRNA (Macmaster et al., 2010; Schmitt et al., 2009). Interestingly, consensus amino acid residues are also conserved among the N1-A1408 16S-RMTases including NpmA, but no considerable similarity in the amino acid alignments was seen between N7-G1405 and N1-A1408 16S-RMTases (Fig. 3B and C). Further determination of fine 3D structures of the 16S-RMTases would provide additional knowledge useful for development of the potent specific inhibitors which can be used to establish the screening method for detection of the 16S-RMTase-producing bacteria in clinical and veterinary microbiology laboratories, and will be a useful tool for better antimicrobial chemotherapy.

8.3. Strategic surveillance of 16S-RMTase-producers

As described above, the 16S-RMTase-producing Gram-negative pathogenic bacteria have been disseminated worldwide and these microbes tend to show multidrug-resistant phenotypes though acquiring various antimicrobial resistance genes such as bla_{NDM-1} and $bla_{CTX-M-15}$. Moreover, genes for 16S-RMTases have been acquired by highly pathogenic microbes like *Salmonella* spp. and *Shigella* spp. Furthermore, these pathogens have already been recovered from livestock, pets, and food (Table 4, Fig. 6), as well as from human. Thereby, it would be very important to continue monitoring the trend of 16S-RMTase producers under countrywide surveillance programs in both human and animal to prevent their further global spread.

9. Conclusion

The era of multidrug-resistant pathogenic Gram-negative bacteria including the members of family *Enterobacteriaceae* and glucose non-fermentative bacilli has arrived. These so-called invincible microbes have been fully armed with various newly emerged antimicrobial resistance mechanisms such as 16S-RMTases and MBLs like NDM-1, and spreading worldwide. As described above, various sets of multifarious antimicrobial resistance genetic determinants have been highly organized and often mediated by diverse mobile genetic elements and integrons embedded usually into a variety of transferable large plasmids, predicting further continuous global proliferation of such stubborn microorganisms. Therefore, we should confront the stern realities and devote our utmost knowledge and skill to cope with such threatening superbugs.

Acknowledgments

A series of our studies on plasmid-mediated 16S-RMTases identified in pathogenic Gram-negative bacteria has been supported by the grants (H18-Shinkou-Ippan-011, H21-Shinkou-Ippan-008, and H24-Shinkou-Ippan-010) from the Ministry of Health Labour and Welfare, Japan.

References

Armstrong, E.S., Miller, G.H., 2010. Combating evolution with intelligent design: the neoglycoside ACHN-490. Current Opinion in Microbiology 13, 565–573.

Arpin, C., Quentin, C., Grobost, F., Cambau, E., Robert, J., Dubois, V., Coulange, L., André, C., 2009. Scientific Committee of ONERBA. Nationwide survey of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in the French community setting. Journal of Antimicrobial Chemotherapy 63, 1205–1214.

Beauclerk, A.A., Cundliffe, E., 1987. Sites of action of two ribosomal RNA methylases responsible for resistance to aminoglycosides. Journal of Molecular Biology 193, 661–671.

Bercot, B., Poirel, L., Nordmann, P., 2008. Plasmid-mediated 16S rRNA methylases among extended-spectrum β-lactamase-producing *Enterobacteriaceae* isolates. Antimicrobial Agents and Chemotherapy 52, 4526–4527.

Bercot, B., Poirel, L., Ozdamar, M., Hakko, E., Turkoglu, S., Nordmann, P., 2010. Low prevalence of 16S methylases among extended-spectrum-β-lactamase-producing *Enterobacteriaceae* from a Turkish hospital. Journal of Antimicrobial Chemotherapy 65, 797–798.

Bogaerts, P., Bouchahrouf, W., de Castro, R.R., Deplano, A., Berhin, C., Pierard, D., Denis, O., Glupczynski, Y., 2011. Emergence of NDM-1-producing *Enterobacteriaceae* in Belgium. Antimicrobial Agents and Chemotherapy 55, 3036–3038.

Bogaerts, P., Galimand, M., Bauraing, C., Deplano, A., Vanhoof, R., De Mendonca, R., Rodriguez-Villalobos, H., Struelens, M., Glupczynski, Y., 2007. Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. Journal of Antimicrobial Chemotherapy 59, 459–464.

Borovinskaya, M.A., Pai, R.D., Zhang, W., Schuwirth, B.S., Holton, J.M., Hirokawa, G., Kaji, H., Kaji, A., Cate, J.H., 2007. Structural basis for aminoglycoside inhibition of bacterial ribosome recycling. Nature Structural & Molecular Biology 14, 727–732.

Bouzidi, N., Aoun, L., Dekhil, M., Granier, S.A., Poirel, L., Brisabois, A., Nordmann, P., Millemann, Y., 2011. Co-occurrence of aminoglycoside resistance gene armA in non-Typhi *Salmonella* isolates producing CTX-M-15 in Algeria. Journal of Antimicrobial Chemotherapy 66, 2180–2181.

Brigante, G., Migliavacca, R., Bramati, S., Motta, E., Nucleo, E., Manenti, M., Migliorino, G., Pagani, L., Luzzaro, F., Vigano, F.E., 2012. Emergence and spread of a multidrug-resistant *Acinetobacter baumannii* clone producing both the carbapenemase OXA-23 and the 16S rRNA methylase ArmA. Journal of Medical Microbiology 61, 653–661.

Carattoli, A., Villa, L., Poirel, L., Bonnin, R.A., Nordmann, P., 2012. Evolution of IncA/C *bla*cmy-2-carrying plasmids by acquisition of the *bla*ndm-1 carbapenemase gene. Antimicrobial Agents and Chemotherapy 56, 783–786.

Castanheira, M., Fritsche, T.R., Sader, H.S., Jones, R.N., 2008. RmtD 16S RNA methylase in epidemiologically unrelated SPM-1-producing *Pseudomonas aeruginosa* isolates from Brazil. Antimicrobial Agents and Chemotherapy 52, 1587–1588, author reply 1588.

Chen, L., Chen, Z.L., Liu, J.H., Zeng, Z.L., Ma, J.Y., Jiang, H.X., 2007. Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. Journal of Antimicrobial Chemotherapy 59, 880–885.

Choi, M.J., Lim, S.K., Nam, H.M., Kim, A.R., Jung, S.C., Kim, M.N., 2011. Apramycin and gentamicin resistances in indicator and clinical *Escherichia coli* isolates from farm animals in Korea. Foodborne Pathogens and Disease 8, 119–123.

Cundliffe, E., 1989. How antibiotic-producing organisms avoid suicide. Annual Review of Microbiology 43, 207–233.

Davis, M.A., Baker, K.N., Orfe, L.H., Shah, D.H., Besser, T.E., Call, D.R., 2010. Discovery of a gene conferring multiple-aminoglycoside resistance in *Escherichia coli*. Antimicrobial Agents and Chemotherapy 54, 2666–2669.

Demydchuk, J., Oliynyk, Z., Fedorenko, V., 1998. Analysis of a kanamycin resistance gene (*kmr*) from *Streptomyces kanamyceticus* and a mutant with increased aminoglycoside resistance. Journal of Basic Microbiology 38, 231–239.

Deng, Y., He, L., Chen, S., Zheng, H., Zeng, Z., Liu, Y., Sun, Y., Ma, J., Chen, Z., Liu, J.H., 2011a. F33:A-:B- and F2:A-:B- plasmids mediate dissemination of *rmtB*-$bla_{CTX-M-9}$ group genes and *rmtB*-*qepA* in *Enterobacteriaceae* isolates from pets in China. Antimicrobial Agents and Chemotherapy 55, 4926–4929.

Deng, Y., Zeng, Z., Chen, S., He, L., Liu, Y., Wu, C., Chen, Z., Yao, Q., Hou, J., Yang, T., Liu, J.H., 2011b. Dissemination of IncFII plasmids carrying *rmtB* and *qepA* in *Escherichia coli* from pigs, farm workers and the environment. Clinical Microbiology and Infection 17, 1740–1745.

Doi, Y., Adams, J.M., Yamane, K., Paterson, D.L., 2007a. Identification of 16S rRNA methylase-producing *Acinetobacter baumannii* clinical strains in North America. Antimicrobial Agents and Chemotherapy 51, 4209–4210.

Doi, Y., Adams-Haduch, J.M., Paterson, D.L., 2008. Genetic environment of 16S rRNA methylase gene *rmtD*. Antimicrobial Agents and Chemotherapy 52, 2270–2272.

Doi, Y., Arakawa, Y., 2007. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clinical Infectious Diseases 45, 88–94.

