Published in final edited form as:

Infect Dis Clin North Am. 2016 June ; 30(2): 523–537. doi:10.1016/j.idc.2016.02.011.

Aminoglycoside Resistance:

The Emergence of Acquired 16S Ribosomal RNA Methyltransferases

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Keywords

Aminoglycoside; 16S ribosomal RNA; Posttranscriptional methylation; Carbapenemease

INTRODUCTION

Antimicrobial resistance has been recognized as one of the most pressing public health and societal issues of our times. The problem is most acute in gram-negative bacteria, where strains resistant to multiple (multidrug-resistant [MDR]) or almost all (extensively drug-resistant [XDR]) available agents are emerging.[1] Of particular concern has been the spread of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae in the 1990s, which was followed closely by the emergence and rapid dissemination of carbapenemase-producing organisms.

The three key classes of antimicrobial agents with gram-negative activity include β-lactams (especially β-lactam-β-lactamase inhibitor combinations, later-generation cephalosporins, and carbapenems), fluoroquinolones, and aminoglycosides. Aminoglycosides were identified through systematic screening of soil Actinobacteria that started in the 1940s. The first aminoglycoside streptomycin was discovered from *Streptomyces griseus* and successfully used for the treatment of tuberculosis and then infections with gram-negative bacteria. A typical aminoglycoside possesses an amino-containing or non-amino-containing sugars linked to six-membered rings with amino group substituents, hence the name aminoglycoside. Numerous aminoglycosides have since been identified or semisynthesized and used in clinical practice.

Aminoglycosides are grouped into 4,6-disubstituted 2-deoxystreptamine (DOS), 4,5-disubstituted DOS, and 4-monosubstituted DOS based on their chemical structures (Figs. 1 and 2). Representative 4,6-disubstituted DOS agents include gentamicin, tobramycin, and

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Potential Conflicts of Interest: Y. Doi has served on advisory boards for Shionogi, Meiji, Tetra-phase, consulted for Melinta Therapeutics, received a speaker fee from Merck, and received research funding from Merck and The Medicines Company for studies unrelated to this work. The other authors report no potential conflicts of interest.

amikacin, which are widely used as intravenous or nebulized formulations for the treatment of infections caused by gram-negative bacteria (usually in combination with a β-lactam agent), gram-positive bacteria (for synergistic activity with a β-lactam or peptidoglycan), and atypical mycobacteria (again in combination with other active agents). 4,5-Disubstituted DOS agents, represented by neomycin, are limited in their utility by toxicity and are administered either orally or topically but not intravenously. Monosubstituted DOS agents are represented by apramycin, which is used in veterinary medicine.

MECHANISMS OF AMINOGLYCOSIDE RESISTANCE

Aminoglycosides bind to the aminoacyl-tRNA recognition site (A-site) of the 16S rRNA that constitutes the 30S ribosomal subunit, leading to inhibition of polypeptide synthesis and subsequent cell death. Resistance to aminoglycosides may occur based on several mechanisms: (1) enzymatic modification and inactivation of the aminoglycosides, mediated by aminoglycoside acetyltransferases, nucleotidyltransferases, or phosphotransferases and commonly observed across gram-positive and -negative bacteria[2,3]; (2) increased efflux; (3) decreased permeability; and (4) modifications of the 30S ribosomal subunit that interferes with binding of the aminoglycosides. For the latter, both mutations (nucleotide replacement) and posttranscriptional modifications have been associated with aminoglycoside resistance. Examples include point mutations in the 16S rRNA and the *rpsL* gene encoding the S12 protein in *Mycobacterium tuberculosis* leading to streptomycin resistance.[4] However, mutations within the 30S ribosomal subunit do not seem to be a common aminoglycoside resistance mechanism among fast-growing pathogenic bacteria in general.

However, posttranscriptional modification of the 16S rRNA is commonly observed among aminoglycoside-producing Actinobacteria, including *Streptomyces* spp and *Micromonospora* spp, which are naturally resistant to these metabolites of their own. This process is mediated by posttranscriptional methylation of either the N-7 position of nucleotide G1405 or the N-1 position of nucleotide A1408 on the 16S rRNA by various 16S rRNA methyltransferases (16S-RMTases) (Fig. 3).[5] Intrinsic N7-G1405 16S-RMTases are found in both *Streptomyces* spp and *Micromonospora* spp, whereas intrinsic N1-A1408 16S-RMTases have only been identified in *Streptomyces* spp.[6] N7-G1405 16S-RMTases confer resistance to 4,6-disubstituted DOS agents, such as gentamicin, tobramycin, and amikacin, but not 4,5-disubstituted and monosubstituted DOS agents. In contrast, N1-A1408 16S-RMTases are capable of conferring resistance all these groups.

