We evaluated the ability of the VITEK 2 system to determine the antimicrobial susceptibility of recent VRE isolates using penicillin G, erythromycin, vancomycin and teicoplanin as test antibiotics. The strains which were determined to be resistant to penicillin G, erythromycin and vancomycin by the VITEK 2 system were also resistant according to both reference methods employed. There were differences between the MICs of these antibiotics by the VITEK 2 system and the reference methods, but susceptible strains were not identified as being resistant and resistant strains were not indicated to be susceptible.

There was one 'very major' error and seven 'minor' errors when the MICs of teicoplanin were calculated using the VITEK 2 system, as compared with the reference method results. The failure of VITEK 2 to determine resistance to teicoplanin has already been reported (van den Braak *et al.*, 2001; Garcia-Garrote *et al.*, 2000). Some vancomycin-resistant strains of *Enterococcus* were phenotypically classified as VanB by the VITEK 2 system in the study by van den Braak *et al.* (2001).

In the present study, there was one 'very major' error in the teicoplanin MIC for one strain (VRE no. 14), i.e. the MIC was 1 $\mu g\,ml^{-1}$ using the VITEK 2 system but 32 $\mu g\,ml^{-1}$ by the two reference methods. This error was considered to be the same as that reported by van den Braak *et al.* (2001). However, the VITEK 2 system detected VRE accurately and distinguished them from the 11 teicoplanin-susceptible strains; the VITEK 2 system results corresponded well with those obtained using the two reference methods.

The VITEK 2 system is easy to use and provides accurate results in detecting resistance of *Enterococcus* species to penicillin G, erythromycin and glycopeptides; this system can also be used to determine the antimicrobial susceptibility of *Enterococcus* including vancomycin-resistant isolates. One of the major advantages of the VITEK 2 system is the significant reduction in handling time as compared with conventional test procedures.

A 24 h incubation period is needed to determine the MICs of vancomycin or teicoplanin for VRE by either the micro broth-dilution or the agar-dilution method according to NCCLS guidelines (2000b). However, the MICs of these antibiotics for 35 VRE isolates were determined within 13 h by the VITEK 2 system, with the phenotypes of all 35 isolates being simultaneously determined during this period. The VITEK 2 system promises to expedite work in clinical microbiological laboratories. Furthermore, the VITEK 2 system may play an important role in the investigation of nosocomial VRE infections.

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PROTEKT 1999–2000: a multicentre study of the antimicrobial susceptibility of respiratory tract pathogens in Japan

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Summary

Design: A six-centre study in Japan during the winter of 1999–2000 assessed the in vitro activity of >20 antimicrobial agents against the common respiratory pathogens *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. The minimum inhibitory concentrations (MIC) of each antimicrobial was determined against these isolates using National Committee for Clinical Laboratory Standards (NCCLS) methodology.
Results: Among *S. pneumoniae* isolates, 44.5% were penicillin resistant. The macrolide resistance rate was 77.9% with 90.5% of penicillin-resistant strains also being macrolide resistant. Resistance mechanisms in macrolide-resistant isolates were identified as *mef*(A) or *erm*(B) in 42.5% and 52.5%, respectively. Of the fluoroquinolone-resistant isolates (1.3%), most were also penicillin and macrolide resistant. All strains were inhibited by telithromycin at ≤1 mg/L. Among *S. pyogenes* isolates, erythromycin resistance was 17.5% overall but showed considerable variation among the six centres. For *H. influenzae*, 8.5% produced β-lactamase and a single β-lactamase-negative, ampicillin-resistant isolate (0.36%) was obtained, and there was no fluoroquinolone resistance. All isolates were susceptible to telithromycin.

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Most antimicrobials showed good activity against *M. catarrhalis*, although 96.7% were β-lactamase positive.
Conclusion: The prevalence of antimicrobial resistance to macrolides, penicillin and the fluoroquinolones among the common respiratory pathogens is high in Japan.
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Introduction

The prevalence of resistant isolates of common bacterial respiratory tract pathogens is increasing, and nowhere more so than in Asia. In some Asian countries, penicillin resistance may be as high as 70%.[1–3] In the last decade, macrolide resistance has also increased dramatically, exceeding penicillin resistance in some areas,[2] and growing resistance to chloramphenicol, co-trimoxazole and tetracycline continues relentlessly.[4]

Most respiratory tract infections are viral in origin but are frequently followed by secondary infections resulting from opportunistic invasion by commensal respiratory bacteria. The four most important bacterial pathogens associated with community-acquired upper and lower respiratory tract infections (RTIs – acute/chronic sinusitis, acute/chronic otitis media, acute/chronic pharyngitis, community-acquired pneumonia, acute bacterial exacerbation of chronic bronchitis and acute bacterial exacerbation of chronic obstructive airways disease) are *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Less commonly, atypical and intracellular pathogens including *Legionella pneumophila*, *Mycoplasma pneumoniae*, and *Chlamydophila (Chlamydia) pneumoniae* are also found as causes of community-acquired RTIs.[5–7]

S. pneumoniae in particular has acquired resistance to several classes of antimicrobial compounds, including penicillins, macrolides and fluoroquinolones, by a variety of mechanisms.[8] For *Haemophilus* species and *M. catarrhalis*, β-lactamase production is the principal mechanism of resistance to penicillins and cephalosporins. The choice of antimicrobial therapy in community-acquired RTIs is generally empirical and complicated by increasing bacterial resistance. Effective strategies for ensuring adequate antimicrobial therapy are therefore necessary but may only be achieved through an understanding of the geographic variation in resistance and by monitoring trends in resistance development.

Established in 1999, PROTEKT (Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin) is an international surveillance study to chart the prevalence of important resistance phenotypes and examine the susceptibility of community-acquired RTI pathogens to a range of antimicrobial compounds. Telithromycin is the first ketolide antibacterial to be approved for clinical use for the treatment of upper and lower RTIs. With over 35 countries and 500 centres now participating, PROTEKT is able to concentrate on defining trends in specific regions and countries. Detailed data from the examination of isolates of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. pyogenes* collected during the 1999–2000 winter season in Japan are now presented and, where possible, related to trends seen in previous studies.[2,9,10]

Materials and methods

Participating centres

During the 1999–2000 winter season, six centres took part in the study: Kanagawa, Sendai, Tokyo (two centres), Nagasaki and Osaka.

Bacterial isolates

Centres were asked to collect the following isolates from patients with community-acquired upper and lower RTIs: ≥40 isolates each of *S. pneumoniae* and *H. influenzae*, ≥25 of *S. pyogenes*, and ≥20 of *M. catarrhalis*. Sources for isolates were cultures from blood, sputum, bronchoalveolar lavage, middle ear fluid, nasopharyngeal swab or aspirate, and sinus aspirate. Duplicate strains or strains originating from previous collections were not accepted.

Identification and antimicrobial susceptibility testing

Isolates were identified at source and re-identified at the central laboratory by methods previously described in detail.[11] Minimum inhibitory concentrations (MICs) were determined using previously described broth microdilution methods,[11] according to the National Committee for Clinical Laboratory Standards (NCCLS) of the USA guidelines, for the following antimicrobial agents: amoxicillin–clavulanate, cefaclor, cefcapene, cefdinir, cefditoren,

cefixime, cefpodoxime, cefuroxime, telithromycin, erythromycin, roxithromycin, clarithromycin, azithromycin, rokitamycin, minocycline, tetracycline, ciprofloxacin, levofloxacin, sparfloxacin and tosufloxacin. MICs were also determined for penicillin and clindamycin against *S. pneumoniae* and *S. pyogenes* isolates and for ampicillin and amoxicillin against *H. influenzae* and *M. catarrhalis* isolates. Test results were acceptable only if the MICs for the control strains were within performance range. The following control strains were used: *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and ATCC 35218, *H. influenzae* ATCC 49766, *H. influenzae* ATCC 49247, and *S. pneumoniae* ATCC 49619.

Breakpoint concentrations used to interpret MIC data qualitatively were based upon those published by the NCCLS of the USA,[12] where available. For telithromycin, NCCLS approved (SAST 2003) breakpoints were applied: *S. pneumoniae*: susceptible ≤1 mg/L, intermediate 2 mg/L, resistant ≥4 mg/L; and for *H. influenzae*: susceptible ≤4 mg/L, intermediate 8 mg/L, resistant ≥16 mg/L. No NCCLS breakpoints are available for *S. pyogenes* or *M. catarrhalis*.

β-lactamase detection

β-lactamase activity was detected using the chromogenic cephalosporin (nitrocefin) test (Unipath Ltd. Basingstoke, UK).

Macrolide resistance mechanism detection

For *S. pneumoniae*, the presence of resistance mechanisms for both MLS_B (*erm*) and M-resistance (*mef*) was analysed using a rapid-cycle multiplex PCR method with probe detection. This method detects *erm*(A), *erm*(A) subclass *erm*(TR), *erm*(B), *erm*(C), and *mef*(A) genes.[13]

Results

Streptococcus pneumoniae

A total of 308 *S. pneumoniae* isolates from the six participating centres were tested. The prevalence of penicillin resistance (MIC ≥2 mg/L) was 44.5% overall and ranged narrowly between 44.2% and 48.4% for five of the six centres, with Osaka lower at 36.4%. Penicillin-intermediate (MIC 0.12–1 mg/L) isolates (19.8% overall) were less evenly distributed, with centres reporting between 7.7% (Kanagawa) and 31.6% (Sendai) (Table 1). The prevalence of macrolide resistance (erythromycin MIC ≥1 mg/L) was 77.9%, far exceeding that of penicillin resistance, and ranged from 67.3% (Kanagawa) to 86.4% (Osaka) (Table 1). Only one strain was of the intermediate type (erythromycin MIC 0.5 mg/L). Almost half of all *S. pneumoniae* isolates (40.3%) were co-resistant to penicillin and erythromycin (macrolide) (Table 1).

Of the 239 macrolide-resistant isolates of *S. pneumoniae* analysed for their resistance mechanism, 52.7% carried *erm*(B) (MLS_B resistance) and 42.7% carried *mef*(A) (efflux resistance), with 3.3% ($n = 8$) of isolates carrying both mechanisms (*mef*(A)+*erm*(B)) (Table 2). *erm*B isolates were evenly distributed across the three penicillin resistance phenotypes, whereas *mef*(A) resistance was associated predominantly with penicillin-resistant (70.6%) rather than penicillin-susceptible (16.7%) isolates.

Among the β-lactams, the most active were cefditoren (MIC_{90} 1 mg/L, 98.4% of all isolates susceptible) and amoxicillin–clavulanate (MIC_{90} 2 mg/L, 96.4% of all isolates susceptible). Both retained >90% activity among the penicillin- and macro-

Table 1 Penicillin and macrolide susceptibility and cross-resistance of *Streptococcus pneumoniae* isolates from Japan.