Doi, Y., de Oliveira Garcia, D., Adams, J., Paterson, D.L., 2007b. Coproduction of novel 16S rRNA methylase RmtD and metallo-β-lactamase SPM-1 in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. Antimicrobial Agents and Chemotherapy 51, 852–856.

Doi, Y., Ghilardi, A.C., Adams, J., de Oliveira Garcia, D., Paterson, D.L., 2007c. High prevalence of metallo-β-lactamase and 16S rRNA methylase coproduction among imipenem-resistant *Pseudomonas aeruginosa* isolates in Brazil. Antimicrobial Agents and Chemotherapy 51, 3388–3390.

Doi, Y., Yokoyama, K., Yamane, K., Wachino, J., Shibata, N., Yagi, T., Shibayama, K., Kato, H., Arakawa, Y., 2004. Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. Antimicrobial Agents and Chemotherapy 48, 491–496.

Dortet, L., Poirel, L., Al Yaqoubi, F., Nordmann, P. NDM-1, OXA-48 and OXA-181 carbapenemase-producing *Enterobacteriaceae* in Sultanate of Oman. Clinical Microbiology and Infection, in press.

Du, X.D., Li, D.X., Hu, G.Z., Wang, Y., Shang, Y.H., Wu, C.M., Liu, H.B., Li, X.S., 2012. Tn*1548*-associated *armA* is co-located with *qnrB2*, *aac(6′)-Ib-cr* and $bla_{CTX-M-3}$ on an IncFII plasmid in a *Salmonella enterica* subsp. *enterica* serovar Paratyphi B strain isolated from chickens in China. Journal of Antimicrobial Chemotherapy 67, 246–248.

Du, X.D., Wu, C.M., Liu, H.B., Li, X.S., Beier, R.C., Xiao, F., Qin, S.S., Huang, S.Y., Shen, J.Z., 2009. Plasmid-mediated ArmA and RmtB 16S rRNA methylases in *Escherichia coli* isolated from chickens. Journal of Antimicrobial Chemotherapy 64, 1328–1330.

Folster, J.P., Rickert, R., Barzilay, E.J., Whichard, J.M., 2009. Identification of the aminoglycoside resistance determinants *armA* and *rmtC* among non-Typhi *Salmonella* isolates from humans in the United States. Antimicrobial Agents and Chemotherapy 53, 4563–4564.

Fontes, L.C., Neves, P.R., Oliveira, S., Silva, K.C., Hachich, E.M., Sato, M.I., Lincopan, N., 2011. Isolation of *Pseudomonas aeruginosa* coproducing metallo-β-lactamase SPM-1 and 16S rRNA methylase RmtD1 in an urban river. Antimicrobial Agents and Chemotherapy 55, 3063–3064.

Fritsche, T.R., Castanheira, M., Miller, G.H., Jones, R.N., Armstrong, E.S., 2008. Detection of methyltransferases conferring high-level resistance to aminoglycosides in *Enterobacteriaceae* from Europe, North America, and Latin America. Antimicrobial Agents and Chemotherapy 52, 1843–1845.

Galani, I., Souli, M., Panagea, T., Poulakou, G., Kanellakopoulou, K., Giamarellou, H., 2012. Prevalence of 16S rRNA methylase genes in *Enterobacteriaceae* isolates from a Greek University Hospital. Clinical Microbiology and Infection 18, E52–E54.

Galimand, M., Courvalin, P., Lambert, T., 2003. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. Antimicrobial Agents and Chemotherapy 47, 2565–2571.

Galimand, M., Sabtcheva, S., Courvalin, P., Lambert, T., 2005. Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn*1548*. Antimicrobial Agents and Chemotherapy 49, 2949–2953.

Golebiewski, M., Kern-Zdanowicz, I., Zienkiewicz, M., Adamczyk, M., Zylinska, J., Baraniak, A., Gniadkowski, M., Bardowski, J., Ceglowski, P., 2007. Complete nucleotide sequence of the pCTX-M3 plasmid and its involvement in spread of the extended-spectrum β-lactamase gene $bla_{CTX-M-3}$. Antimicrobial Agents and Chemotherapy 51, 3789–3795.

Gonzalez-Zorn, B., Catalan, A., Escudero, J.A., Dominguez, L., Teshager, T., Porrero, C., Moreno, M.A., 2005a. Genetic basis for dissemination of *armA*. Journal of Antimicrobial Chemotherapy 56, 583–585.

Gonzalez-Zorn, B., Teshager, T., Casas, M., Porrero, M.C., Moreno, M.A., Courvalin, P., Dominguez, L., 2005b. *armA* and aminoglycoside resistance in *Escherichia coli*. Emerging Infectious Diseases 11, 954–956.

Granier, S.A., Hidalgo, L., San Millan, A., Escudero, J.A., Gutierrez, B., Brisabois, A., Gonzalez-Zorn, B., 2011. ArmA methyltransferase in a monophasic *Salmonella enterica* isolate from food. Antimicrobial Agents and Chemotherapy 55, 5262–5266.

Gurung, M., Moon, D.C., Tamang, M.D., Kim, J., Lee, Y.C., Seol, S.Y., Cho, D.T., Lee, J.C., 2010. Emergence of 16S rRNA methylase gene *armA* and cocarriage of bla_{IMP-1} in *Pseudomonas aeruginosa* isolates from South Korea. Diagnostic Microbiology and Infectious Disease 68, 468–470.

Ho, P.L., Lo, W.U., Yeung, M.K., Lin, C.H., Chow, K.H., Ang, I., Tong, A.H., Bao, J.Y., Lok, S., Lo, J.Y., 2011. Complete sequencing of pNDM-HK encoding NDM-1 carbapenemase from a multidrug-resistant *Escherichia coli* strain isolated in Hong Kong. PLoS One 6, e17989.

Hou, J., Huang, X., Deng, Y., He, L., Yang, T., Zeng, Z., Chen, Z., Liu, J.H., 2012. Dissemination of the fosfomycin resistance gene *fosA3* with CTX-M β-lactamase genes and *rmtB* carried on IncFII plasmids among *Escherichia coli* isolates from pets in China. Antimicrobial Agents and Chemotherapy 56, 2135–2138.

Hopkins, K.L., Escudero, J.A., Hidalgo, L., Gonzalez-Zorn, B., 2010. 16S rRNA methyltransferase RmtC in *Salmonella enterica* serovar Virchow. Emerging Infectious Diseases 16, 712–715.

Husain, N., Tkaczuk, K.L., Tulsidas, S.R., Kaminska, K.H., Cubrilo, S., Maravic-Vlahovicek, G., Bujnicki, J.M., Sivaraman, J., 2010. Structural basis for the methylation of G1405 in 16S rRNA by aminoglycoside resistance methyltransferase Sgm from an antibiotic producer: a diversity of active sites in m7G methyltransferases. Nucleic Acids Research 38, 4120–4132.

Islam, M.A., Talukdar, P.K., Hoque, A., Huq, M., Nabi, A., Ahmed, D., Talukder, K.A., Pietroni, M.A., Hays, J.P., Cravioto, A., Endtz, H.P. Emergence of multidrug-resistant NDM-1-producing Gram-negative bacteria in Bangladesh. European Journal of Clinical Microbiology & Infectious Diseases, in press.

Jensen, V.F., Jakobsen, L., Emborg, H.D., Seyfarth, A.M., Hammerum, A.M., 2006. Correlation between apramycin and gentamicin use in pigs and an

increasing reservoir of gentamicin-resistant *Escherichia coli*. Journal of Antimicrobial Chemotherapy 58, 101–107.

Jiang, Y., Yu, D., Wei, Z., Shen, P., Zhou, Z., Yu, Y., 2010. Complete nucleotide sequence of *Klebsiella pneumoniae* multidrug resistance plasmid pKP048, carrying bla_{KPC-2}, bla_{DHA-1}, *qnrB4*, and *armA*. Antimicrobial Agents and Chemotherapy 54, 3967–3969.

Jin, J.S., Kwon, K.T., Moon, D.C., Lee, J.C., 2009. Emergence of 16S rRNA methylase *rmtA* in colistin-only-sensitive *Pseudomonas aeruginosa* in South Korea. International Journal of Antimicrobial Agents 33, 490–491.

Kang, H.Y., Kim, K.Y., Kim, J., Lee, J.C., Lee, Y.C., Cho, D.T., Seol, S.Y., 2008. Distribution of conjugative-plasmid-mediated 16S rRNA methylase genes among amikacin-resistant *Enterobacteriaceae* isolates collected in 1995 to 1998 and 2001 to 2006 at a university hospital in South Korea and identification of conjugative plasmids mediating dissemination of 16S rRNA methylase. Journal of Clinical Microbiology 46, 700–706.