ACQUIRED 16S RIBOSOMAL RNA METHYLTRANSFERASES IN GRAM-NEGATIVE BACTERIA

The first 16S-RMTases mediating aminoglycoside resistance and reported from outside aminoglycoside-producing Actinobacteria were ArmA in *Klebsiella pneumoniae* and RmtA in *Pseudomonas aeruginosa*.[7,8] ArmA was first identified in an isolate of *K pneumoniae* from the urine of a patient admitted to a hospital in Paris in 2000,[7] but its nucleotide sequence could be found on a plasmid carried by a *Citrobacter freundii* clinical strain identified in Poland in 1996.[9,10] ArmA was subsequently confirmed to function as N7-

G1405 16S-RMTase.[11] RmtA was identified in a sputum isolate of *P aeruginosa* in Japan in 1997.[8]

With the subsequent discovery of additional enzymes, a total of 9 N7-G1405 16S-RMTases, some with proven function (ArmA, RmtB, and RmtC) and others with putative function based on amino acid sequence similarity and resistance phenotype (ie, resistance to gentamicin, tobramycin, and amikacin but susceptibility to neomycin and apramycin), have been identified (Table 1). They include ArmA, RmtA, RmtB (which includes RmtB1 and RmtB2 alleles), RmtC, RmtD (which includes RmtD1 and RmtD2 alleles), RmtE, RmtF, RmtG, and RmtH (Table 2). These acquired N7-G1405 16S-RMTases share modest to high amino acid sequence similarities with each other.

In contrast, only a single acquired N1-A1408 16S-RMTase has been discovered to date. This enzyme, named NpmA, was identified from an *Escherichia coli* clinical strain in Japan in 2007.[12] NpmA confers a broader spectrum of aminoglycoside resistance that includes neomycin and apramycin in addition to gentamicin, tobramycin, and amikacin, and possesses an amino acid sequence that is distinct from those of the N7-G1405 16S-RMTases (see Table 1). NpmA has subsequently been shown to catalyze modification of nucleotide A1408.[13]

Reviewed next are the genetic context and epidemiology of the acquired 16S-RMTases that have been identified to date.

ArmA

The *armA* gene was initially identified on plasmid pCTX-M3 in *C freundii* and plasmid pIP1204 in *K pneumoniae*, both of which also carried an ESBL gene $bla_{\text{CTX-M-3}}$.[10,14] In these plasmids, *armA* is located downstream of insertion sequence IS*CR1*, which follows a class 1 integron comprising *dhfrA12* (trimethoprim resistance), *aadA2* (streptomycin resistance), and *sul1* (sulfonamide resistance). Typically, *mel* and *mph2* (macrolide resistance) are located downstream of *armA*. Although the incompatibility groups of the plasmids and the gene cassette contents of the class 1 integron may be variable, this overall genetic context of *armA* seems to be well conserved in the family Enterobacteriaceae and *Acinetobacter baumannii*.

armA is one of the most frequently encountered 16S-RMTase genes along with *rmtB*, and is widely distributed in Enterobacteriaceae and *A baumannii*. In Enterobacteriaceae, it has been found mostly in species, such as *K pneumoniae*, involved in health care–associated infections, but there are also reports of *armA* found in species implicated in food-borne and diarrheal illnesses, including *Salmonella enterica*[14,15] and *Shigella flexneri*.[14] In *A baumannii*, *armA* is often identified in carbapenem-resistant strains that also carry the acquired carbapenemase gene $bla_{\text{OXA-23}}$, although these two genes are located on separate plasmids.[16] In a study of carbapenem-nonsusceptible *A baumannii* isolates collected from hospitals across the United States, 49% of the isolates carried *armA*, suggesting a high prevalence in this species (Doi Y, 2011, unpublished data).[17]