Centre	No. of isolates	Pen-I[a]		Pen-R[b]		Mac-R[c]		Pen-R/Mac-R	
		n	%	*n*	%	*n*	%	*n*	%
Kanagawa	52	4	7.7	23	44.2	35	67.3	18	34.6
Sendai	38	12	31.6	18	47.4	32	84.2	18	47.4
Tokyo 1	54	11	20.4	24	44.4	43	79.6	22	40.7
Tokyo 2	62	10	16.1	30	48.4	48	77.4	27	43.5
Nagasaki	58	14	24.1	26	44.8	44	75.9	23	39.7
Osaka	44	10	22.7	16	36.4	38	86.4	16	36.4
Total	308	61	19.8	137	44.5	240	77.9	124	40.3

[a] Penicillin-intermediate: MIC 0.12–1 mg/L.
[b] Penicillin-resistant: MIC ≥ 2 mg/L.
[c] Erythromycin-resistant: MIC ≥ 1 mg/L.

Table 2 Effect of specific macrolide-resistance mutations for 239 macrolide-resistant isolates of *Streptococcus pneumoniae* from Japan and classified by penicillin susceptibility phenotype.

Genotype	MAC-R[a]		MIC range (mg/L)	PEN-S[b]		PEN-I[c]		PEN-R[d]	
	n	%		*n*	%	*n*	%	*n*	%
mef(a)	102	42.7	1–≥128	17	16.7	13	12.7	72	70.6
erm(b)	126	52.7	32–≥128	44	34.9	34	27.0	48	38.1
mef(a) + *erm*(b)	8	3.3	64–≥128	2	25	2	25	4	50
None specified	3	1.3	64–≥128	3	100	0	0	0	0

[a] Macrolide-resistant (erythromycin MIC ≥1 mg/L).
[b] Penicillin-susceptible: MIC ≤ 0.06 mg/L.
[c] Penicillin-intermediate: MIC 0.12–1 mg/L.

lide-resistant isolates (Table 3). With the exception of telithromycin and the fluoroquinolones, susceptibility to non-β-lactams was low (Table 3). Among the penicillin-resistant isolates, <10% were susceptible to macrolides and tetracycline.

Erythromycin, roxithromycin, azithromycin and clarithromycin gave typical trimodal MIC distributions with clusters of isolates inhibited by 0.06–0.12 mg/L, 2–4 mg/L and >32–>64 mg/L (Figure 1). Small numbers of isolates were inhibited

Table 3 Comparative in vitro activity and percentage susceptibility of various antimicrobials against penicillin-intermediate, penicillin-resistant and erythromycin-resistant isolates of *Streptococcus pneumoniae* from Japan using NCCLS (2002) interpretative breakpoints.

Antimicrobial	All isolates (*n* = 308)			PEN-I[a] (*n* = 61)			PEN-R[b] (*n* = 137)			MAC-R[c] (*n* = 240)		
	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[d]	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[d]	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[d]	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[d]
Penicillin	0.5	4	35.7	0.25	1	0	2	4	0	2	4	27.9
Amoxicillin–clavulanate[e]	0.5	2	96.4	0.25	0.5	100	2	2	92.0	0.5	2	95.8
Cefaclor	16	>64	20.8	16	64	8.2	64	>64	0	2	4	11.3
Cefcapene	2	4	NA	2	4	NA	4	4	NA	2	4	NA
Cefdinir	4	8	44.5	2	4	41.0	8	8	2.2	4	8	35.8
Cefditoren	0.5	1	98.4	0.5	1	96.7	1	1	97.8	0.5	1	97.9
Cefixime	16	32	–[f]	16	64	–[f]	32	64	–[f]	32	64	–[f]
Cefpodoxime	2	4	40.3	2	4	36.1	2	4	0	2	4	32.1
Cefuroxime	4	8	41.2	4	8	37.7	8	8	0	4	8	33.3
Telithromycin	0.06	0.25	100[g]	0.06	0.5	100[g]	0.06	0.12	100[g]	0.06	0.25	100[g]
Erythromycin	8	>64	21.8	64	>64	19.7	4	>64	9.5	64	>64	0
Roxithromycin	8	>32	NA	>32	>32	NA	8	>32	NA	64	64	NA
Clarithromycin	4	>32	22.1	32	>32	19.7	4	>32	9.5	32	>32	0
Azithromycin	8	>64	21.8	64	>64	18.0	8	>64	9.5	64	>64	0
Rokitamycin	0.12	>32	NA	1	>32	NA	0.12	>32	NA	1	64	NA
Clindamycin	0.12	>4	54.9	4	>4	41.0	0.12	>4	62.0	4	>4	42.1
Minocycline	8	16	NA	16	16	NA	8	16	NA	16	16	NA
Tetracycline	>16	>16	20.8	16	>16	18.0	>16	>16	9.5	>16	>16	4.2
Ciprofloxacin	1	2	NA	1	2	NA	1	2	NA	1	2	NA
Levofloxacin	1	1	96.4	0.5	1	100	1	1	97.1	1	1	96.3
Sparfloxacin	0.25	0.5	96.1	0.25	0.25	100	0.25	0.25	96.4	0.25	0.5	95.8
Tosufloxacin	0.12	0.12	NA	0.06	0.12	NA	0.06	0.12	NA	0.12	0.12	NA

[a] Penicillin-intermediate: MIC 0.12–1 mg/L.
[b] Penicillin-resistant: MIC ≥2 mg/L.
[c] Erythromycin-resistant: MIC ≥1 mg/L.
[d] % of isolates susceptible.
[e] Also applies to amoxicillin.
[f] Susceptibility predicted from penicillin.
[g] NCCLS (SAST Jan 2003) approved breakpoint for telithromycin: susceptible ≤1 mg/L; NA = NCCLS breakpoints not available.

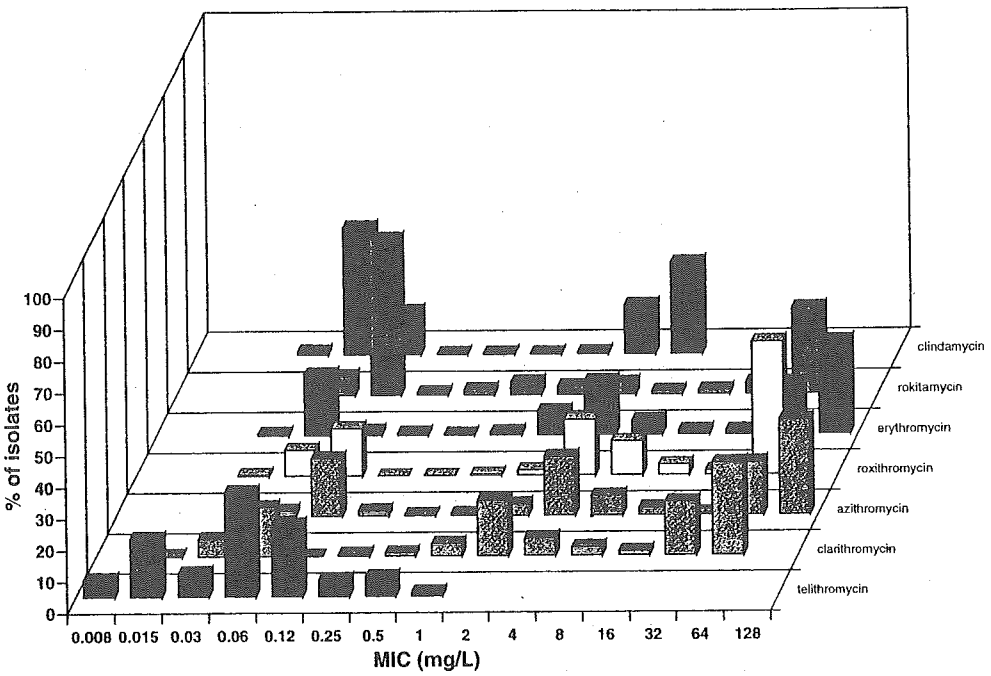

Figure 1 MIC distribution for macrolide-lincosamide-streptogramin (MLS) class antimicrobials against *Streptococcus pneumoniae* from Japan.

by each inter-mode concentration of each antimicrobial. Rokitamycin and clindamycin showed two obvious clusters in their MIC distributions, with just under half the isolates inhibited within the lowest concentration cluster. This was reflected in the MIC_{50} (0.12 mg/L) for rokitamycin and clindamycin, which differed considerably from the four macrolides with typical trimodal MIC distributions (MIC_{50} 4–8 mg/L).

Telithromycin showed much lower mode MIC (0.06 mg/L) and MIC_{90} (0.25 mg/L) than the macrolides (Figure 1). Among the macrolide-resistant isolates, the telithromycin MIC_{90} value was markedly higher for the *erm*(B) genotype (0.5 mg/L) than the *mef*(A) genotype (0.12 mg/L). Despite a shift upwards in the distribution of telithromycin MIC values among the macrolide-resistant isolates (particularly among the eight *erm*(B)+*mef*(A) strains (Figure 2)) compared with macrolide-susceptible isolates (telithromycin MIC_{90} 0.015 mg/L), all isolates were susceptible to telithromycin at ≤1 mg/L.

Fluoroquinolone resistance (levofloxacin MIC ≥8 mg/L) was 1.3% overall, with little variation among centres. Of the four fluoroquinolone-resistant isolates, three were penicillin-resistant and one was penicillin-susceptible. All four fluoroquinolone-resistant isolates were also macrolide- and tetracycline-resistant. Susceptibility to telithromycin was unaffected by fluoroquinolone resistance. Overall, of those antibacterial agents tested, the most active against *S. pneumoniae* in the winter season 1999–2000 in Japan (in terms of potency and susceptibility percentage) were telithromycin, sparfloxacin, levofloxacin, cefditoren and amoxicillin–clavulanate.

Streptococcus pyogenes

The most potent antimicrobial against *S. pyogenes* isolates was penicillin (MIC_{90} 0.008 mg/L) against which all 120 isolates were susceptible. Macrolide resistance showed considerable variation among the six centres, with the highest prevalence (42.1%) in Sendai and 0% in Nagasaki (although this centre collected only three isolates). Overall, 82.5% of isolates were erythromycin-susceptible. Among the 21 (17.5%) erythromycin-resistant isolates, the mechanisms of resistance detected were *mef*(A) in 15 isolates, *erm*(A) subclass *erm*(TR) in five isolates and *erm*(B) in one isolate. Telithromycin had mode MIC (0.015 mg/L) and MIC_{90} (0.25 mg/L), values which were 16- to 32-fold lower than those of the tested macrolides.