Karah, N., Haldorsen, B., Hermansen, N.O., Tveten, Y., Ragnhildstveit, E., Skutlaberg, D.H., Tofteland, S., Sundsfjord, A., Samuelsen, Ø., 2011. Emergence of OXA-carbapenemase- and 16S rRNA methylase-producing international clones of *Acinetobacter baumannii* in Norway. Journal of Medical Microbiology 60, 515–521.

Karthikeyan, K., Thirunarayan, M.A., Krishnan, P., 2010. Coexistence of bla_{OXA-23} with bla_{NDM-1} and *armA* in clinical isolates of *Acinetobacter baumannii* from India. Journal of Antimicrobial Chemotherapy 65, 2253–2254.

Kharel, M.K., Subba, B., Basnet, D.B., Woo, J.S., Lee, H.C., Liou, K., Sohng, J.K., 2004. A gene cluster for biosynthesis of kanamycin from *Streptomyces kanamyceticus*: comparison with gentamicin biosynthetic gene cluster. Archives of Biochemistry and Biophysics 429, 204–214.

Kholodii, G.Y., Yurieva, O.V., Gorlenko, Z., Mindlin, S.Z., Bass, I.A., Lomovskaya, O.L., Kopteva, A.V., Nikiforov, V.G., 1997. Tn*5041*: a chimeric mercury resistance transposon closely related to the toluene degradative transposon Tn*4651*. Microbiology 143 (Pt 8), 2549–2556.

Kim, J.W., Heo, S.T., Jin, J.S., Choi, C.H., Lee, Y.C., Jeong, Y.G., Kim, S.J., Lee, J.C., 2008. Characterization of *Acinetobacter baumannii* carrying bla_{OXA-23}, bla_{PER-1} and *armA* in a Korean hospital. Clinical Microbiology and Infection 14, 716–718.

Kojic, M., Topisirovic, L., Vasiljevic, B., 1992. Cloning and characterization of an aminoglycoside resistance determinant from *Micromonospora zionensis*. Journal of Bacteriology 174, 7868–7872.

Landman, D., Babu, E., Shah, N., Kelly, P., Backer, M., Bratu, S., Quale, J., 2010. Activity of a novel aminoglycoside, ACHN-490, against clinical isolates of Escherichia coli and Klebsiella pneumoniae from New York City. Journal of Antimicrobial Chemotherapy 65, 2123–2127.

Lee, H., Yong, D., Yum, J.H., Roh, K.H., Lee, K., Yamane, K., Arakawa, Y., Chong, Y., 2006. Dissemination of 16S rRNA methylase-mediated highly amikacin-resistant isolates of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in Korea. Diagnostic Microbiology and Infectious Disease 56, 305–312.

Lee, H.W., Kang, H.Y., Shin, K.S., Kim, J., 2007. Multidrug-resistant *Providencia* isolates carrying bla_{PER-1}, bla_{VIM-2}, and *armA*. Journal of Microbiology 45, 272–274.

Li, D.X., Zhang, S.M., Hu, G.Z., Wang, Y., Liu, H.B., Wu, C.M., Shang, Y.H., Chen, Y.X., Du, X.D., 2012. Tn3-associated *rmtB* together with *qnrS1*, *aac(6′)-Ib-cr* and $bla_{CTX-M-15}$ are co-located on an F49:A-:B- plasmid in an *Escherichia coli* ST10 strain in China. Journal of Antimicrobial Chemotherapy 67, 236–238.

Liou, G.F., Yoshizawa, S., Courvalin, P., Galimand, M., 2006. Aminoglycoside resistance by ArmA-mediated ribosomal 16S methylation in human bacterial pathogens. Journal of Molecular Biology 359, 358–364.

Liu, J.H., Deng, Y.T., Zeng, Z.L., Gao, J.H., Chen, L., Arakawa, Y., Chen, Z.L., 2008. Coprevalence of plasmid-mediated quinolone resistance determinants QepA, Qnr, and AAC(6′)-Ib-cr among 16S rRNA methylase RmtB-producing Escherichia coli isolates from pigs. Antimicrobial Agents and Chemotherapy 52, 2992–2993.

Livermore, D.M., Mushtaq, S., Warner, M., Zhang, J.C., Maharjan, S., Doumith, M., Woodford, N., 2011. Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant *Enterobacteriaceae* isolates. Journal of Antimicrobial Chemotherapy 66, 48–53.

Macmaster, R., Zelinskaya, N., Savic, M., Rankin, C.R., Conn, G.L., 2010. Structural insights into the function of aminoglycoside-resistance A1408 16S rRNA methyltransferases from antibiotic-producing and human pathogenic bacteria. Nucleic Acids Research 38, 7791–7799.

Magnet, S., Blanchard, J.S., 2005. Molecular insights into aminoglycoside action and resistance. Chemical Reviews 105, 477–498.

McGann, P., Hang, J., Clifford, R.J., Yang, Y., Kwak, Y.I., Kuschner, R.A., Lesho, E.P., Waterman, P.E., 2012. Complete Sequence of a Novel 178-Kilobase Plasmid Carrying bla_{NDM-1} in a *Providencia stuartii* Strain Isolated in Afghanistan. Antimicrobial Agents and Chemotherapy 56, 1673–1679.

Moissenet, D., Weill, F.X., Arlet, G., Harrois, D., Girardet, J.P., Vu-Thien, H., 2011. *Salmonella enterica* serotype Gambia with CTX-M-3 and *armA* resistance markers: nosocomial infections with a fatal outcome. Journal of Clinical Microbiology 49, 1676–1678.

Mushtaq, S., Irfan, S., Sarma, J.B., Doumith, M., Pike, R., Pitout, J., Livermore, D.M., Woodford, N., 2011. Phylogenetic diversity of *Escherichia coli* strains producing NDM-type carbapenemases. Journal of Antimicrobial Chemotherapy 66, 2002–2005.

Naas, T., Bentchouala, C., Cuzon, G., Yaou, S., Lezzar, A., Smati, F., Nordmann, P., 2011. Outbreak of *Salmonella enterica* serotype Infantis producing ArmA 16S RNA methylase and CTX-M-15 extended-spectrum β-lactamase in a neonatology ward in Constantine, Algeria. International Journal of Antimicrobial Agents 38, 135–139.

Ohta, T., Hasegawa, M., 1993a. Analysis of the nucleotide sequence of *fmrT* encoding the self-defense gene of the istamycin producer, *Streptomyces tenjimariensis* ATCC 31602; comparison with the squences of *kamB of Streptomyces tenebrarius* NCIB 11028 and *kamC of Saccharopolyspora hirsuta* CL102. Journal of Antibiotics 46, 511–517.

Ohta, T., Hasegawa, M., 1993b. Analysis of the self-defense gene (*fmrO*) of a fortimicin A (astromicin) producer, *Micromonospora olivasterospora*: comparison with other aminoglycoside-resistance-encoding genes. Gene 127, 63–69.

Oteo, J., Domingo-Garcia, D., Fernandez-Romero, S., Saez, D., Guiu, A., Cuevas, O., Lopez-Brea, M., Campos, J. Abdominal abscess due to NDM-1-producing *Klebsiella pneumoniae* in Spain. Journal of Medical Microbiology, in press.

Park, Y.J., Lee, S., Yu, J.K., Woo, G.J., Lee, K., Arakawa, Y., 2006. Co-production of 16S rRNA methylases and extended-spectrum β-lactamases in AmpC-producing *Enterobacter cloacae*, *Citrobacter freundii and Serratia marcescens* in Korea. Journal of Antimicrobial Chemotherapy 58, 907–908.

Park, Y.J., Yu, J.K., Kim, S.I., Lee, K., Arakawa, Y., 2009. Accumulation of plasmid-mediated fluoroquinolone resistance genes, *qepA* and *qnrS1*, in *Enterobacter aerogenes* co-producing RmtB and class A β-lactamase LAP-1. Annals of Clinical and Laboratory Science 39, 55–59.

Partridge, S.R., Tsafnat, G., Coiera, E., Iredell, J.R., 2009. Gene cassettes and cassette arrays in mobile resistance integrons. FEMS Microbiology Review 33, 757–784.

Perichon, B., Bogaerts, P., Lambert, T., Frangeul, L., Courvalin, P., Galimand, M., 2008. Sequence of conjugative plasmid pIP1206 mediating resistance to aminoglycosides by 16S rRNA methylation and to hydrophilic fluoroquinolones by efflux. Antimicrobial Agents and Chemotherapy 52, 2581–2592.