Another specific group of bacteria where *armA* seems to have high prevalence is Enterobacteriaceae producing NDM (New Delhi Metallo-β-lactamase)-type carbapenemase. NDM-1, the first NDM-type carbapenemase, was initially reported in *K pneumoniae* and *E coli* strains that were isolated from a patient who had traveled from New Delhi, India to Sweden in 2009.[18] NDM-producing Enterobacteriaceae has since spread worldwide rapidly causing many outbreaks, including recent ones in Denver and Chicago.[19,20] Soon after the discovery of NDM-1 it became clear that many Enterobacteriaceae strains producing NDM-1 or its variants were also highly resistant to 4,6-disubstituted DOS agents including gentamicin, tobramycin, and amikacin.[21] Investigations into the bla_{NDM}-carrying plasmids have revealed that bla_{NDM} is frequently colocated with *armA*, or other 16S-RMTase genes (especially *rmtB*, *rmtC*, and *rmtF*), on the same plasmids.[22] Some of these plasmids additionally carry plasmid-mediated fluoroquinolone-resistance genes, such as *qnrB1*.[23] Acquisition of these MDR plasmids would therefore simultaneously confer resistance to most β-lactams including carbapenems, aminoglycosides, and fluoroquinolones, the three key groups of agents with activity against gram-negative bacteria. *armA* has also been found in *P aeruginosa* carrying another metallo-β-lactamase gene bla_{IMP-1} in Korea,[24] and *K pneumoniae* or other Enterobacteriaceae carrying a *Klebsiella pneumoniae* carbapenemase (KPC)-type carbapenemase gene bla_{KPC-2} in Italy and China.[25,26]

Finally, there seems to be a reservoir of *armA* in food animals. For instance, *armA* has been reported in *E coli* from chickens in China,[27] and *S enterica* in chicken meat at a supermarket in La Réunion Island.[28] However, the extent of spread of *armA* in food animals is unclear at this point.

RmtA

RmtA was first identified in Japan in a *P aeruginosa* clinical isolate that had been isolated in 1997 and showed high-level resistance to the 4,6-disubstituted DOS aminoglycosides.[8] The 6.2-kb genetic region including *rmtA* of *P aeruginosa* is located between two copies of a kappa-gamma element, a 262-bp possible mobile element.[29]

Compared with ArmA, the occurrence of RmtA has been sporadic so far, with a limited number of reports of *rmtA*-carrying *P aeruginosa* coming from Japan and Korea.[30,31] However, a plasmid carrying bla_{NDM-1} and *rmtA* was reported in a *K pneumoniae* clinical strain that was isolated from a patient who was hospitalized in India and later sought care in Switzerland.[32] This association suggests that there may be an unrecognized reservoir of *rmtA* in India, where other 16S-RMTase genes are frequently found with bla_{NDM-1}.

RmtB

RmtB was first reported from a *Serratia marcescens* clinical strain that was isolated in Japan in 2002.[33] *rmtB* is located on a plasmid and downstream of a Tn*3*-like transposon, and bla_{TEM-1}. Although the sequences downstream of *rmtB* are variable, a fluoroquinolone efflux gene *qepA1* or its variants can sometimes be found adjacent to *rmtB*.

rmtB has revealed a worldwide distribution among Enterobacteriaceae, with reports of its identification coming from Asia, the Americas, Europe, the Middle East, Africa, and Oceania.[6,34–36] Like *armA*, *rmtB* is frequently associated with bla_{NDM-1} on the same

plasmids.[37] Therefore, the ongoing spread of *bla*$_{NDM-1}$-carrying Enterobacteriaceae likely contributes significantly to further dissemination of *rmtB*.

Another unique feature of RmtB, as with ArmA, is its association with food animals, but RmtB seems to be more prevalent than ArmA in this ecological niche. Most reports come from China, with *rmtB* identified in high rates in *E coli* from pigs, farm workers and their environment,[38,39] chickens,[27] and pets.[40]

RmtC

RmtC was first reported in a *Proteus mirabilis* clinical strain that was isolated from a hospitalized patient in Japan in 2003.[41] Located on a nonconjugative plasmid, *rmtC* is adjacent to an IS*Ecp1*-like element, which also provides the promoter sequence for the expression *rmtC*.[42]