Haemophilus influenzae

β-lactamase production amongst *H. influenzae* isolates (n = 281) had an overall incidence of 8.5% and variation among centres of 5.1% to 11.5%. A single β-lactamase-negative, ampicillin-resistant (MIC ≥4 mg/L) strain (BLNAR) was identified (from Sendai). A further nine β-lactamase-negative isolates,

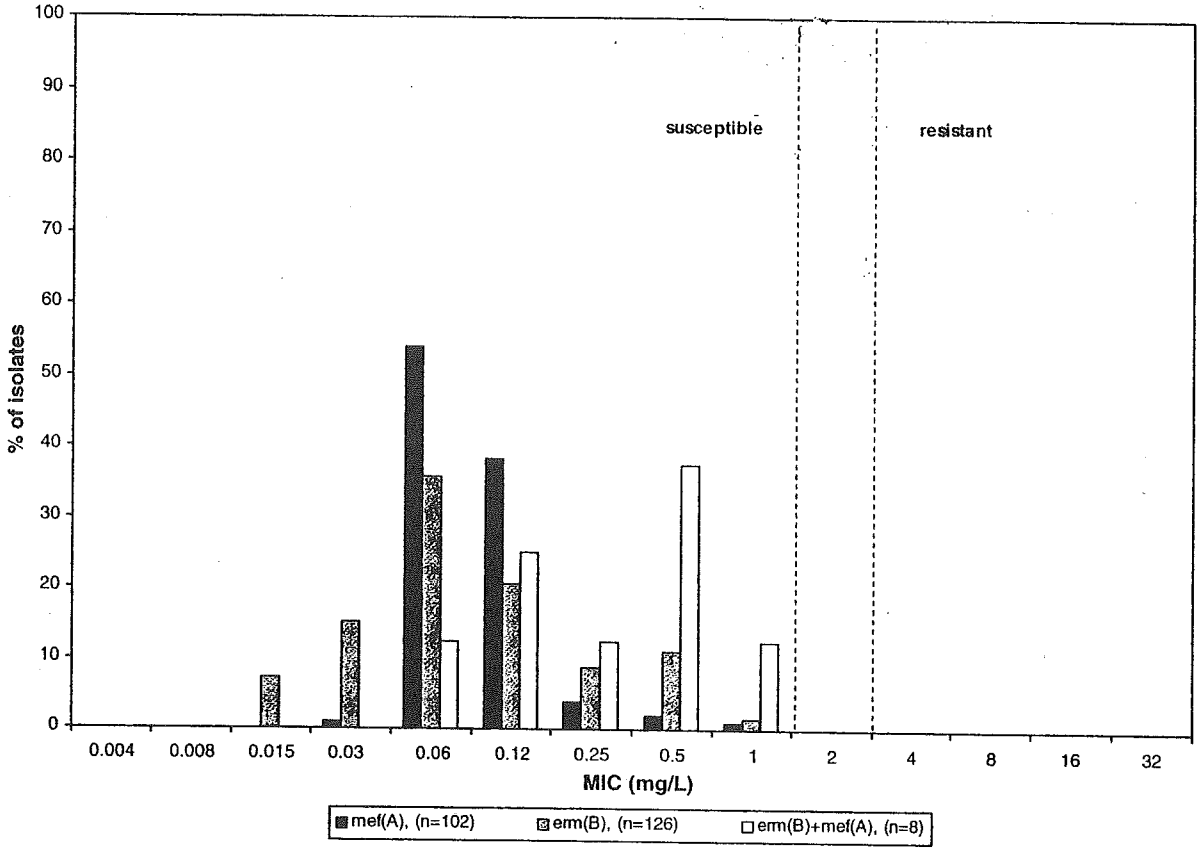

Figure 2 Telithromycin MIC distribution for macrolide-resistant genotypes of *Streptococcus pneumoniae* from Japan.

however, had ampicillin MICs of 2 mg/L (intermediate resistance according to NCCLS breakpoints).

Comparative in vitro activity of all antimicrobial compounds tested against *H. influenzae* and categorised by β-lactamase production is shown in Table 4. Of the β-lactams tested, cefditoren (MIC_{90} 0.06 mg/L; no NCCLS breakpoint) and cefixime (MIC_{90} 0.25 mg/L; 100%) were the most active.

Chloramphenicol resistance had low prevalence (3.6%), with nine of the ten nonsusceptible isolates also β-lactamase-positive. Similarly, tetracycline resistance was low (6.4%) with resistant isolates predominantly β-lactamase-positive (12/18). The MIC_{90} values for both chloramphenicol and tetracycline among β-lactamase-positive *H. influenzae* isolates (16 mg/L) were 16 times greater than for β-lactamase-negative isolates (Table 4).

The MICs of the macrolides and telithromycin to *H. influenzae* isolates followed unimodal distributions in the rank order: azithromycin (MIC_{90} 1 mg/L) > telithromycin (MIC_{90} 2 mg/L) > rokitamycin (MIC_{90} 8 mg/L) > clarithromycin and roxithromycin (MIC_{90} 16 mg/L), (Table 4). There was no correlation between ketolide/macrolide susceptibility and β-lactamase production.

Moraxella catarrhalis

Of the 122 *M. catarrhalis* isolates, 118 (96.7%) were β-lactamase-positive. With the exception of some β-lactams (ampicillin, cefaclor, cefuroxime and cefcapene), all antimicrobials tested showed good activity (MIC_{90} values of ≤1 mg/L) against *M. catarrhalis* isolates (Table 4). Cefixime was the most active β-lactam (MIC_{90} 0.25 mg/L), followed by cefdinir and cefditoren (both, MIC_{90} 0.5 mg/L) (Table 4). The rank order of activity of the MLS class of antimicrobials was azithromycin (MIC_{90} 0.06 mg/L) > telithromycin, clarithromycin, and rokitamycin (MIC_{90} 0.25 mg/L) > roxithromycin (MIC_{90} 0.5 mg/L). Sparfloxacin and tosufloxacin were the most potent (MIC_{90} 0.008 mg/L) fluoroquinolones.

Discussion

Streptococcus pneumoniae

Previous reports have demonstrated the increasing prevalence of penicillin resistance of both intermediate (MIC 0.12–1 mg/L) and resistant (MIC

Table 4 Comparative in vitro activity of various antimicrobials against isolates of *Haemophilus influenzae* and *Moraxella catarrhalis* from Japan.

Antimicrobial	*Haemophilus influenzae*									*Moraxella catarrhalis*[a]	
	All isolates (n = 281)			β-lactamase positive (n = 24)			β-lactamase negative (n = 257)			All isolates (n = 122)	
	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[b]	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[b]	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[b]	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Ampicillin	0.25	2	87.9	>16	>16	0	0.25	1	96.1	8	16
Amoxicillin–clavulanate	0.5	2	99.3	1	2	100	0.5	2	99.2	0.12	0.25
Cefaclor	4	16	86.5	16	32	45.8	4	8	90.3	2	16
Cefcapene	0.5	1	NA	1	16	NA	0.5	4	NA	8	16
Cefdinir	0.25	1	91.8	0.5	2	79.2	0.25	1	93.0	0.12	0.5
Cefditoren	0.015	0.06	NA	0.015	0.12	NA	0.015	0.03	NA	0.12	0.5
Cefixime	0.03	0.25	100	0.12	0.5	100	0.03	0.25	100	0.25	0.25
Cefpodoxime	0.06	0.5	99.3	0.12	1	100	0.06	0.5	99.2	0.5	1
Cefuroxime	1	4	95.4	2	4	95.8	1	4	95.3	2	4
Telithromycin	1	2	100[c]	1	2	100[c]	1	2	100[c]	0.06	0.25
Roxithromycin	8	16	NA	8	8	NA	8	16	NA	0.25	0.5
Clarithromycin	8	16	88.3	8	16	75.0	8	16	89.5	0.25	0.25
Azithromycin	1	1	100	1	2	100	1	1	100	0.06	0.06
Rokitamycin	4	8	NA	4	8	NA	4	8	NA	0.25	0.25
Minocycline	1	2	NA	1	2	NA	1	2	NA	0.06	0.06
Tetracycline	0.5	1	93.6	1	16	50.0	0.5	1	97.7	0.25	0.5
Co-trimoxazole	0.06	0.06	97.9	0.06	4	87.5	0.06	0.06	98.8	0.12	0.25
Chloramphenicol	0.5	1.0	96.4	0.5	8	62.5	0.5	0.5	99.6	0.5	0.5
Ciprofloxacin	0.015	0.015	100	0.015	0.03	100	0.015	0.015	100	0.03	0.03
Levofloxacin	0.015	0.015	100	0.015	0.03	100	0.015	0.015	100	0.03	0.03
Sparfloxacin	0.004	0.008	99.3	0.008	0.008	100	0.004	0.008	99.2	0.008	0.008
Tosufloxacin	0.004	0.008	NA	0.008	0.008	NA	0.004	0.008	NA	0.008	0.008

[a] NCCLS breakpoints not available for *M. catarrhalis*.
[b] % of isolates susceptible according to NCCLS breakpoints.
[c] NCCLS (SAST 2003) approved breakpoint for *H. influenzae*: susceptible ≤4 mg/L; NA = NCCLS breakpoints not available.

≥2 mg/L) phenotypes amongst isolates of *S. pneumoniae*.[2,9,14] During the 1999–2000 winter season, 44.5% of *S. pneumoniae* RTI isolates from Japan were penicillin resistant and 19.8% were penicillin intermediate, a pattern with small geographic variation throughout Japan (Table 1). In previous studies, Yoshida et al.[15] found that penicillin resistance increased from 4.3% in 1988 to 9.8% in 1992 and Sahm et al.[10] reported 10.1% penicillin resistance for the 1997–98 winter season. Therefore, penicillin resistance in Japan is increasing and current data strongly suggest that the trend has accelerated in recent years.

Resistance to penicillin in *S. pneumoniae* is mediated by changes in the affinity of high molecular weight penicillin binding proteins (PBPs) for their substrates. As these PBPs are also targets for other β-lactams, the activity of aminopenicillins, cephalosporins and carbapenems is also reduced against penicillin-resistant strains. This is most evident with compounds considered active only against penicillin-susceptible *S. pneumoniae*, such as cefaclor and cefixime. Cefuroxime, cefpodoxime and cefdinir retained some activity against penicillin-intermediate isolates (approximately 40%), but little or no activity against resistant isolates. This perhaps reflects the trend towards greater resistance as previous work has shown that cefuroxime, among other cephalosporins, can retain activity against many penicillin-resistant strains.[16,17] The most effective β-lactams for the 1999–2000 winter season in Japan were cefditoren and amoxicillin–clavulanate, with over 90% susceptibility among penicillin-resistant strains. The amoxicillin–clavulanate results can be extrapolated to include amoxicillin as an effective β-lactam (92% susceptibility among penicillin-resistant strains), although amoxicillin itself was not tested against *S. pneumoniae*.