Perichon, B., Courvalin, P., Galimand, M., 2007. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. Antimicrobial Agents and Chemotherapy 51, 2464–2469.

Poirel, L., Bonnin, R.A., Nordmann, P., 2011a. Analysis of the resistome of a multidrug-resistant NDM-1-producing *Escherichia coli* strain by high-throughput genome sequencing. Antimicrobial Agents and Chemotherapy 55, 4224–4229.

Poirel, L., Al Maskari, Z., Al Rashdi, F., Bernabeu, S., Nordmann, P., 2011b. NDM-1-producing *Klebsiella pneumoniae* isolated in the Sultanate of Oman. Journal of Antimicrobial Chemotherapy 66, 304–306.

Poirel, L., Dortet, L., Bernabeu, S., Nordmann, P., 2011c. Genetic features of bla_{NDM-1}-positive *Enterobacteriaceae*. Antimicrobial Agents and Chemotherapy 55, 5403–5407.

Poirel, L., Lagrutta, E., Taylor, P., Pham, J., Nordmann, P., 2010. Emergence of metallo-β-lactamase NDM-1-producing multidrug-resistant *Escherichia coli* in Australia. Antimicrobial Agents and Chemotherapy 54, 4914–4916.

Poirel, L., Revathi, G., Bernabeu, S., Nordmann, P., 2011d. Detection of NDM-1-producing *Klebsiella pneumoniae* in Kenya. Antimicrobial Agents and Chemotherapy 55, 934–936.

Poirel, L., Schrenzel, J., Cherkaoui, A., Bernabeu, S., Renzi, G., Nordmann, P., 2011e. Molecular analysis of NDM-1-producing enterobacterial isolates from Geneva, Switzerland. Journal of Antimicrobial Chemotherapy 66, 1730–1733.

Poole, K., 2005. Aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 49, 479–487.

Ramirez, M.S., Tolmasky, M.E., 2010. Aminoglycoside modifying enzymes. Drug Resistance Updates 13, 151–171.

Sabtcheva, S., Saga, T., Kantardjiev, T., Ivanova, M., Ishii, Y., Kaku, M., 2008. Nosocomial spread of *armA*-mediated high-level aminoglycoside resistance in *Enterobacteriaceae* isolates producing CTX-M-3 β-lactamase in a cancer hospital in Bulgaria. Journal of Chemotherapy 20, 593–599.

Salabi, A.E., Toleman, M.A., Weeks, J., Bruderer, T., Frei, R., Walsh, T.R., 2010. First report of the metallo-β-lactamase SPM-1 in Europe. Antimicrobial Agents and Chemotherapy 54, 582.

Samuelsen, Ø., Thilesen, C.M., Heggelund, L., Vada, A.N., Kümmel, A., Sundsfjord, A., 2011a. Indentification of NDM-1-producing *Enterobacteriaceae* in Norway. Journal of Antimicrobial Chemotherapy 66, 670–672.

Samuelsen, Ø., Toleman, M.A., Hasseltvedt, V., Fuursted, K., Leegaard, T.M., Walsh, T.R., Sundsfjord, A., Giske, C.G., 2011b. Molecular characterization of VIM-producing *Klebsiella pneumoniae* from Scandinavia reveals genetic relatedness with international clonal complexes encoding transferable multidrug resistance. Clinical Microbiology and Infection 17, 1811–1816.

Schmitt, E., Galimand, M., Panvert, M., Courvalin, P., Mechulam, Y., 2009. Structural bases for 16S rRNA methylation catalyzed by ArmA and RmtB methyltransferases. Journal of Molecular Biology 388, 570–582.

Sekizuka, T., Matsui, M., Yamane, K., Takeuchi, F., Ohnishi, M., Hishinuma, A., Arakawa, Y., Kuroda, M., 2011. Complete sequencing of the bla_{NDM-1}-positive IncA/C plasmid from *Escherichia coli* ST38 isolate suggests a possible origin from plant pathogens. PLoS One 6, e25334.

Sheng, J.F., Li, J.J., Tu, S., Sheng, Z.K., Bi, S., Zhu, M.H., Shen, X.M., Li, L.J. bla_{KPC} and *rmtB* on a single plasmid in *Enterobacter amnigenus* and *Klebsiella pneumoniae* isolates from the same patient. European Journal of Clinical Microbiology & Infectious Diseases, in press.

Sole, M., Pitart, C., Roca, I., Fabrega, A., Salvador, P., Munoz, L., Oliveira, I., Gascon, J., Marco, F., Vila, J., 2011. First description of an *Escherichia coli* strain producing NDM-1 carbapenemase in Spain. Antimicrobial Agents and Chemotherapy 55, 4402–4404.

Sung, J.Y., Kwon, K.C., Cho, H.H., Koo, S.H., 2011. Antimicrobial resistance determinants in imipenem-nonsusceptible *Acinetobacter calcoaceticus-baumannii* complex isolated in Daejeon, Korea. The Korean Journal of Laboratory Medicine 31, 265–270.

Tenover, F.C., Tickler, I., Armstrong, E.S., Kubo, A., Lopez, S., Persing, D.H., Miller, G.H., 2011. Activity of ACHN-490 against methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from patients in US hospitals. International Journal of Antimicrobial Agents 38, 352–354.

Tian, G.B., Rivera, J.I., Park, Y.S., Johnson, L.E., Hingwe, A., Adams-Haduch, J.M., Doi, Y., 2011. Sequence type ST405 *Escherichia coli* isolate producing QepA1, CTX-M-15, and RmtB from Detroit, Michigan. Antimicrobial Agents and Chemotherapy 55, 3966–3967.

Tijet, N., Andres, P., Chung, C., Lucero, C., Low, D.E., Galas, M., Corso, A., Petroni, A., Melano, R.G., 2011. *rmtD2*, a new allele of a 16S rRNA methylase gene, has been present in *Enterobacteriaceae* isolates from Argentina for more than a decade. Antimicrobial Agents and Chemotherapy 55, 904–909.

Toleman, M.A., Walsh, T.R., 2011. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. FEMS Microbiology Review 35, 912–935.

Tolmasky, M.E., Crosa, J.H., 1987. Tn*1331*, a novel multiresistance transposon encoding resistance to amikacin and ampicillin in *Klebsiella pneumoniae*. Antimicrobial Agents and Chemotherapy 31, 1955–1960.

Unwin, J., Standage, S., Alexander, D., Hosted Jr., T., Horan, A.C., Wellington, E.M., 2004. Gene cluster in *Micromonospora echinospora* ATCC15835 for the biosynthesis of the gentamicin C complex. Journal of Antibiotics 57, 436–445.

Wachino, J., Shibayama, K., Kimura, K., Yamane, K., Suzuki, S., Arakawa, Y., 2010. RmtC introduces G1405 methylation in 16S rRNA and confers high-level aminoglycoside resistance on Gram-positive microorganisms. FEMS Microbiology Letters 311, 56–60.

Wachino, J., Shibayama, K., Kurokawa, H., Kimura, K., Yamane, K., Suzuki, S., Shibata, N., Ike, Y., Arakawa, Y., 2007. Novel plasmid-mediated 16S rRNA m^1A1408 methyltransferase, NpmA, found in a clinically isolated Escherichia coli strain resistant to structurally diverse aminoglycosides. Antimicrobial Agents and Chemotherapy 51, 4401–4409.

Wachino, J., Yamane, K., Kimura, K., Shibata, N., Suzuki, S., Ike, Y., Arakawa, Y., 2006a. Mode of transposition and expression of 16S rRNA methyltransferase gene *rmtC* accompanied by *ISEcp1*. Antimicrobial Agents and Chemotherapy 50, 3212–3215.

Wachino, J., Yamane, K., Shibayama, K., Kurokawa, H., Shibata, N., Suzuki, S., Doi, Y., Kimura, K., Ike, Y., Arakawa, Y., 2006b. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. Antimicrobial Agents and Chemotherapy 50, 178–184.

Wu, Q., Liu, Q., Han, L., Sun, J., Ni, Y., 2010. Plasmid-mediated carbapenem-hydrolyzing enzyme KPC-2 and ArmA 16S rRNA methylase conferring high-level aminoglycoside resistance in carbapenem-resistant *Enterobacter cloacae* in China. Diagnostic Microbiology and Infectious Disease 66, 326–328.

Wu, Q., Zhang, Y., Han, L., Sun, J., Ni, Y., 2009. Plasmid-mediated 16S rRNA methylases in aminoglycoside-resistant *Enterobacteriaceae* isolates in Shanghai, China. Antimicrobial Agents and Chemotherapy 53, 271–272.