The second RmtC-producing isolate was reported from Australia in *P mirabilis*, which was isolated from the urine of a patient who had recently returned from India.[43] Then, 13 clonally related *S enterica* Virchow strains among the 2004 to 2008 culture collection at the Health Protection Agency in the United Kingdom were found to possess *rmtC*.[44] Remarkably, 4 of the 12 patients affected by these *S enterica* Virchow isolates reported recent travel to India. Soon thereafter, *rmtC* began to appear in conjunction with *bla*$_{NDM-1}$, just as had been observed with *armA* and *rmtB*. *rmtC* was found in 12 of 18 *bla*$_{NDM-1}$-carrying *E coli* isolates from the United Kingdom, India, and Pakistan.[45] In New Zealand, all 5 *bla*$_{NDM-1}$-carrying Enterobacteriaceae isolates referred to a national reference laboratory possessed *rmtC*, with all cases associated with recent health care contact in India.[46] In a survey at a hospital in India, 3.7% of Enterobacteriaceae isolates had *rmtC*, often along with *bla*$_{NDM}$.[47] *rmtC* has also been found among *K pneumoniae* clinical isolates in Nepal.[48] These data suggest that *rmtC* likely originated in the Indian subcontinent and *rmtC* is being incorporated by MDR/XDR Enterobacteriaceae, in particular those producing NDM-type carbapenemase.

RmtD

RmtD is unique in that it has only been reported from South America. First identified in *P aeruginosa* producing SPM-1 metallo-β-lactamase in Brazil,[49] *rmtD* (now also termed *rmtD1* because of the identification of its variant *rmtD2*) seems to have been mobilized by putative transposase IS*CR14* (formerly Orf494).[50] *rmtD1* and *rmtD2* have since been identified in various Enterobacteriaceae species in Brazil, Chile, and Argentina.[51,52] More recently, cocarriage of *rmtD1* or *rmtD2* with *bla*$_{KPC-2}$ has been reported in *K pneumoniae* from Brazil.[53] Unlike with NDM-1-producing Enterobacteriaceae, *rmtD1* or *rmtD2* and *bla*$_{KPC}$ are located on separate plasmids.

RmtE

Only two RmtE-producing strains have been reported to date, both *E coli*, one from a cow and another from a patient in the United States.[54,55] *rmtE* is located on a class 1 integron, but as an independent gene and not an integron gene cassette, on a large plasmid that also carries *bla*$_{CMY-2}$ encoding an AmpC β-lactamase (cephalosporinase).[56]

RmtF

More so than any other acquired 16S-RMTases that have been identified to date, RmtF is very closely associated with NDM. The first RmtF-producing strain identified was *K pneumoniae* coproducing NDM-1 that was isolated from a patient in La Réunion Island.[57] *rmtF* has so far been found on class 1 integrons downsteam of bla_{NDM}.[58]

In a single hospital surveillance study in India, 3.4% of Enterobacteriaceae carried *rmtF*, 59% of them along with bla_{NDM}.[47] Isolates carrying *rmtF* have since been reported from Nepal, Australia, and Minnesota.[16,36,48,59]

RmtG

Like RmtD, RmtG is largely unique to South America. First identified in KPC-producing *K pneumoniae* clinical isolates in Brazil,[53] it has been found in Chile and Miami, all in *K pneumoniae*.[60,61] *rmtG* is flanked downstream by IS*Vsa3*, but this insertion sequence likely belongs to the site of insertion of the module carrying *rmtG* rather than the *rmtG*-containing module itself.[62] There are currently no data on the epidemiology of *rmtG*.

RmtH

RmtH was recently identified in an ESBL-producing *K pneumoniae* strain that was isolated from a US soldier who suffered wound infection from an explosion during deployment in Iraq.[63] *rmtH* is bracketed by two copies of IS*CR2*, which likely played a role in the initial mobilization of this unusual 16S-RMTase gene.

NpmA

NpmA was discovered in an *E coli* clinical strain that was isolated from a patient in Japan in 2003 and is the only acquired N1-A1408 16S-RMTases known to date.[12] Because of its unique site of action, NpmA confers resistance to 4,5-disubstituted and monosubstituted DOS agents (eg, neomycin and apramycin, respectively). *npmA* and its flaking sequences are bracketed by the two copies of IS*26*, suggesting their involvement in the mobilization of *npmA*. *K pneumoniae* and *Enterobacter* spp carrying *npmA* were recently reported from Saudi Arabia.[35]

PREVALENCE OF 16S RIBOSOMAL RNA METHYLTRANSFERASES

Prevalence data on acquired 16S-RMTases remain relatively scarce, and low prevalence rates of 1% or less have been reported among Enterobacteriaceae from Europe, Japan, and Argentina.[30,52,64–67] However, some studies, most of them single-center, have found alarmingly high rates of 16S-RMTase genes among Enterobacteriaceae. In Korea, rates of 2.8% to 11.4% have been reported among Enterobacteriaceae.[68,69] In China, of 680 and 337 unique *E coli* and *K pneumoniae* clinical isolates collected at a hospital between 2006 and 2008, 5.4% and 6.2% were positive for *rmtB* or *armA*, respectively.[70,71]