Macrolides form the principal alternative to β-lactams for the treatment of lower RTIs involving *S. pneumoniae*. However, it is now clear that this class of compounds, including erythromycin, clarithromycin and azithromycin, is seriously compromised by the development of resistance not only as a result of

the increasing prevalence of penicillin-resistant pneumococci but also, in Japan, among penicillin-susceptible strains.

Typical of the Far East, *S. pneumoniae* macrolide resistance in Japan is high (77.9%) with some centre variation (67.3–86.4%). This finding of 77.9% is considerably higher than the 66.5% reported for the 1997–1998 winter season.[10] The proportion of penicillin-resistant isolates (n = 137) that are also macrolide-resistant has not increased over the same period (124/137, 90.5%) and is slightly lower than the previous study (1997–1998, 95.5%).

Two main mechanisms are known to account for macrolide resistance in *S. pneumoniae*. With the first, resistance is associated with specific mutation within the *erm* gene that confers resistance to most macrolides, lincosamides and streptogramin B antibiotics.[18] With the second, the so-called M phenotype, resistance is mediated by an efflux mechanism due to the presence of the *mef*(A) gene that confers resistance to 14- and 15-membered macrolides.[19] Growing macrolide resistance is of increasing concern, especially that dependent upon the *erm*(B) genotype; not only because it is the more potent macrolide resistance, but because resistance to other antimicrobial compounds appear preferentially to be associated with it. This study shows that in Japan, the distribution of *erm*(B) and *mef*(A) are similar.

Telithromycin, a synthetic ketolide derived by chemical modification of desclarithromycin, was designed to maintain potent antimicrobial activity against community-acquired respiratory tract infection (CARTI) pathogens, even macrolide-resistant pneumococci, and not to induce resistance due to *erm*(B).[20] There was, however, an upward shift in telithromycin MICs among the isolates with *erm*(B)-mediated macrolide resistance compared with *mef*(A) strains. This effect of *erm*(B) resistance on the activity of telithromycin has been reported previously although, as in this study, all the isolates were still found to be inhibited by telithromycin at ≤1 mg/L.

Worldwide incidence of fluoroquinolone-resistant *S. pneumoniae* (levofloxacin MIC ≥8 mg/L) is rare, although it tends to be concentrated in pockets of Asia (specifically Hong Kong) and North America. The four (1.3%) resistant isolates from Japan were obtained from four different centres, and would therefore suggest random distribution and independent origin.

Streptococcus pyogenes

Streptococcus pyogenes was susceptible to most of the antimicrobials tested with the notable exception of the macrolides (17.5% resistant, mostly *mef*(A)). Telithromycin was 16- to 32-fold more potent than the macrolides although penicillin remains the most potent antimicrobial.

Haemophilus influenzae

There is considerable variability worldwide in the prevalence of β-lactamase production by *H. influenzae*, with previous studies showing values of 19% for Europe, 42% for the USA and around 14% for Japan.[10,21,22] The value for Japan is slightly higher than the finding here of 8.5%. Only a single (0.36%) β-lactamase-negative ampicillin-resistant (BLNAR) (ampicillin MIC ≥4 mg/L) strain was isolated in Japan during the winter season 1999–2000, although 3.2% of isolates were β-lactamase-negative with low-level resistance to ampicillin (MIC 2 mg/L). These values are considerably lower that those published for Japan by Hasegawa et al.[23]

Of the β-lactams tested, cefixime (100%), cefpodoxime (99.3%), cefuroxime (95.4%), and cefdinir (91.8%) were the most active, followed by ampicillin (87.9%), cefaclor (86.5%), and amoxicillin (81.5%), (Table 4). β-lactamase production conferred resistance to ampicillin and amoxicillin for all isolates, but had little or no effect on susceptibility to cefixime, cefpodoxime, and cefuroxime. For cefdinir and cefaclor the effect was partial, susceptibility being reduced by approximately 15% and 50%, respectively.

Similar partial co-resistance was observed for chloramphenicol and tetracycline, where 99.6% and 97.7% β-lactamase-negative isolates were susceptible compared with 62.5% and 50% β-lactamase-positive isolates, respectively.

All isolates were susceptible to azithromycin, with 88.3% susceptible to clarithromycin. For the 1997–1998 winter season, Sahm et al. also found 100% susceptibility of isolates to azithromycin,[10] with 93.2% susceptible to clarithromycin, indicating a slightly increased resistance towards this macrolide. In 1999–2000, the azithromycin MICs for the Japanese isolates were all ≤2 mg/L. All isolates of *H. influenzae* were susceptible to the ketolide telithromycin at ≤4 mg/L.

Moraxella catarrhalis

β-lactamase production was observed in 96.7% of *M. catarrhalis* isolates tested in Japan, a figure almost identical to 97.5% reported by Sahm et al.[10] for the 1997–98 winter season. β-lactamase-producing strains of *M. catarrhalis* were first reported in the late 1970s and by the late 1980s, these strains were predominant, accounting for more than 80% of clinical isolates in a number of studies.[8,24,25]

The β-lactamases of *M. catarrhalis* are inhibited by clavulanic acid and the combination of amoxicillin–clavulanic acid has been shown to be highly active against this species.[25–29] Indeed, in this study, among β-lactamase-positive *M. catarrhalis* the MIC_{90} for unprotected ampicillin was high at 16 mg/L, in contrast with 0.25 mg/L for amoxicillin–clavulanate.

Summary

Despite growing public awareness Japan has witnessed increased and even accelerating resistance to the macrolides and to β-lactams. Fluoroquinolone resistance, albeit at a low level, would also appear to be endemic. This study documents the high prevalence of antimicrobial resistance and co-resistance among respiratory pathogens in Japan.

For a great proportion of respiratory infections that require antimicrobial therapy, amoxicillin remains largely effective; however, in Japan, the preference is for the use of newer drugs as first-line treatment. This study reinforces the necessity for judicious use of old and new antimicrobial compounds and, with the technical ability that is now available, to evaluate resistance at a genetic level to monitor more detailed patterns of emergence.

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Mechanisms of disease

Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*

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Summary

Background Bacteria develop resistance to aminoglycosides by producing aminoglycoside-modifying enzymes such as acetyltransferase, phosphorylase, and adenyltransferase. These enzymes, however, cannot confer consistent resistance to various aminoglycosides because of their substrate specificity. Notwithstanding, a *Pseudomonas aeruginosa* strain AR-2 showing high-level resistance (minimum inhibitory concentration >1024 mg/L) to various aminoglycosides was isolated clinically. We aimed to clone and characterise the genetic determinant of this resistance.

Methods We used conventional methods for DNA manipulation, susceptibility testing, and gene analyses to clone and characterise the genetic determinant of the resistance seen. PCR detection of the gene was also done on a stock of *P aeruginosa* strains that were isolated clinically since 1997.

Findings An aminoglycoside-resistance gene, designated *rmtA*, was identified in *P aeruginosa* AR-2. The *Escherichia coli* transformant and transconjugant harbouring the *rmtA* gene showed very high-level resistance to various aminoglycosides, including amikacin, tobramycin, isepamicin, arbekacin, kanamycin, and gentamicin. The 756-bp nucleotide *rmtA* gene encoded a protein, RmtA. This protein showed considerable similarity to the 16S rRNA methylases of aminoglycoside-producing actinomycetes, which protect bacterial 16S rRNA from intrinsic aminoglycosides by methylation. Incorporation of radiolabelled methyl groups into the 30S ribosome was detected in the presence of RmtA. Of 1113 clinically isolated *P aeruginosa* strains, nine carried the *rmtA* gene, as shown by PCR analyses.

Interpretation Our findings strongly suggest intergeneric lateral gene transfer of 16S rRNA methylase gene from some aminoglycoside-producing microorganisms to *P aeruginosa*. Further dissemination of the *rmtA* gene in nosocomial bacteria could be a matter of concern in the future.

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Introduction

Acquisition of multidrug resistance in nosocomial pathogens such as *Pseudomonas aeruginosa* has become a global concern.[1] For the treatment of infectious diseases caused by *P aeruginosa*, fluoroquinolones, broad-spectrum β lactams including carbapenems, and aminoglycosides such as the anti-pseudomonal drug amikacin, are the drugs of last resort. In Japan, however, about 20% of clinically isolated *P aeruginosa* have acquired resistance to imipenem or ciprofloxacin, while about 5% of clinical isolates also show resistance to amikacin.[2] Therefore, continuing amplification of resistance rates and levels, and expansion of resistance profiles to aminoglycosides in *P aeruginosa*, is becoming a general and genuine threat in clinical settings.[3]

Various aminoglycosides—such as gentamicin, kanamycin, amikacin, tobramycin, and isepamicin—have been developed and used for chemotherapy since the 1950s.[4] These drugs have high affinities for 16S rRNA of the bacterial 30S ribosome, and they block protein synthesis.[5] Over the past few decades, results of many studies on the mechanisms of resistance to aminoglycosides have shown self-modification of drugs to be the most typical mechanism; impermeability caused by upregulation of the active multidrug efflux system MexXY-OprM also confers broad but low-level resistance to aminoglycosides.[6] Several aminoglycoside-modifying enzymes—such as acetyltransferase, phosphorylase, and adenyltransferase—that catalyse covalent modification of specific amino or hydroxyl groups have been identified.[7] These enzymes have been noted in various nosocomial bacteria and are generally associated with transposable elements mediated by transferable R-plasmids. To overcome these modifying enzymes, a novel semisynthetic aminoglycoside, arbekacin, a derivative of kanamycin, was developed in Japan; this drug shows strong activity against various bacterial species and is rarely inactivated by single 6′ acetylation or 2″-phosphorylation.[8] Arbekacin showed effective antibacterial activity against various gram-positive and gram-negative bacteria by inhibition of 16S rRNA in bacterial 30S ribosome.[9,10]

Arbekacin has been used in Japan since 1990,[11] although this drug was approved only for control of infections caused by meticillin-resistant *Staphylococcus aureus* (MRSA) for prudent antibiotic use. However, several arbekacin-resistant MRSA strains have emerged in Japan, which produce the bifunctional enzyme, aminoglycoside-6′-*N*-acetyltransferase-2″-*O*-phospho-transferase, which mediates both 6′-acetylation and 2″-phosphorylation; this type of modification, however, confers only low-level drug resistance (minimum inhibitory concentration [MIC] between 4 and 32 mg/L).[12]

GLOSSARY

16S rRNA METHYLASES
Enzymes essential for folding and stabilisation of rRNA by methylation in bacterial ribosomes. Aminoglycoside-producing actimomycetes produce enzymes that mediate methylation of ribonucleotide residues at the aminoglycoside-binding site of 16S rRNA to protect their own 16S rRNAs from intrinsic aminoglycosides.