Xia, L.N., Tao, X.Q., Shen, J.Z., Dai, L., Wang, Y., Chen, X., Wu, C.M., 2011. A survey of β-lactamase and 16S rRNA methylase genes among fluoroquinolone-resistant *Escherichia coli* isolates and their horizontal transmission in Shandong, China. Foodborne Pathogens and Disease 8, 1241–1248.

Yamane, K., Doi, Y., Yokoyama, K., Yagi, T., Kurokawa, H., Shibata, N., Shibayama, K., Kato, H., Arakawa, Y., 2004. Genetic environments of the *rmtA* gene in *Pseudomonas aeruginosa* clinical isolates. Antimicrobial Agents and Chemotherapy 48, 2069–2074.

Yamane, K., Rossi, F., Barberino, M.G., Adams-Haduch, J.M., Doi, Y., Paterson, D.L., 2008a. 16S ribosomal RNA methylase RmtD produced by *Klebsiella pneumoniae* in Brazil. Journal of Antimicrobial Chemotherapy 61, 746–747.

Yamane, K., Wachino, J., Suzuki, S., Arakawa, Y., 2008b. Plasmid-mediated *qepA* gene among *Escherichia coli* clinical isolates from Japan. Antimicrobial Agents and Chemotherapy 52, 1564–1566.

Yamane, K., Wachino, J., Suzuki, S., Kimura, K., Shibata, N., Kato, H., Shibayama, K., Konda, T., Arakawa, Y., 2007a. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrobial Agents and Chemotherapy 51, 3354–3360.

Yamane, K., Wachino, J., Suzuki, S., Shibata, N., Kato, H., Shibayama, K., Kimura, K., Kai, K., Ishikawa, S., Ozawa, Y., Konda, T., Arakawa, Y., 2007b. 16S rRNA methylase-producing, gram-negative pathogens, Japan. Emerging Infectious Diseases 13, 642–646.

Yan, J.J., Wu, J.J., Ko, W.C., Tsai, S.H., Chuang, C.L., Wu, H.M., Lu, Y.J., Li, J.D., 2004. Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from two Taiwanese hospitals. Journal of Antimicrobial Chemotherapy 54, 1007–1012.

Yao, Q., Zeng, Z., Hou, J., Deng, Y., He, L., Tian, W., Zheng, H., Chen, Z., Liu, J.H., 2011. Dissemination of the *rmtB* gene carried on IncF and IncN plasmids among *Enterobacteriaceae* in a pig farm and its environment. Journal of Antimicrobial Chemotherapy 66, 2475–2479.

Yokoyama, K., Doi, Y., Yamane, K., Kurokawa, H., Shibata, N., Shibayama, K., Yagi, T., Kato, H., Arakawa, Y., 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet 362, 1888–1893.

Yoshizawa, S., Fourmy, D., Puglisi, J.D., 1998. Structural origins of gentamicin antibiotic action. EMBO Journal 17, 6437–6448.

Yu, F.Y., Yao, D., Pan, J.Y., Chen, C., Qin, Z.Q., Parsons, C., Yang, L.H., Li, Q.Q., Zhang, X.Q., Qu, D., Wang, L.X., 2010. High prevalence of plasmid-mediated 16S rRNA methylase gene *rmtB* among *Escherichia coli* clinical isolates from a Chinese teaching hospital. BMC Infectious Diseases 10, 184.

Zacharczuk, K., Piekarska, K., Szych, J., Zawidzka, E., Sulikowska, A., Wardak, S., Jagielski, M., Gierczynski, R., 2011. Emergence of *Klebsiella pneumoniae* coproducing KPC-2 and 16S rRNA methylase ArmA in Poland. Antimicrobial Agents and Chemotherapy 55, 443–446.

Zhao, W.S., Liu, G.Y., Mi, Z.H., Zhang, F., 2011. Coexistence of bla_{OXA-23} with *armA* and novel *gyrA* mutation in a pandrug-resistant *Acinetobacter baumannii* isolate from the blood of a patient with haematological disease in China. Journal of Hospital Infection 77, 278–279.

Zong, Z., Partridge, S.R., Iredell, J.R., 2008. RmtC 16S rRNA methyltransferase in Australia. Antimicrobial Agents and Chemotherapy 52, 794–795.

Eur J Clin Microbiol Infect Dis (2012) 31:1095–1100
DOI 10.1007/s10096-011-1412-x

ARTICLE

Regional spread and control of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* in Kyoto, Japan

A. Matsushima · S. Takakura · M. Yamamoto · Y. Matsumura · M. Shirano · M. Nagao · Y. Ito · Y. Iinuma · T. Shimizu · N. Fujita · S. Ichiyama

Received: 4 August 2011 / Accepted: 30 August 2011 / Published online: 4 October 2011

Abstract The purpose of this investigation was to control the post-outbreak prevalence of vancomycin-resistant enterococci (VRE) in the affected Kyoto region. The study period was from 2005 to 2010. Faecal samples were subjected to VRE screening, and vancomycin resistance genes were detected by polymerase chain reaction (PCR). The genotype was determined by pulsed-field gel electrophoresis (PFGE) of genomic DNA digested with *Sma*I and by multilocus sequence typing (MLST). A VRE control programme was established in 2006, consisting of a laboratory-based faecal VRE screening system, annual surveillance of hospital inpatients and the promotion of adequate infection control measures. *vanA-Enterococcus faecium*, *vanB-E. faecium* and *vanB-E. faecalis* were detected at 35, 12 and 5 hospitals, respectively. Genotype analysis revealed that all of the vancomycin-resistant *E. faecium* isolates obtained since 2005 belonged to ST78, and that clonally related *vanB-E. faecalis* of ST64 had spread to three hospitals. The rate of faecal VRE carriage among the patients enrolled in the annual surveillance increased until 2007, when it reached 24 (1.2%) of the 2,035 enrolled patients. The rate began to decrease in 2008 and, by 2010, reached a low of 4 (0.17%) of the 2,408 enrolled patients. While VRE did spread within the Kyoto region, the VRE control programme succeeded in controlling the overall VRE spread.

Introduction

Vancomycin-resistant enterococci (VRE) infections arose as a global problem in the 1990s. They rapidly increased the prevalence in the hospital settings in the United States and became one of the major nosocomial pathogens by 2000 [1]. VRE-infected patients experience prolonged illness with extended hospital stays, increased costs of care and increased

A. Matsushima (✉) · S. Takakura · M. Yamamoto · Y. Matsumura · M. Shirano · M. Nagao · S. Ichiyama
Department of Infection Control and Prevention,
Kyoto University Hospital,
54 Shogoin-Kawaharacho, Sakyo,
Kyoto 6068507, Japan
e-mail: akimat@kuhp.kyoto-u.ac.jp

A. Matsushima · S. Takakura · M. Yamamoto · Y. Matsumura · M. Shirano · M. Nagao · S. Ichiyama
Department of Clinical Laboratory Medicine,
Kyoto University Graduate School of Medicine,
54 Shogoin-Kawaharacho, Sakyo,
Kyoto 6068507, Japan

Y. Ito
Department of Respiratory Medicine,
Kyoto University Graduate School of Medicine,
54 Shogoin-Kawaharacho, Sakyo,
Kyoto 6068507, Japan

Y. Iinuma
Department of Infection Diseases, Kanazawa Medical University,
1-1 Daigaku, Uchinada,
Kahoku-gun, Ishikawa 9200293, Japan

T. Shimizu
Department of Infectious Diseases, Kyoto City Hospital,
1-2 Mibu-Higashitakadacho, Nakagyo,
Kyoto 6048845, Japan

N. Fujita
Department of Infection Control and Laboratory Medicine,
Kyoto Prefectural University of Medicine,
465 Kajii-cho, Kamigyo,
Kyoto 602-8566, Japan

mortality [2, 3]. Because a period of colonisation precedes the VRE bloodstream infection, the control and prevention of VRE colonisation are important [4]. In the past several decades, an exponential increase in VRE prevalence has been observed in the United States, Korea and Greece [1, 5, 6]. In some countries, including Sweden, the spread of a predominant VRE clone has been reported, which prompted concerns about VRE becoming an endemic hospital pathogen [5].

The first *vanA-Enterococcus faecium* in Japan was reported in 1996 in Kyoto [7]. According to the Infectious Diseases Surveillance Center (IDSC), fewer than 90 confirmed cases of VRE infection have occurred per year [8]. However, the actual prevalence of VRE, including both symptomatic cases and faecal carriers, is suspected to be much higher. To date, no major epidemiological studies have been conducted in Japan. Some studies based on faecal specimens were conducted following sporadic nosocomial outbreaks in early the 2000s; however, the regional spread of VRE was not proven [9].