The prevalence rates may be even higher in India and Saudi Arabia. Of 1000 consecutive Enterobacteriaceae clinical isolates collected at a hospital in India between 2010 and 2011, a total of 14% carried at least one 16S-RMTase gene, including *armA*, *rmtB*, *rmtC*, and

rmtF.[47] Of 330 unique Enterobacteriaceae clinical isolates collected at a hospital in Saudi Arabia in 2011, a total of 37% carried at least one 16S-RMTase gene.[35]

CLINICAL IMPLICATIONS

To date, no data are available on the impact of acquired 16S-RMTase production and clinical outcome of patients when they are treated with aminoglycosides, largely because aminoglycosides by themselves are not considered the first-line agents for either empiric or definitive therapy for infections from gram-negative bacteria in most clinical settings, and also because prevalence rates of 16S-RMTases are relatively low in developed countries.

Nonetheless, the real threat of 16S-RMTases is the loss of a potential treatment option for the salvage therapy for MDR/XDR gram-negative bacterial infections where treatment options are already limited. 16S-RMTase genes are increasingly identified along with other significant resistance genes, especially carbapenemase genes, in the same isolates. Given the extremely high level of aminoglycoside resistance conferred by the acquired 16S-RMTases, this precludes the use of key aminoglycosides (gentamicin, tobramycin, and amikacin) even when carbapenems have already been excluded from the treatment option. For instance, plazomicin, a new aminoglycoside agent that is under clinical development specifically for use against carbapenem-resistant Enterobacteriaceae, is not active against isolates that produce acquired 16S-RMTase. This is most prominently observed for NDM-producing Enterobacteriaceae that frequently coproduces 16S-RMTase.[72] Although most KPC-producing *K pneumoniae* remain susceptible to plazomicin at this point, increasing reports of KPC and 16S-RMTase coproduction are a cause for concern.[25,26,53,73]

SUMMARY

Antimicrobial resistance in gram-negative pathogens has become one of the most challenging issues faced in daily clinical practice. Especially troublesome is the global spread of carbapenem-resistant Enterobacteriaceae, which has accelerated since the appearance of KPC and NDM-type carbapenemases. Aminoglycoside resistance mediated by acquired 16S-RMTase is a relatively new mechanism that was described in the early 2000s, but it now seems to be converging with the carbapenemase epidemic, thereby facilitating the emergence of XDR and, in some instances, pandrug-resistant organisms. Although not first-line in many clinical scenarios, aminoglycosides remain an important class of agents with excellent bactericidal activity when the organisms of interest are resistant to other classes, especially β-lactams and fluoroquinolones. Therefore, the ongoing dissemination of 16S-RMTases among already MDR organisms is an unwelcome event.

Acknowledgments

Y. Doi was supported by research grants from the National Institutes of Health (R01AI104895, R21AI107302, and R21AI123747).

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KEY POINTS

- Aminoglycoside-producing Actinobacteria produce 16S ribosomal RNA methyltransferase (16S-RMTase) to protect themselves.
- High-level aminoglycoside resistance caused by production of acquired 16S-RMTase in pathogenic gram-negative bacteria was first reported in the early 2000s.
- Bacteria that produce 16S-RMTases frequently coproduce ESBL, and more recently, carbapenemase, especially NDM-1.
- Spread of 16S-RMTase-producing bacteria further compromises the already limited treatment options for infections caused by MDR/XDR pathogens.