ACTINOMYCETES
A group of morphologically diverse gram-positive bacteria (order Actinomycetales) that produce various bioactive agents including antibiotics, enzymes, and vitamins. *Streptomyces* spp and *Micromonospora* spp belong to this bacterial order.

CONJUGATION
Transmission of bacterial plasmids through direct contact between bacterial cells.

SHINE-DALGARNO SEQUENCE
A specific nucleotide sequence essential for initiation of bacterial protein synthesis in bacterial ribosome, according to information encoded by mRNA. The 3'-terminal region of 16S rRNA in bacterial 30S ribosomal subunit recognises and attaches to this sequence. The ATG codon locating just downstream of the Shine-Dalgarno sequence generally functions as the initiation codon for formyl-methionine, which is usually the forefront aminoacid residue at the N-terminal of peptides.

TRANSCONJUGANTS
Bacterial cells that accept foreign plasmid by conjugation.

In this study, we aimed to characterise the genetic determinant for multiple and high-level aminoglycoside resistance in a clinically isolated *P aeruginosa* strain showing consistent and very high-level resistance to all clinically useful aminoglycosides, including amikacin and arbekacin. We also aimed to characterise the prevalence of the molecular mechanism of very high-level resistance to arbekacin found in *P aeruginosa* strain AR-2 among clinically isolated *P aeruginosa* strains.

Methods

Procedures

DNA manipulation, susceptibility testing, and gene analyses

We isolated *P aeruginosa* strain AR-2 from a clinical sample (sputum) taken in 1997. We used *E coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA) as the transformation host and for propagation of plasmids. We used *P aeruginosa* strain 105 (ciprofloxacin-resistant, arbekacin-sensitive, amikacin-sensitive) as recipient in a CONJUGATION experiment. The plasmid pBC-SK+ (Stratagene) was used as the cloning vector, and pTO001—an *E coli-P aeruginosa* shuttle-cloning vector—was also used. *P aeruginosa* PAO1 served as the host for subcloning experiments. Unless noted otherwise, we grew cultures at 37°C in Luria-Bertani broth. We established MICs of aminoglycosides by an agar dilution method with Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA), according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines M7-A5.[13]

Primers used

RMTA-forward
5'-CTAGCGTCCATCCTTTCCTC-3'

RMTA-reverse
5'-TTTGCTTCCATGCCCTTGCC-3'

DNA prepared from *P aeruginosa* AR-2 was digested with HindIII and ligated into the HindIII site of pBC-SK+ with T4 DNA ligase (Nippon Gene, Tokyo, Japan); the resulting recombinant plasmid was named pBCH9, and the deleted plasmid was named pBCH9-13 (figure 1). We selected *E coli* strain XL1-Blue transformants carrying a roughly 8-kb insert on Luria-Bertani agar plates containing both arbekacin (2 mg/L) and chloramphenicol (30 mg/L). We assayed MICs on both the parent strain and transformants, according to the guidelines of the NCCLS. We established the nucleotide sequence by the dideoxy-chain termination method with a model 3100 DNA sequencer (Applied Biosystems Japan, Tokyo, Japan). We did nucleotide and aminoacid sequence homology searches with the internet program FASTA (National Institute of Genetics, Mishima, Japan).[14] We aligned nucleotide and aminoacid sequences with GENETYX-MAC software, version 10.1.1 (Software Development, Tokyo, Japan).

To ascertain the transferability of the *rmtA* gene for arbekacin resistance, we did conjugation experiments with *P aeruginosa* strain 105 as a recipient. TRANSCONJUGANTS were selected on Mueller-Hinton agar

	P aeruginosa AR-2	*E coli* XL1-blue			*P aeruginosa* PAO1		*P aeruginosa*	
		pBCH9	pBCH9-13	pBC-SK+	pTORmtA	pTO001	Transconjugant	105*
4,6-substituted deoxystreptamine antimicrobials								
Kanamycin groups								
Arbekacin	>1024	>1024	>1024	0·5	>1024	1	>1024	4
Amikacin	>1024	>1024	>1024	1	>1024	8	>1024	4
Kanamycin	>1024	>1024	>1024	2	>1024	128	>1024	>1024
Tobramycin	>1024	>1024	512	1	>1024	1	>1024	256
Gentamicin groups								
Gentamicin	>1024	>1024	1024	0·5	>1024	256	>1024	>1024
Sisomicin	>1024	512	>1024	0·5	>1024	256	>1024	>1024
Isepamicin	>1024	>1024	>1024	0·5	>1024	4	>1024	8
4,5-substituted deoxystreptamine antimicrobials								
Neomycin	>1024	4	>1024	4	512	16	>1024	>1024
Other aminoglycosides								
Streptomycin	128	4	2	4	32	32	>1024	>1024
Hygromycin B	1024	64	2	32	512	512	1024	512
Others								
Ceftazidime	2	0·5	0·25	0·25	ND	ND	128	32
Imipenem	1	0·25	0·25	0·125	ND	ND	16	16
Ciprofloxacin	0·25	0·125	0·125	0·125	ND	ND	64	64

ND=not determined. pBCH9, pBCH9-13, pBC-SK+, pTORmtA, and pTO001 are the plasmids harboured by each transformant. pBC-SK+, pTO00=cloning vector, expression vector. pBCH9=pBC-SK+ + 8 kb insert fragment. pBCH9-13=pBC-SK+ + 1–2 kb insert fragment. pTORmtA=pTO001+rmtA.*105 was recipient for the conjugation study.

MICs (mg/L) for parental strain, transformants, and transconjugant

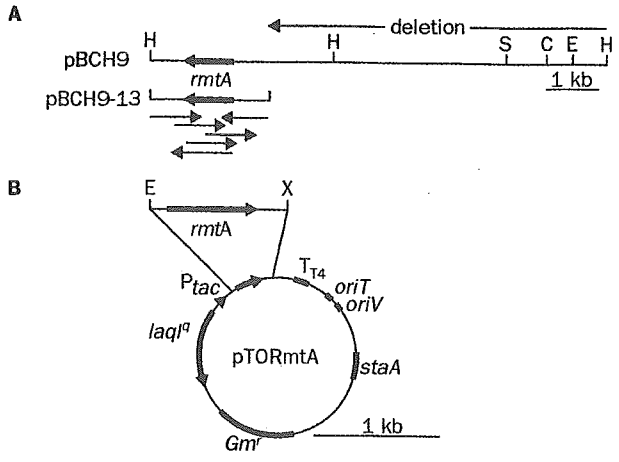

Figure 1: **Sequencing strategy (A) and restriction map (B) of the DNA insert**

Black bar represents the coding region of the *rmtA* gene and the arrow the direction of transcription. Horizontal thin arrows show the sequencing strategy. H=HindIII; C=ClaI; S=SacI; E=EcoRI; X=XbaI.

containing arbekacin (64 mg/L) and ciprofloxacin (5 mg/L). We did genomic DNA analysis of the parent, recipient, and transconjugants with pulsed-field gel electrophoresis. SpeI-digested genomic DNA fragments were separated for 22 h at 6 V/cm and 14°C with a CHEF-DR system (BioRad Laboratories, Tokyo, Japan). We did electrophoresis in two ramps as follows: pulse times were linearly increased from 4 s to 8 s for 12 h during the first ramp and from 8 s to 50 s for 10 h during the second ramp.

Assay of gene product

For thin layer chromatography analyses, we harvested bacterial cells grown in Luria-Bertani broth at the middle of the logarithmic phase. Cells were washed and resuspended with 0·1 mol/L phosphate buffer (pH 7·0). We disrupted the cell suspension by a French press (Ohtake, Tokyo, Japan), and then centrifuged it at 7700 *g* for 10 min at 4°C. The supernatant was ultracentrifuged at 100 000 *g* for 3 h at 4°C with a 65 Ti rotor (Beckman Instruments, Fullerton, CA, USA), and we stored the resulting cell-free extract at –20°C before use. We did acetylation or phosphorylation under the following conditions: 0·5 mmol/L arbekacin, 0·1 mol/L phosphate buffer (pH 7·0), 10% (volume/volume) cell-free extract, and 4 mmol/L acetylCoA or ATP in a 50 μL reaction mixture. After incubation for 3 h at 37°C, we monitored every reaction mixture by thin layer chromatography, which we did with a silica gel plate (Merck silica gel 60 F254; Merck, Darmstadt, Germany) developed with 5% KH_2PO_4 and stained with ninhydrin reagent.

Preparation of ribosomes, ribosomal subunits, and post-ribosomal supernatant containing material removed from 70S ribosomes by high salt washing (S100) was done as described by Skeggs and others.[15] For assay of methylase activity, the extract (S100) from a *P aeruginosa* clone that harbours a recombinant plasmid, pTORmtA, carrying the *rmtA* genes was used as a source of methylase together with S-adenosyl-L-methionine as cofactor. Radiolabelled methyl groups were incorporated into 16S rRNA at 35°C in reaction mixtures (100 μL total volume) made up in a buffer containing 50 mmol/L Hepes-KOH (pH 7·5 at 20°C), 7·5 mmol/L $MgCl_2$, 37·5 mmol/L NH_4Cl, and 3 mmol/L 2-mercaptoethanol. Such mixtures contained 20 pmol of *P aeruginosa* PAO1 (pTO001) 30S ribosomal subunits (substrates for methylation) together with 50 μL S100 from the clone of *P aeruginosa* (controls received S100 from *P aeruginosa* PAO1 [pTO001]) plus $9{\cdot}25\times10^4$ Bq S-adenosyl-[methyl-^{3}H]-L-methionine (18·5 GBq/mmol). We removed samples (10 μL) at intervals (0, 10, 30, and 50 min) and allowed them to permeate a DEAE (diethylaminoethyl) filtermat (glass fibre filter, with DEAE active groups; Wallac, Turku, Finland), and the filtermat was washed three times with ice-cold 50 mmol/L glycine hydrochloride (pH 4·5) and a further two times with ice-cold ethanol. The filtermat was dried and soaked in a scintillator, MeltiLexTM (Wallac), and then radioreactivity was counted by MicroBeta plus (Wallac).