In 2005, a large nosocomial outbreak of *vanA-E. faecium* occurred in a single Kyoto hospital, and more than 100 faecal *vanA-E. faecium* carriers were identified. A task force was formed in order to establish a regional VRE control programme. Vancomycin-resistant *E. faecium* and *E. faecalis* did spread in the Kyoto region; however, our infection control programme successfully controlled their spread.

Materials and methods

Study settings

The study period was from the beginning of 2005 to the end of 2010. The region of interest in this study is the Kyoto Prefecture, which, in 2010, contained 177 hospitals and a population of ~2.6 million. Kyoto City (population ~1.5 million) is located in the middle of the Prefecture.

VRE definition

VRE was defined as an *E. faecium* or *E. faecalis* isolate positive for the *vanA* or *vanB* gene.

Microbiology

The screening of clinical faecal specimens or routine faecal surveillance samples (e.g. screening on admission) was performed by spreading the sample on a VRE selective agar plate (Nippon Becton Dickinson Company, Tokyo, Japan). For increased sensitivity, faecal swab samples, taken during the annual regional surveillance, were enriched with liquid media and spread on VRE selective agar plates [10]. Samples were suspended in 1 ml of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4), and 0.5 ml of each sample was inoculated into 10 ml of bile esculin azide broth containing 15 mg of vancomycin per litre (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). After incubation at 35°C for 48 h, broth samples with dark brown or black discolouration were inoculated onto VRE selective agar plates and incubated at 35°C for 48 h. Discrete colonies with enterococci-consistent morphologic features were transferred to heart infusion agar plates supplemented with 5% sheep blood (Eiken Chemical Co., Ltd., Tokyo, Japan). Species were identified using the API 20 Strep system (bioMérieux, St. Louis, MO, USA).

Molecular methods

Total bacterial DNA was extracted using a Qiagen DNA mini kit (Qiagen, Hildesheim, Germany), according to the manufacturer's instructions. Glycopeptide resistance genes (*vanA*, *vanB*, *vanC1* and *vanC2/3*) and *E. faecalis*-specific, *E. faecium*-specific and 16S *rDNA* genes were detected by multiplex polymerase chain reaction (PCR), as previously described [11].

Pulsed-field gel electrophoresis (PFGE)

The first vancomycin-resistant *E. faecium* isolate obtained from each facility was genotyped by the PFGE of genomic DNA digested with *SmaI* (Takara Bio, Otsu, Japan), as previously described [12, 13]. FN-1, a *vanA-E. faecium* isolate obtained in Kyoto in 1996 [7], was also analysed. Electrophoresis was performed using a GenePath system (Bio-Rad Laboratories, Tokyo, Japan), with pulse times increasing from 1.0 to 14.0 s for 18.5 h at 200 V (6 V/cm). Genetic relatedness was analysed using GelCompar II software (Applied Maths, Kortrijk, Belgium). Isolates were considered to be related when their PFGE banding patterns were ≥80% in similarity [14].

Multilocus sequence typing (MLST)

MLST was performed as previously described [15, 16]. The eBURST V3 program (http://efaecium.mlst.net/ or http://efaecalis.mlst.net/) was used to assign a sequence type (ST) to each isolate according to its allelic profile.

VRE control programme

A task force comprising hospital personnel, clinical laboratory chiefs and public health workers was organised and supported by the local authority to establish a VRE control programme.

The faecal VRE screening system started in 2006. Every faecal specimen, with or without an order from clinicians, was screened with VRE selective agar plates supplied by the task force. Positive plates were sent to the task force for further microbiology and molecular analyses.

Regional VRE control guidelines, based on previously published guidelines [17, 18], were established to promote adequate infection control measures. The guidelines emphasised good hand hygiene and barrier precaution in the caring for patients who were positive for VRE and emphasised on-admission surveillance cultures to detect VRE introduced into the hospitals. On-admission screening was especially recommended for patients transferred from other hospitals or non-hospital care facilities, because facility-to-facility transfer has been one of the main routes of VRE spread in the United States [19]. Precise information (i.e. species, vancomycin resistance type, and first and last date of positive tests) was sent from one facility to the next upon patient transfer. Task force members visited facilities where VRE was detected and gave practical infection control advice.

Annual regional surveillance began in 2005 with the approval of the ethics committee of Kyoto University. The purpose was to identify patients with VRE colonisation missed by the routine faecal screening system. Participating hospitals were recruited throughout the region. Patient criteria included those with a history of VRE carriage who had been hospitalised for more than 7 days and who met at least one of the following: urinary and/or faecal incontinence, tube feeding, use of a urethral catheter, received antimicrobial chemotherapy (>2 weeks) or underwent a surgical procedure (<1 month). Oral informed consent was obtained and each participant was anonymised by facility personnel. Surveillance was carried out once per year from 2005 to 2008, and was reduced in 2010 to once every 2 years.

Results

Regional spread of VRE

By the end of 2010, VRE was detected in 44 (25%) of 177 hospitals. Thirty-five (80%) of these 44 hospitals recognised the first cases of VRE colonisation by faecal VRE screening and the other nine (20%) recognised them by annual surveillance. *vanA-E. faecium* was isolated in 35 hospitals, and 31 (89%) of these hospitals were located in Kyoto. *vanB-E. faecium* was isolated in 12 hospitals, with six (50%) of these hospitals located in Kyoto and four located (33%) in northern districts. *vanB-E. faecalis* was detected in five hospitals.

Genotype analysis

Four clusters of clonally related isolates were identified among the 35 *vanA-E. faecium* isolates. The largest cluster (15 isolates) was obtained between 2006 and 2009. KR01 belonged to the cluster consisting of nine isolates obtained by the end of 2006. There were another two clusters: one consisted of four isolates obtained since 2009 and the other consisted of two isolates obtained in 2006 and 2007. Two clusters were identified among the 12 *vanB-E. faecium* isolates. The MLST type of all *E. faecium* isolates obtained since 2005 was ST78, which was different from the FN-1 isolates (ST16). Both ST78 and ST16 belonged to clonal complex 17 (Fig. 1a).

One cluster, consisting of three isolates, was identified among the five *vanB-E. faecalis* isolates. The ST of these isolates was 64, a member of clonal complex 8. The STs of the other two isolates were 4 and 390, neither of which belonged to the major clonal complexes (Fig. 1b).

VRE control programme

Faecal VRE screening began in 2006, with participation from 98 (55%) of the 177 regional hospitals. By the end of 2010, the number of hospitals reached 116 (66%), holding 78% of beds in the area. In 2005, three hospitals recognised their first cases of VRE colonisation by clinically ordered microbiological testing (before the faecal screening system began). Thirty-two (78%) of the 41 hospitals affected by VRE after 2006 detected their first VRE by the faecal screening system and the other nine (22%) hospitals detected them by annual surveillance.

In 2005 (9 months after the hospital outbreak of KR01), *vanB-E. faecalis* carriage was identified by the annual surveillance in only 1 of 2,253 patients (0.044%) in 108 hospitals. The number of faecal carriers detected by this surveillance significantly increased to 14 (0.71%) of 1,961 patients from eight hospitals in 2006, and reached 24 (1.2%) of 2,035 patients from eight hospitals in 2007. This number then decreased to 12 (0.50%) of 2,379 patients from six hospitals in 2008, and, further, to 4 (0.17%) of 2,408 patients from three hospitals in 2010 (Fig. 2).