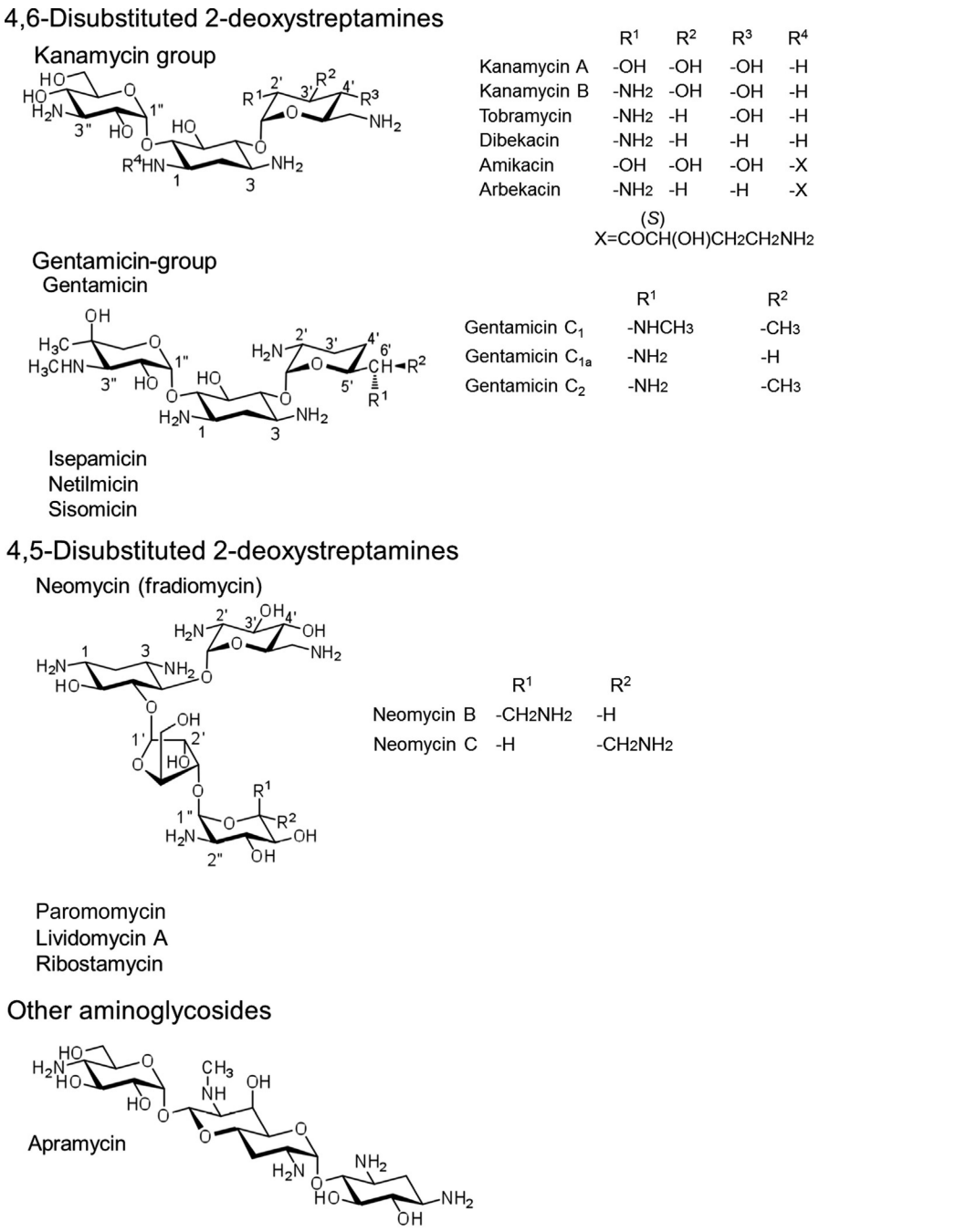

Fig. 1.

Aminoglycosides whose activities are compromised by methylation of nucleotide G1405 or A1408 of 16S rRNA. (From Yonezawa M, Ida T, Umemura E, et al. Antibiotics and chemotherapy (kagakuryohou no ryouiki), 31:(1476)67–73, 2015, a review article written in Japanese; with permission.)

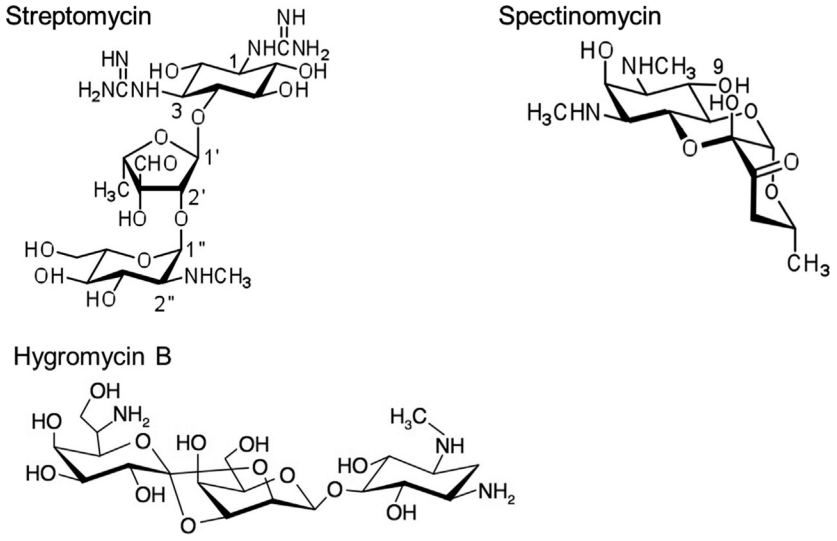

Fig. 2.