PCR screening of rmtA *gene harbouring strains*

We screened a bacterial stock of 1113 clinically isolated *P aeruginosa* strains for the *rmtA* gene. PCR analyses with the primers shown in the panel, which amplify a 635-bp fragment within the *rmtA* gene, were done on strains showing a degree of resistance to gentamicin, amikacin, and arbekacin (MICs ≥32 mg/L) .

```
ACCATCCCGAGTTGTGGCCTTCCTACTGCGATAATTCATATGCTATTCGCCCCGATCTGGTGCTCAGCGGCCATGCGCATGGCGGGCAGA   90
  H  P  E  L  W  P  S  Y  C  D  N  S  Y  A  I  R  P  D  L  V  L  S  G  H  A  H  G  G  Q  I

TCGGCCTGCCATGGATCGGCGGGCTGCTTGCGCCCAATCAAGGGTTCTTCCCTAAGTATACCTCGGGGCACTATGCCGCAGACAATGGCA  180
  R  L  P  W  I  G  G  L  L  A  P  N  Q  G  F  F  P  K  Y  T  S  G  H  Y  A  A  D  N  G  T

          -35                 -10
CCCAAATGATTGTCAGCCGGGGGCTTGGCAATAGCATCTTTCCCGTGCGTGTATTCAATCGCCCCCATTTGCCGGTGATCACGCTAACGG  270
  Q  M  I  V  S  R  G  L  G  N  S  I  F  P  V  R  V  F  N  R  P  H  L  P  V  I  T  L  T  G

GTGGGTAGGGTGAGCCGAGGTATAAGGGATCATTTATGCAATCAGGCGCAGCAATTCCCACCACAACCAAGGAGACACAGTATGAGCTTT  360
  G  *                                                                    M  S  F    3

GACGATGCCCTAGCGTCCATCCTTTCCTCAAAAAAATATCGTTCCCTCTGCCCCGATACCGTACGGCGGATTTTAGATCAGGAATGGGGG  450
D  D  A  L  A  S  I  L  S  S  K  K  Y  R  S  L  C  P  D  T  V  R  R  I  L  D  Q  E  W  G    33

CGGCACAAATCGCCTAAGCTGGCAGTGGAGGCCACTCGCACCCGGCTGCACGGGATTTGCGGGGCCTATGTCACGCCGGAATCGCTCAAG  540
R  H  K  S  P  K  L  A  V  E  A  T  R  T  R  L  H  G  I  C  G  A  Y  V  T  P  E  S  L  K    63

GCTGCAGCAGCGGCATTATCGGTTGGCGATGTGCAAAAGGCACTGTCGCTGCACGCCTCTACCAAGGAGCGGTTGGCCGAATTGGACTGC  630
A  A  A  A  A  L  S  V  G  D  V  Q  K  A  L  S  L  H  A  S  T  K  E  R  L  A  E  L  D  C    93

CTCTACGATTTTATCTTTCTGGCGGGGTGCCCCATCGTGTGTTGGATATCGCTTGCGGCCTAAACCCGCTGGCCCTCTTTATACGTGAC  720
L  Y  D  F  I  F  S  G  G  V  P  H  R  V  L  D  I  A  C  G  L  N  P  L  A  L  F  I  R  D   123

ATAACATCTGTATGGGCGTGCGACATCCATCAGGGGTTGGGCGATGTGATCACCCCCTTTGCCCATCATCAGGGATTGGACTTCACGTTC  810
I  T  S  V  W  A  C  D  I  H  Q  G  L  G  D  V  I  T  P  F  A  H  H  Q  G  L  D  F  T  F   153

GCCCTGCAGGATGTGATGTGTACGCCGCCCACTGAGACGGGGGATTTGGCACTGGTATTTAAATTACTGCCTTTGCTGGAGCGAGAGCAA  900
A  L  Q  D  V  M  C  T  P  P  T  E  T  G  D  L  A  L  V  F  K  L  L  P  L  L  E  R  E  Q   183

GCTGGCGCCGCCATGGCGCTACTGCAGGCACTAGCTACCCCTCGGATTGCCGTCAGCTTCCCCACCCGCAGTTTAGGCGGGCGCGGCAAG  990
A  G  A  A  M  A  L  L  Q  A  L  A  T  P  R  I  A  V  S  F  P  T  R  S  L  G  G  R  G  K   213

GGCATGGAAGCAAACTATTCCGCATGGTTCGAGGGGCCACTGCCTGATGAATTTGAAATTGAGGATACCAAGACCATTGGAATAGAGCTT 1080
G  M  E  A  N  Y  S  A  W  F  E  G  A  L  P  D  E  F  E  I  E  D  T  K  T  I  G  I  E  L   243

GTGTACATGATAAAAAGGAATAAGTGAGCTAAGCCAAGGAGCAAACCCTACCCTGCTATATAAAAAGAGGAACGAAAAAATAGTTTTCGT 1170
V  Y  M  I  K  R  N  K  *                                                                  251

TCCTCTTTTGTTATATCGACTACACAGCTATGATTGCTGTTTCTTGAGTAGGCGTTTATAGGTCAGTTCTATCCCCAACGCCCCCAGCG 1260
                          *  S  Q  Q  K  K  L  L  R  K  Y  T  L  E  I  G  L  A  G  L

GTGCTGTCAGCAAGATAGCGAGAATCGCCACCGCTAGTATCGCTTCCCCGCCGCTCAGCCCCATCGACAGCGGGATTGAACCAATCGCAG 1350
P  A  T  L  L  I  A  L  I  A  V  A  L  I  A  E  G  G  S  L  G  M  S  L  P  I  S  G  I  A
```

Figure 2: **Nucleotide and deduced aminoacid sequences of *rmtA* gene and RmtA protein**

The 1350 bp sequence of the 1662 bp area determined is shown. Stop codons are indicated by asterisks. A putative SHINE-DALGARNO SEQUENCE is boxed. The putative promoter –35 region is marked by a bold line at positions 190–195, and the possible –10 region is indicated by a bold line below the nucleotide sequence at positions 212–217.

```
P. aer. AR-2/RmtA  ----------MSFDDALASILSSKKYRSLCPDIVRRILDQEWGRHKSPKLAVEATR  46
M. ros. /Grm       ---MT-TSTGD--D-RIDQLQQAITKSRRYQIVAPAIVRRLARAALVASRGDVPDAV-KR  52
M. zio. /Sgm       ---MT-APAAD--D-RIDEIERAITKSRRYQIVAPAIVRRLARAALVAARGDVPDAV-KR  52
S. hin. /NbrB      MPHPA-PGPADAEDPRLAEVMAAVRSSRRYQSVAPEIVRRLAANALVASRGDLAEAV-KR  58
S. kan. /Kmr       ----MSQSASDE-DPKLTRVVEAVRGGRRYRSVTDQAVRRLARAALAVEGGDVTRAT-KR  54
S. ten. /KgmB      MPHPA-PGPGDPEDPRLAEVVDAVRSSRRYQSVAPEIVRRLATSALVASRGDLAEAV-KR  58
                                .  .  .  ...*..... .***..  .... ...  .. .*

P. aer. AR-2/RmtA  TR--LHGICGAYV--TPES---L-KAAAAALSVGDVQ--KA-LSLHA-----SIKERLAE  90
M. ros. /Grm       TKRGLHEIYGAFLPPSAPNYTALLRHLDSAVEAGDDEAVVRWD-RRAMSVHMSIRERVPH  111
M. zio. /Sgm       TKRGLHEIYGAFLPPSPPNYAALLRHLDSAVDAGDDEAV-RAALLRAMSVHISIRERLPH  111
S. hin. /NbrB      TKRSLHEVFGAYLPSPP-KYDALLRQLRDAVDAGDDEAVRAVLHRAMSTHAST-RERLPI  116
S. kan. /Kmr       TKRGLHEVFGAFMPMTPK-YEALLRDVPEALELDDPEAIRTAL-KPALGAHSSIRERLPI  112
S. ten. /KgmB      TKRGLHEIFGAYLPSPP-KYDALLRQLRGAVDAATIRPCGHPAPRHVHARLHP-RA-LPI  115
                   *...**.. ** ..  .  .  .*.. .  *. ... ..      .     .......

P. aer. AR-2/RmtA  LDCLYDFIFSGGVPHRVL--DIACGLNPLAL-FIRDITS-V-W-ACDIHQGLGDVITPFA  144
M. ros. /Grm       LDEFYREIFRHVPRPNTL-RDLACGLNPLAVPWM-GLSDEIVYVASDIDARLMDFVGAAL  169
M. zio. /Sgm       LDEFYRELFRHLPRPNTL-RDLACGLNPLAAPWM-GLPAETVYIASDIDARLVGFVDEAL  169
S. hin. /NbrB      LEEFYREVFARLDAPISV-RDLACGMNPLAAPWM-PGSDAFTYHASDIDIRLTEFLAAAL  174
S. kan. /Kmr       LTEMYAEVFDRLDIAPATVRDLACGMNPLTVPWM-PLPAGTTYLASDIDHRLMDFAGIVL  171
S. ten. /KgmB      LDEFYREVFARCADPASV-RDLACGMNPLAAPWM-PGSDAFTYHASDIDIRLMEFLDAAL  173
                   *...*.. *     .  .*.*** ***. ...      . *.**. .*  .   ..

P. aer. AR-2/RmtA  HHQGLDFIFALQDVMCIPPTETGDLALVF-KLLPLLEREQAGAAMALLQALATPRIAVSF  203
M. ros. /Grm       TRLGVAHRTSVVDLLEARLDEP-ADVILLLKTLPCLETQQRGSGWEVIDIVNSPIIVVTF  228
M. zio. /Sgm       TRLNVPHRTNVADLLEDRLDEP-ADVILLLKTLPCLETQQRGSGWEVIDIVNSPNIVVTF  228
S. hin. /NbrB      ETLGVAHDVRVRDLMIGVG-EVATDVILLLKTLPCIEAQGRGQGWDLIDAIRSPVVVVSF  233
S. kan. /Kmr       TALGVPNRVEVRDLLITDPDPEPAD-VIFLFKAVPCLEAQGKGLGWKLLDQINSPVLVVSF  230
S. ten. /KgmB      ETLGVAHDVRVRDLMIGVG-EVEIDVILLLKIVPCIEAQGRGQGWDLIDAIRSPLVVVSF  232
                      ... .  . *.     *  ......*..*..* . .* .. ...   .*  .*.*

P. aer. AR-2/RmtA  PIRSLGGRGKGMEANYSAWFEGALPDE-FEIEDIKTIGIELVYMIKRNK              251
M. ros. /Grm       PIKSLGQRSKGMFQNYSQSFESQASERSCRIQRLEI-GNELIYVIHK--            274
M. zio. /Sgm       PIKSLGQRSKGMFQNYSQSFESQARERSCRIQRLEI-GNELIYVIQK--            274
S. hin. /NbrB      PIKSLGQRSKGMFNTYSANFGAWLENRPHDVEQVEF-RNELVYFVRKNA            281
S. kan. /Kmr       PIKTLGRRSRGMYQTHSAEFARRVAERPWKCDEIEF-GDELVYVVIKG-            277
S. ten. /KgmB      PIKSLGQRSKGMFNTYSANFDAWLENRPHDVEQLEF-RNELVYFVRKNA            280
                   **..**.*..**.  .*. *      .       . ..**.*  .
```