Discussion

While no regional spread of vancomycin-resistant bacteria had been reported in Japan prior to 2005, VRE has become a major hospital pathogen in many other countries. However, *vanA-E. faecium* isolates were detected in three hospitals in urban Kyoto City in 2005, and one of them experienced a large outbreak that affected more than 100 patients. This resembled a situation reported from the

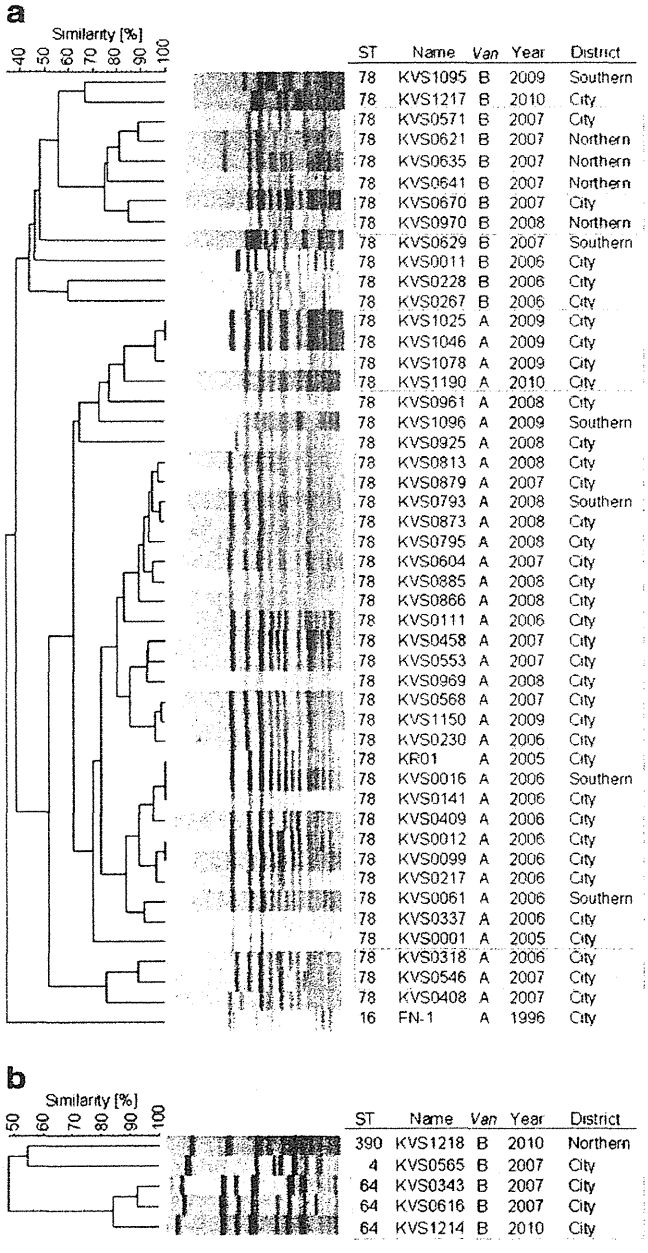

Fig. 1 Genotype analysis of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* isolates. **a** Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) analysis of vancomycin-resistant *E. faecium* isolates. Thirty-five *vanA*-positive isolates and 12 *vanB*-positive isolates were included in the analysis. The dendrograms was created using an UPGM (unweighted pair group method, Dice coefficient) algorithm, with optimisation 0.54% and band-tolerance 0.91%. This analysis revealed four and two clusters of genetically related isolates (PFGE pattern ≥80% similarity) among the *vanA*- and *vanB*-positive isolates, respectively. FN-1, a *vanA*-type *E. faecium* (Kyoto, 1996) was included as a control. Both ST78 and ST16 belonged to clonal complex 17. **b** PFGE and MLST analysis of vancomycin-resistant *E. faecalis* isolates. The dendrogram was created with an optimisation of 0.75% and a band-tolerance of 1.0%. One cluster of genetically related isolates, consisting of three ST64 isolates, was identified among the five *vanB-E. faecalis* isolates. Abbreviations, ST: sequence type; *Van*: the type of vancomycin resistance gene; City: Kyoto City; Southern: Southern districts; Northern: Northern districts

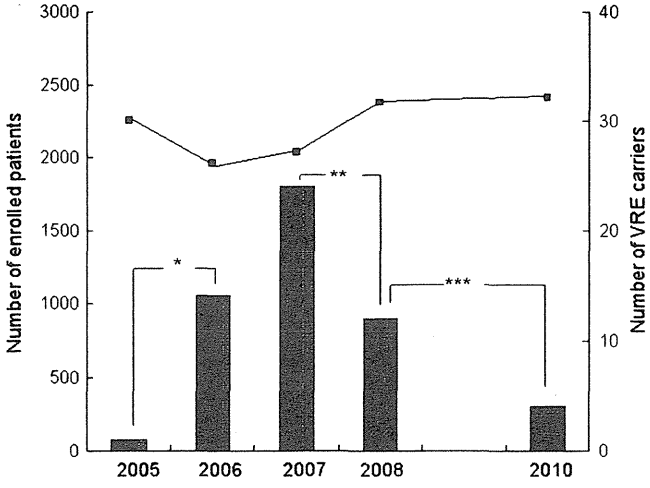

Fig. 2 Number of patients enrolled and faecal carriers of vancomycin-resistant enterococci (VRE) in the annual regional surveillance programme. VRE was identified by the annual surveillance in 2005 in only 1 of 2,253 enrolled patients (0.044%). The number of faecal carriers significantly increased to 14 (0.71%) of 1,961 patients in 2006 (*$p<0.001$, Fisher's exact probability test) and reached 24 (1.2%) of 2,035 patients in 2007. This number then decreased to 12 (0.50%) of 2,379 patients in 2008 (**$p<0.05$, Chi-square test), and, further, to 4 (0.17%) of 2,408 patients in 2010 (***$p<0.05$, Fisher's exact probability test). Regional surveillance was skipped in 2009. The *solid squares* represent enrolled patients and the *bars* represent faecal VRE carriers for each year

United States in the 1990s, where *vanA-E. faecium* had broken out in limited facilities, while the majority of the hospital-acquired enterococci remained vancomycin-susceptible [20]. The prevalence of VRE in the United States then rose exponentially in the following years [21, 22], prompting the urgent need for an effective control programme in Kyoto following initial reports of increasing VRE.

The dissemination of vancomycin-resistant *E. faecium* with preferred hospital association was, as Bonten et al. described, the pattern of VRE increase in the United States, and this pattern was recently reported from many countries, including those where VRE had not been a major problem [1, 23, 24]. MLST analysis revealed the predominance of strains belonging to CC17 among these nosocomial isolates worldwide [15]. The spread of CC17 vancomycin-resistant *E. faecium* is considered to be based on a high prevalence of CC17 vancomycin-susceptible ampicillin-resistant isolates that have adapted well to the hospital settings [25, 26]. CC17 vancomycin-resistant *E. faecium* may become more apparent in Japan in the future, because the ampicillin resistance rate among *E. faecium* isolates has become already high [27].

We also found that ST64 *E. faecalis* (belonging to CC8) was distributed to three facilities in the City area. Vancomycin-resistant *E. faecalis* belonging to CC2 and CC9 have, so far, been reported to be associated with high-risk clonal complexes, which are especially well

adapted to the hospital environment [28]. Our observation, together with a report from Cuba that there were three isolates of ST64 obtained from hospital clinical specimens, suggests that CC8 may be, at least in some countries, another high-risk clonal complex [29]. Further epidemiological and molecular study is needed. This observation is consistent with the report that these pathogens have increased ability to infect human hosts, which confer selective advantages for different genogroups [30].

Both the number of VRE-affected hospitals and patients, as determined by the annual surveillance, increased linearly until 2007. In addition, most of the *vanA-E. faecium* isolates obtained by the end of 2007 belonged to the two largest genetic clusters. These observations suggest that faecal VRE carriers had not been adequately identified until 2007, and that unnoticed hospital-to-hospital spread had continued. This study indicates that, since 2008, the VRE control programme has worked effectively. The laboratory-based faecal screening system detected the faecal carriers earlier, and the annual regional surveillance worked as a safety net to detect the cases from whom no clinical faecal specimens had been taken or from whom no VRE was detected by the routine screening. These two surveillance systems may have also led to a Hawthorne effect (a form of reactivity whereby subjects improve their behaviour simply in response to the fact that they are being studied, not in response to any particular intervention) among hospital caregivers, resulting in better infection control practices.

Limitations in this study should be noted. First, the enrichment broth in this study used a comparably high vancomycin concentration of 15 mg of vancomycin per litre. Although Novicki et al. reported that bile esculin azide broth with this concentration of vancomycin supported the growth of *E. faecalis* ATCC 51299, a strain having a measured vancomycin minimum inhibitory concentration (MIC) of 16 μg/ml, we might have missed some isolates with lower MICs [31]. Another limitation of our method is that we defined VRE in this study as the isolates that were positive for the *vanA* or *vanB* gene by PCR. We might have missed some PCR-negative isolates, as molecular testing may not always detect VRE.

Second, not all hospitals were prospectively recruited to participate in the faecal screening system or in the annual surveillance. Unrecognised faecal carriers may still exist, particularly in non-participating hospitals. The spread of VRE from these hospitals can still be monitored indirectly by the on-admission screening of transferred patients to the participating hospitals.