Aminoglycosides whose activities are not affected by methylation of nucleotide G1405 or A1408 of 16S rRNA. (From Yonezawa M, Ida T, Umemura E, et al. Antibiotics and chemotherapy (kagakuryohou no ryouiki), 31:(1476)67–73, 2015, a review article written in Japanese; with permission.)

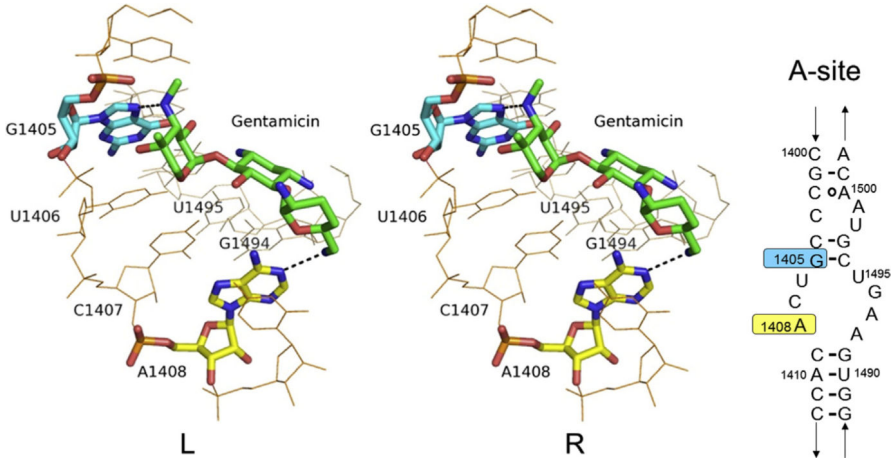

Fig. 3.

Three-dimensional structure of the A-site of 16S rRNA bound to gentamicin.

Table 1

Resistance phenotype of aminoglycosides conferred by acquired N7-G1405 and N1-A1408 16S-RMTases

	N7-G1405 16S-RMTase	N1-A1408 16S-RMTase
Aminoglycosides	**RmtA through RmtH ArmA**	**NpmA**
4,6-Disubstituted DOS (gentamicin, tobramycin, amikacin)	R	R
4,5-Disubstituted DOS (neomycin)	S	R
Monosubstituted DOS (apramycin)	S	R
No DOS ring (streptomycin)	S	S

Table 2

Overview of acquired N7-G1405 and N1-A1408 16S-RMTases

16S-RMTase	Common Species	Common Coresistance	Prevalence	Distribution
ArmA	*Klebsiella pneumoniae* *Acinetobacter baumannii*	CTX-M ESBL NDM carbapenemase OXA-23 carbapenemase	Very high in *A baumannii* High among NDM producers	Worldwide
RmtA	*Pseudomonas aeruginosa*	—	Low	Japan, Korea
RmtB	*Escherichia coli* *K pneumoniae*	CTX-M ESBL NDM carbapenemase	High in China High among NDM producers	Worldwide
RmtC	*K pneumoniae* *Proteus mirabilis*	NDM carbapenemase	High among NDM producers	India, United Kingdom
RmtD	*P aeruginosa* *K pneumoniae*	CTX-M ESBL KPC carbapenemase	Low	South America
RmtE	*E coli*	CMY-2 AmpC	Very low	United States
RmtF	*K pneumoniae*	NDM carbapenemase	High among NDM producers	India, United Kingdom
RmtG	*K pneumoniae*	CTX-M ESBL KPC carbapenemase	Low	South America
RmtH	*K pneumoniae*	CTX-M ESBL	Very low	Iraq
NpmA	*E coli* *K pneumoniae* *Enterobacter* spp	—	Very low	Japan, Saudi Arabia

Abbreviation: NDM, New Delhi Metallo-β-lactamase.