Figure 3: **Comparison of aminoacid sequences of known 16S rRNA methylases with *P aeruginosa* AR-2 RmtA**

Proteins in comparison: GrmB, *Micromonospora rosea*; Sgm, *M zionensis*; NbrB, *Streptoalloteichus hindustanus*; Kmr, *Streptomyces kanamyceticus*; and KgmB, *Streptomyces tenebrarius*. Identical aminoacid residues among all six enzymes are indicated by asterisks, and aminoacids with similar properties are indicated by dots. Dashes represent gaps introduced to improve alignment.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

P aeruginosa strain AR-2 showed very high-level resistance to various aminoglycosides (table). Arbekacin resistance was transferred from AR-2 to *P aeruginosa* PAO1 by conjugation, and the *E coli* clone (XL1-Blue) and transconjugant of *P aeruginosa* strain 105 showed similar resistance profiles to AR-2 against various aminoglycosides, as shown in the table. The pattern on pulsed-field gel electrophoresis of SpeI-digested genomic DNA fragments of the transconjugant was closely similar to that of the *P aeruginosa* recipient strain 105 (not shown). This finding suggested that the transconjugant was not a ciprofloxacin-resistant mutant of the donor strain *P aeruginosa* AR-2. By thin layer chromatography, however, no detectable conversion was noted in the rate of flow value of arbekacin after in-vitro acetylation or phosphorylation reactions (data not shown). Therefore, the mechanism underlying the wide range of resistance to various aminoglycosides is difficult to establish, since it is not merely production of known aminoglycoside-modifying enzymes. These findings suggest that in strain AR-2, novel molecular mechanisms determine multiple aminoglycoside resistance.

By sequencing of the plasmid carrying the *rmtA* gene, we determined a 1662-bp nucleotide sequence carrying arbekacin resistance (figure 1). An open reading frame of 756 bp was noted, with the initiation codon ATG at position 352 and the stop codon TGA at position 1105. The G+C content of the open reading frame was 55%. By part sequencing of the flanking region of the *rmtA* gene, the gene was suggested to be carried by Tn5041, which mediates Hg^{+}-resistance in *Pseudomonas* spp. The nucleotide sequence has been submitted to the EMBL, GenBank, and DDBJ databases and assigned accession number AB083212.

The open reading frame encoded a putative protein, RmtA, with 251 aminoacids (molecular weight 27 430; figure 2). The predicted aminoacid sequence of RmtA showed considerable similarity to the 16S rRNA METHYLASES produced by aminoglycoside-producing ACTINOMYCETES (figure 3).[16,17] The deduced 251 aminoacid sequence of RmtA was closely similar to GrmB and Sgm methylases found in sisomicin-producing

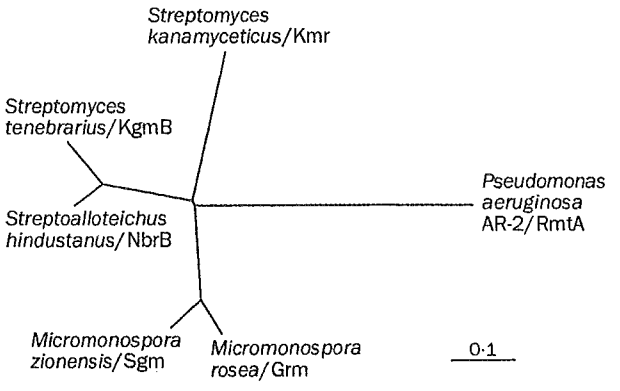

Figure 4: **Dendrogram of 16S rRNA methylases**

Units for bar are genetic units calculated with the CLUSTAL W program, which reflects the number of aminoacid exchanges.

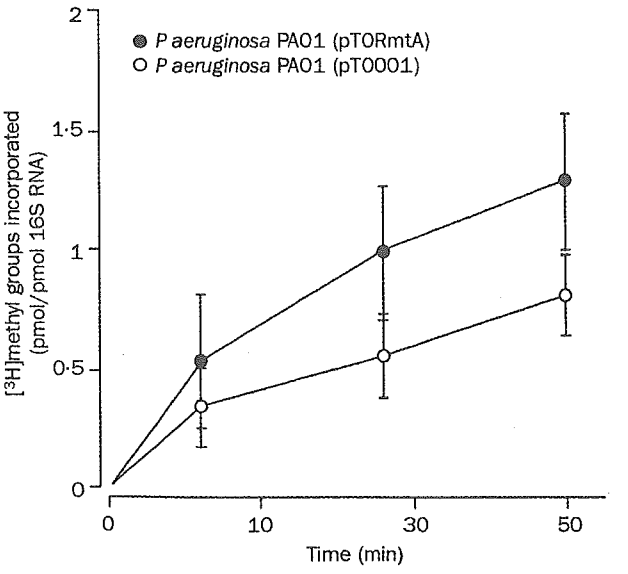

Figure 5: **Methylation of 30S ribosomal subunit by RmtA**

Error bars indicate SD.

Micromonospora rosea (35%; SWISS-PROT accession number P24619) and *M zionensis* (34%; EMBL accession number JG0018), respectively. RmtA also showed similarities to the NbrB methylase of the nebramycin-complex-producing *Streptoalloteichus hindustanus* (33%; EMBL accession number AF038408), to the Kmr methylase from the kanamycin-producing *Streptomyces kanamyceticus* (30%; EMBL accession number CAA75800), and to the KgmB methylase from the nebramycin complex-producing *Streptomyces tenebrarius* (31%; EMBL accession number AAB20100). The dendrogram in figure 4 suggests the evolutionary relation between the 16S rRNA methylases and RmtA, implying a potential intergeneric transfer of the gene from some aminoglycoside-producing actinomycetes to *P aeruginosa*. The dendrogram was calculated with the CLUSTAL W program.[14]

Incorporation of a radiolabelled methyl group into the 30S ribosomal subunits prepared from *P aeruginosa* PAO1 (pTO001) was seen (figure 5).

Of 1113 clinically isolated *P aeruginosa* strains that have been isolated from Japanese hospitals and stocked in our laboratory since 1997, nine strains were shown to carry the *rmtA* gene by PCR. These strains were isolated from seven separate hospitals in five prefectures in Japan.

Discussion

We have reported a completely new mechanism for multiple aminoglycoside resistance—that is, enzymatic methylation of the 16S rRNA found in gram-negative bacteria.

Although intergeneric lateral gene transfer has been regarded as a method of acquisition of new phenotypes for bacteria to survive in hazardous environments,[18,19] its rate and background are not well known. The *rmtA* gene product, RmtA, showed considerable similarity to 16S rRNA methylases that protect 16S rRNA in aminoglycoside-producing actinomycetes such as *Streptomyces* spp and *Micromonospora* spp. In fact, a cell-free cytosolic fraction of *P aeruginosa* PAO1 (pTORmtA) containing RmtA accelerated uptake of the ^{3}H-labelled methyl group into the 30S ribosome of *P aeruginosa* PAO1. Moreover, the *rmtA* gene was suggested to be carried by the transposon Tn5041, which mediates mercury resistance. These results suggest that traces of the 16S rRNA methylase gene have moved by intergeneric lateral gene transfer from some aminoglycoside-producing bacteria into *P aeruginosa* because of the increasingly heavy clinical use of arbekacin, which is rarely inactivated by ordinary aminoglycoside-modifying enzymes generally found in gram-negative bacteria.

Since arbekacin resistance of strain AR-2 can be easily transferred to *P aeruginosa* strain 105 by conjugation (10^{-4}–10^{-5}), the *rmtA* gene could be contained on a self-transmissible large plasmid, though more precise characterisation is now underway. This finding indicates that further widespread dissemination of the *rmtA* gene in gram-negative bacteria is possible as an important ecological result of heavy antibiotic use in clinical settings.[20] In this study, nine *P aeruginosa* strains that carry the *rmtA* gene were isolated from seven separate hospitals located in five prefectures across Japan. This finding indicates that in Japanese clinical settings there has been stealthy multifocal proliferation of *P aeruginosa* strains that have acquired consistent and very high-level resistance to various clinically important aminoglycosides through production of the newly identified 16S rRNA methylase. Since resistance to fluoroquinolones and carbapenems has already developed in gram-negative bacteria including *P aeruginosa*,[2,21] emergence of multidrug resistant superbug strains through further acquisition of the *rmtA* gene threatens to become a serious clinical problem. Further global transmission of the *rmtA* gene in gram-negative bacteria could become a matter of grave concern in the future. Like vancomycin-resistant *S aureus* strains[22] and plasmid-mediated quinolone-resistant *Klebsiella pneumoniae*,[23] bacteria readily cope with hazardous environments by accepting any genes, even those from hereditarily distant microorganisms.[24,25]

Contributors

K Yokoyama cloned and characterised the *rmtA* gene and the product, RmtA. H Kurokawa obtained clinical isolates and initially isolated *P aeruginosa* AR-2. Y Doi, K Yamane, N Shibata, T Yagi, K Shibayama, and H Kato contributed to the characterisation of RmtA. Y Arakawa contributed to coordination of the study and writing and editing of the report.

Conflict of interest statement

None declared.

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PCR Typing of Genetic Determinants for Metallo-β-Lactamases and Integrases Carried by Gram-Negative Bacteria Isolated in Japan, with Focus on the Class 3 Integron

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From January 2001 to December 2002, 587 strains of gram-negative bacterial isolates demonstrating resistance to ceftazidime and a combination of sulbactam and cefoperazone were subjected to a disk diffusion screening test using sodium mercaptoacetic acid; 431 strains (73.4%) appeared to produce metallo-β-lactamase (MBL). Of these 431 strains, 357 were found by PCR to carry genes for IMP-1 type MBL (bla_{IMP-1}), while only 7 and 67 strains carried the IMP-2 gene (bla_{IMP-2}) and the VIM-2 gene (bla_{VIM-2}), respectively. Neither VIM-1 nor SPM-1 type MBL genes were found among the strains tested. Of 431 strains, 427 carried the *intI1* gene, and 4 strains carrying both the *intI1* and *intI3* genes were reidentified as *Pseudomonas putida* harboring bla_{IMP-1}. Of these four *P. putida* strains, three strains and one strain, respectively, were separately isolated from two hospitals located in the same prefecture, and the three strains showed very similar pulsed-field gel electrophoresis patterns. Of 357 bla_{IMP-1} carriers, 116, 53, 51, 47, and 30 strains were identified as *Pseudomonas aeruginosa*, *Alcaligenes xylosoxidans*, *P. putida*/*fluorescens*, *Serratia marcescens*, and *Acinetobacter baumannii*, respectively. Four strains carrying bla_{IMP-2} were reidentified as *P. putida*. Sixty-three *P. aeruginosa* strains and four *P. putida* strains carried bla_{VIM-2}. Of 427 *intI1*-positive strains, 180, 53, 51, 47, and 35 were identified as *P. aeruginosa*, *A. xylosoxidans*, *P. putida*/*fluorescens*, *S. marcescens*, and *A. baumannii*, respectively. In the present study, it was confirmed that strains carrying bla_{IMP-1} with a class 1 integron are the most prevalent type in Japan, although several *intI3* carriers have also been identified sporadically in this country.