Third, only 6 years have passed since the initial spread of VRE, and VRE prevalence has remained low during these years. This observation may partially account for the success of our "passive" faecal screening system and our on-admission screening of patients transferred from other facilities in controlling VRE spread; however, these methods may be insufficient in upcoming years. Since more than 25% of hospitals are affected by VRE, an exponential increase in infection prevalence could occur.

In conclusion, VRE has spread in the Kyoto region of Japan since 2005; however, our regional programme has succeeded in its efforts toward VRE control.

Acknowledgements We thank Kyoto City and the Kyoto Prefecture for their practical support. We also appreciate the study and manuscript advice offered by Drs. Yasuyoshi Ike, Haruyoshi Tomita and Yoshichika Arakawa.

Aki Matsushima has received research funding from the Kurozumi Medical Foundation.

Shunji Takakura has received research funding from the Japanese Ministry of Education, Culture, Sports, Science and Technology (Kiban B, 18390172).

Yoshitsugu Iinuma has received research funding from the Japanese Ministry of Health, Labour and Welfare (the Research Grant for Longevity Sciences 21A-20).

Satoshi Ichiyama has received research funding from Kyoto City and the Kyoto Prefecture.

Conflicts of interest None of the authors have any financial relationships with the organisation that sponsored the research.

References

1. Bonten MJ, Willems R, Weinstein RA (2001) Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis 1(5):314–325
2. Salgado CD, Farr BM (2003) Outcomes associated with vancomycin-resistant enterococci: a meta-analysis. Infect Control Hosp Epidemiol 24(9):690–698
3. Carmeli Y, Eliopoulos G, Mozaffari E, Samore M (2002) Health and economic outcomes of vancomycin-resistant enterococci. Arch Intern Med 162(19):2223–2228
4. Salgado CD (2008) The risk of developing a vancomycin-resistant *Enterococcus* bloodstream infection for colonized patients. Am J Infect Control 36(10):S175.e5–S175.e8
5. Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, Klare I, Kristinsson KG, Leclercq R, Lester CH, Lillie M, Novais C, Olsson-Liljequist B, Peixe LV, Sadowy E, Simonsen GS, Top J, Vuopio-Varkila J, Willems RJ, Witte W, Woodford N (2008) Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill 13(47):pii=19046
6. Lee K, Jang SJ, Lee HJ, Ryoo N, Kim M, Hong SG, Chong Y (2004) Increasing prevalence of vancomycin-resistant *Enterococcus faecium*, expanded-spectrum cephalosporin-resistant *Klebsiella pneumoniae*, and imipenem-resistant *Pseudomonas aeruginosa* in Korea: KONSAR study in 2001. J Korean Med Sci 19(1):8–14
7. Fujita N, Yoshimura M, Komori T, Tanimoto K, Ike Y (1998) First report of the isolation of high-level vancomycin-resistant *Enterococcus faecium* from a patient in Japan. Antimicrob Agents Chemother 42(8):2150
8. Infectious Diseases Surveillance Center (IDSC) (2010) Infectious diseases weekly report. National Institute of Infectious Diseases. Available online at: http://idsc.nih.go.jp/idwr/ydata/report-Ea.html. Accessed 21 July 2011
9. Matsumoto T, Muratani T, Okada K, Shiraishi M, Hayashida T, Oki T, Odawara M (2004) No regional spread of vancomycin-resistant enterococci with *vanA* or *vanB* in Kitakyushu, Japan. J Infect Chemother 10(6):331–334

10. Ieven M, Vercauteren E, Descheemaeker P, van Laer F, Goossens H (1999) Comparison of direct plating and broth enrichment culture for the detection of intestinal colonization by glycopeptide-resistant enterococci among hospitalized patients. J Clin Microbiol 37(5):1436–1440
11. Kariyama R, Mitsuhata R, Chow JW, Clewell DB, Kumon H (2000) Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. J Clin Microbiol 38 (8):3092–3095
12. Turabelidze D, Kotetishvili M, Kreger A, Morris JG Jr, Sulakvelidze A (2000) Improved pulsed-field gel electrophoresis for typing vancomycin-resistant enterococci. J Clin Microbiol 38(11):4242–4245
13. Duck WM, Steward CD, Banerjee SN, McGowan JE Jr, Tenover FC (2003) Optimization of computer software settings improves accuracy of pulsed-field gel electrophoresis macrorestriction fragment pattern analysis. J Clin Microbiol 41(7):3035–3042
14. Donabedian SM, Perri MB, Abdujamilova N, Gordoncillo MJ, Naqvi A, Reyes KC, Zervos MJ, Bartlett P (2010) Characterization of vancomycin-resistant *Enterococcus faecium* isolated from swine in three Michigan counties. J Clin Microbiol 48(11):4156–4160
15. Leavis HL, Bonten MJ, Willems RJ (2006) Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. Curr Opin Microbiol 9(5):454–460
16. Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, Van Embden JD, Willems RJ (2002) Multilocus sequence typing scheme for *Enterococcus faecium*. J Clin Microbiol 40(6):1963–1971
17. Muto CA, Jernigan JA, Ostrowsky BE, Richet HM, Jarvis WR, Boyce JM, Farr BM; SHEA (2003) SHEA guideline for preventing nosocomial transmission of multidrug-resistant strains of *Staphylococcus aureus* and *Enterococcus*. Infect Control Hosp Epidemiol 24 (5):362–386
18. Siegel JD, Rhinehart E, Jackson M, Chiarello L; Healthcare Infection Control Practices Advisory Committee (2007) Management of multidrug-resistant organisms in health care settings, 2006. Am J Infect Control 35(10 Suppl 2):S165–S193
19. Martone WJ (1998) Spread of vancomycin-resistant enterococci: why did it happen in the United States? Infect Control Hosp Epidemiol 19 (8):539–545
20. Bopp LH, Schoonmaker DJ, Baltch AL, Smith RP, Ritz WJ (1999) Molecular epidemiology of vancomycin-resistant enterococci from 6 hospitals in New York State. Am J Infect Control 27(5):411–417
21. National Nosocomial Infections Surveillance System (NNIS) (1999) National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1990–May 1999, issued June 1999. Am J Infect Control 27(6):520–532
22. National Nosocomial Infections Surveillance System (NNIS) (2000) National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992–April 2000, issued June 2000. Am J Infect Control 28(6):429–448
23. Hsieh YC, Lee WS, Ou TY, Hsueh PR (2010) Clonal spread of CC17 vancomycin-resistant *Enterococcus faecium* with multilocus sequence type 78 (ST78) and a novel ST444 in Taiwan. Eur J Clin Microbiol Infect Dis 29(1):25–30
24. Stampone L, Del Grosso M, Boccia D, Pantosti A (2005) Clonal spread of a vancomycin-resistant *Enterococcus faecium* strain among bloodstream-infecting isolates in Italy. J Clin Microbiol 43 (4):1575–1580
25. Galloway-Peña JR, Nallapareddy SR, Arias CA, Eliopoulos GM, Murray BE (2009) Analysis of clonality and antibiotic resistance among early clinical isolates of *Enterococcus faecium* in the United States. J Infect Dis 200(10):1566–1573
26. Fallico L, Boldrin C, Grossato A, Franchin E, De Canale E, Tommasini T, Parisi SG, Manganelli R, Palù G (2011) Molecular epidemiology of *Enterococcus faecium* isolates from an Italian hospital. Infection 39(2):127–133
27. Muratani T, Matsumoto T (2004) Bacterial resistance to antimicrobials in urinary isolates. Int J Antimicrob Agents 24(Suppl 1):S28–S31
28. Ruiz-Garbajosa P, Bonten MJ, Robinson DA, Top J, Nallapareddy SR, Torres C, Coque TM, Cantón R, Baquero F, Murray BE, del Campo R, Willems RJ (2006) Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. J Clin Microbiol 44 (6):2220–2228
29. Quiñones D, Kobayashi N, Nagashima S (2009) Molecular epidemiologic analysis of *Enterococcus faecalis* isolates in Cuba by multilocus sequence typing. Microb Drug Resist 15(4):287–293
30. Galloway-Peña JR, Nallapareddy SR, Arias CA, Eliopoulos GM, Murray BE (2009) Analysis of clonality and antibiotic resistance among early clinical isolates of *Enterococcus faecium* in the United States. J Infect Dis 200:1566–1573
31. Novicki TJ, Schapiro JM, Ulness BK, Sebeste A, Busse-Johnston L, Swanson KM, Swanzy SR, Leisenring W, Limaye AP (2004) Convenient selective differential broth for isolation of vancomycin-resistant *Enterococcus* from fecal material. J Clin Microbiol 42(4):1637–1640