Since metallo-β-lactamases (MBLs) can hydrolyze a very wide range of broad-spectrum β-lactams, MBL-producing gram-negative bacteria usually demonstrate consistent resistance to a variety of broad-spectrum β-lactams, including oxyiminocephalosporins, cephamycins, and carbapenems, which are the last resort for control of infections caused by gram-negative bacteria. Thus, MBL-producing gram-negative bacteria have been recognized to be among the most important nosocomial pathogens (3), and further proliferation of these strains in clinical settings will pose a serious global problem in the future (8). For this reason, aggressive surveillance of MBL producers with respect to the classification of the genetic determinant for MBLs as well as the integron will be extremely important.

At least three major groups of plasmid-mediated MBLs—the IMP, VIM, and SPM types—have been recognized worldwide, and their genetic determinants are often associated with integrons (5, 9). IMP-1-producing *Serratia marcescens* was initially identified in Japan in 1991 (14), and in 1997 VIM-1 and SPM-1 producers were also isolated, in Italy (10) and Brazil (23), respectively. After these reports, variants of these MBL types have been reported from almost every global region. For the IMP and VIM types of MBLs, at least 12 and 6 variants, respectively, have been published or reported to date (http://www.lahey.org/studies/other.stm#table 1). Moreover, genes for these MBLs are usually located in integrons that successfully accumulate many antibiotic-resistant gene cassettes as a gene cluster (22).

At least nine genetically different integrons have been identified in various bacterial species (5), and class 1 (9), class 2 (16), and class 3 (1) integrons are often found in pathogenic gram-negative bacilli including *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Acinetobacter* spp., *Escherichia coli*, *S. marcescens*, *Citrobacter freundii*, and *Salmonella* spp. (15). Among these integrons, those in class 1 and class 3 have been reported to carry genetic determinants for MBLs. An IMP-1 type MBL associated with a class 1 integron was initially found in an *S. marcescens* clinical isolate in Japan (14), and integron-associated IMP-1 type MBLs were subsequently found in various gram-negative bacterial species such as *Klebsiella pneumoniae*, *C. freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *E. coli*, *Proteus vulgaris*, *Acinetobacter* spp., *Alicaligenes* spp., and *P. putida* (2, 20, 21).

In 1993, a class 3 integron-mediating IMP-1 was first identified by Arakawa et al. in an *S. marcescens* strain isolated in Japan (1). The organization of a class 1 integron that carries the gene for IMP-1 type MBL was characterized for *P. aeruginosa* (9). Although various class 1 integrons that convey MBL genes have been reported from a variety of gram-negative bacterial species isolated from different geographical regions, only a few isolates carrying class 3 integrons have been reported to date. Recently, *intI3*, a genetic determinant for class 3 integrons, was identified in a class A β-lactamase (GES-1)-producing *K. pneumoniae* strain isolated in Portugal (EMBL accession no. AY219651). This finding may suggest the poten-

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TABLE 1. PCR primers for detection of MBL and integrase genes

Gene	Primer name (sequence)	Expected size of amplicon (bp)	Reference or source
MBL genes			
bla_{IMP-1}	F1 (5′-ACC GCA GCA GAG TCT TTG CC-3′) R1 (5′-ACA ACC AGT TTT GCC TTA CC-3′)	587	This study
bla_{IMP-2}	F2 (5′-GTT TTA TGT GTA TGC TTC C-3′) R2 (5′-AGC CTG TTC CCA TGT AC-3′)	678	This study
bla_{VIM-1}	F3 (5′-AGT GGT GAG TAT CCG ACA G-3′) R3 (5′-ATG AAA GTG CGT GGA GAC-3′)	261	24
bla_{VIM-2}	F4 (5′-ATG TTC AAA CTT TTG AGT AAG-3′) R4 (5′-CTA CTC AAC GAC TGA GCG-3′)	801	17[a]
bla_{SPM-1}	F5 (5′-GCG TTT TGT TTG TTG CTC-3′) R5 (5′-TTG GGG ATG TGA GAC TAC-3′)	786	This study
Integrase genes			
intI1	F6 (5′-GCA TCC TCG GTT TTC TGG-3′) R6 (5′-GGT GTG GCG GGC TTC GTG-3′)	457	This study
intI2	F7 (5′-CAC GGA TAT GCG ACA AAA AGG T-3′) R7 (5′-GTA GCA AAC GAG TGA CGA AAT G-3′)	789	This study
intI3	F8 (5′-ATC TGC CAA ACC TGA CTG-3′) R8 (5′-CGA ATG CCC CAA CAA CTC-3′)	922	This study
Coamplification of the *intI3*–bla_{IMP-1} region	F9 (5′-GGT CTT GTA GGC TGT AAT TG-3′) R9 (5′-TTG TGG CTT GGA ACC TTT AC-3′)	609	This study

[a] Since the nucleotide sequence appearing in reference 17 has a typographical error, the accurate sequence is shown in this table.

tial for a future worldwide dissemination of bacteria carrying class 3 integrons in addition to the widely dispersal of class 1 integrons. In the present study, we characterized the types of MBLs and integrons found in various gram-negative bacteria isolated in Japanese clinical environments.

MATERIALS AND METHODS

Bacterial strains. From January 2001 to December 2002, 978 strains belonging to gram-negative bacterial species were submitted to the reference laboratory for antibiotic resistance at the Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo, Japan, for typing and/or characterization of β-lactamases, including extended-spectrum β-lactamases (ESBLs), AmpC or CMY type class C cephamycinases, and class B MBLs. Of these strains, 587 demonstrating high-level resistance to both ceftazidime and sulbactam-cefoperazone (MICs, ≥128 μg/ml) were selected for screening of MBL production, because, from our experience, most MBL-producing gram-negative bacteria demonstrate very high levels of resistance to these agents. In the case of *Acinetobacter baumannii*, however, strains for which MICs of ceftazidime and sulbactam-cefoperazone were ≥16 μg/ml were also selected for screening in order to prevent overlooking of MBL producers. This is because several MBL-producing *Acinetobacter* strains were found to demonstrate low-level resistance to these agents in our preliminary study, and no data were available on the distribution of MICs of ceftazidime and sulbactam-cefoperazone for MBL-producing *Acinetobacter* spp. *Stenotrophomonas maltophilia* and *Chryseobacterium* spp., which produce chromosomal MBLs, were excluded from this study. Strains to be tested were collected from 108 Japanese hospitals, with only one strain selected from each patient.

Screening of MBL producers. Strains selected by the criteria described above were subjected to a screening test for MBL production by using disks containing an MBL inhibitor (2). Two Kerby-Bauer disks containing 30 μg of ceftazidime and one disk containing 3 mg of sodium mercaptoacetic acid (SMA) (Eiken Chemical Co. Ltd., Tokyo, Japan) were used in the screening test. When the strains produce MBL together with a large amount of ESBL, AmpC cephalosporinase, or CMY type enzymes as well as bacterial membrane alterations, no evident expansion of the growth inhibition zone around the ceftazidime disk tends to appear. For such strains, a disk containing 10 μg of imipenem or meropenem was used instead of the ceftazidime disk. However, disks containing 10 μg of imipenem or meropenem are not suitable for screening all strains that produce MBL only, because the MICs of imipenem and meropenem for several MBL producers are lower than 8 μg/ml, as reported in previous studies (2, 20). Appearance of a large growth inhibition zone around the disks containing imipenem or meropenem sometimes leads to an incorrect judgment. Several samples of the SMA-test results are shown in Fig. 1.

PCR detection of genes for MBLs and integrases. Although PCR analysis can predict only the approximate types of genes, it is suitable for testing a large number of samples at the same time. In the present study, therefore, we used this method for the rough classification of genetic determinants for MBLs and integrases. PCR analyses for the detection of MBL genes were carried out for all strains for which the screening test using SMA disks gave positive results. PCR amplification for the detection of genes for MBL and integrases was performed according to the method reported by Senda et al. (20), using each positive-control strain to avoid false-negative results. The PCR primers for bla_{VIM-1} and bla_{VIM-2} were used with the same PCR conditions as those for the other types of MBL genes. The five primer sets used in this study for amplifying MBL genes are shown in Table 1. PCR amplification of integrase genes was also performed as described above, by using three primer sets shown in Table 1.

Sequencing analyses of PCR amplicons. Sequencing analyses on both strands were performed on five amplicons in each bla_{IMP-1}-, bla_{IMP-2}-, or bla_{VIM-2}-positive strain and on five amplicons in the *intI1*-positive strain. All four amplicons from *intI3*-positive strains were sequenced from both sides. PCR amplicons were purified by use of the Qiaquick PCR purification kit (Qiagen K.K., Tokyo, Japan) prior to the labeling reaction. DNA sequences were determined by using BigDye Primer Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, Calif.) and an ABI model 377 DNA sequence analyzer (Perkin-Elmer Applied Biosystems, Foster City, Calif.). Nucleotide and amino acid sequences were analyzed by the GENETYX program (version 11.0, available at http://www.sdc.co.jp/genetyx/; SDC Co. Ltd., Tokyo, Japan) and were submitted to the DNA Data Bank of Japan (DDBJ) database to check the identity or similarity of each sequence by using FASTA (http://www.ddbj.nig.ac.jp/E-mail/homology-j.html).

PCR analysis of the relationship between the *intI3* and bla_{IMP-1} genes. A PCR primer set (Table 1) was made for coamplification of the *intI3* and bla_{IMP-1} genes. By this PCR analysis, a fragment containing the 5′ regions of both the *intI3* and bla_{IMP-1} genes is able to be amplified, so that the distance and relationship between the *intI3* and bla_{IMP-1} genes can be measured.

Reidentification of bla_{VIM-2}- or *intI3*-positive strains of *P. putida*/*fluorescens*. It is sometimes difficult to distinguish *P. putida* from *Pseudomonas fluorescens* by the routine identification protocol that depends on biochemical profiles. Therefore, four strains of *P. putida*/*fluorescens* carrying bla_{VIM-2} were reidentified by a sequencing analysis of their 16S rRNA according to the method described previously (19). Four *intI3*-positive strains of *P. putida*/*fluorescens* were also reidentified in the same manner.

Pulsed-field gel electrophoretic (PFGE) analysis of *intI3*-positive strains.