Latency of *Klebsiella* species and *Escherichia coli* strains that harbor a conjugative IncA/C type plasmid carrying both *rmtB* and $bla_{CTX-M-14}$

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ABSTRACT Six high-level aminoglycoside and third generated cephalosporin of cefotaxime resistance *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Escherichia coli* isolated from clinical specimens in the same Japanese hospital since 2001. These 6 strains were resistant to all 4,6-disubstituted deoxystreptamine including albekacin. The results of multiplex PCR for 16S rRNA methylase and bla_{TEM}, bla_{SHV}, bla_{CTX-M} specific PCR reaction show that these strains harbor 16S rRNA methylase of *rmtB* and β-lactamase resistance gene of $bla_{CTX-M-9}$ group in the same large plasmid. Sequence analysis of $bla_{CTX-M-9}$ group PCR products shows this β-lactamase is $bla_{CTX-M-14}$. *rmtB*-harbored plasmids are classified the IncA/C by PCR and restriction pattern by EcoRI show only one or two band differences. Moreover, southern hybridization of EcoRI digested *rmtB* harbored plasmid by *rmtB* probe show the same hybridization pattern of 4.8 kbp bands. PFGE fingerprinting analysis of four *K. pneumoniae* revealed two fingerprinting patterns. These facts suggested that *rmtB*-positive starains not only spread horizontal transfer of *rmtB*-harbored plasmid but also clonal spread of the same strain.

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Key words : **Aminoglycoside resistance, 16S rRNA methylase, Enterobacteriacae**

INTRODUCTION

Among antimicrobial agents, aminoglycosides are among the efficacious groups of antibiotics against pathogenic gram-negative bacteria which usually cause life-threatening infections. Aminoglycosides block the peptide bind-forming translocation process through binding to the A-site of the 16S rRNA that constitutes bacterial 30S ribosome[1]. The most frequently encountered resistance mechanism against aminoglycosides among pathogenic bacteria is enzymatic modifications of the -OH or $-NH_2$ groups of these agents. Three modification enzymes such as aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotidyltransferase/adenylyltransferases (ANT/AAD), and aminoglycoside phosphotransferases (APH)[2] have been identified in various bacterial species. However, these enzymes usually have relatively narrow

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substrate specificities, and almost all of them hardly inactivate multiple aminoglycosides at the same time.

Mutations at the A-site of 16S rRNA also provide a type of resistance to aminoglycosides for various bacterial species, but this kind of mutation usually has adverse effects on bacterial growth through reduction of protein synthesis. Thus, mutations at 16S rRNA usually become problematic, especially in slow-growing bacteria such as *Mycobacterium* spp.[3, 4]. The third molecular mechanism for aminoglycoside-resistance is alteration in bacterial membrane including active efflux pump systems such as MexXY-OprM found in *Pseudomonas aeruginosa*[5, 6]. The fourth aminoglycoside-resistance mechanism is the methylation of bacterial 16S rRNA, a main target of aminoglycosides. Indeed, the 16S rRNA protection by methylation gives a very high level of resistance against intrinsic aminoglycosides, but this resistance mechanism has exclusively been found only among aminoglycoside-producing bacteria[7], and no such kind of 16S rRNA protection had been identified in clinically isolated pathogenic bacteria before 2003. Since 2003, seven plasmid-mediated 16S rRNA methylase such as RmtA[8], RmtB[9], RmtC[10], RmtD[11], RmtE[12], RmtF[13], RmtG (JX486113), ArmA[14], and NpmA[15] have identified *Enterobacteriaceae, Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Our study, it was found that various gram-negative bacterial species harboring some of these 16S rRNA methylases have already been proliferating in separate medical facilities in Japan[16, 17].

From 2001 to 2003, six strains including four *Klebsiella pneumoniae*, one *Klebsiella oxytoca*, and one *Escherichia coli* that possessed a very high level of resistance against various amimoglycosides, as well as cefotaxime, were isolated in a Japanese hospital. In the present study, the genetic relations among six bacterial strains and their plasmids were elucidated.

MATERIALS AND METHODS

Bacterial strains

From 2001 to 2003, six strains highly resistant to both amikacin and gentamicin were isolated clinically in a hospital in Japan. These were four *K. pneumoniae* strains MRY05-304, MRY05-305, MRY05-306, and MRY05-307, one K. *oxytoca* MRY05-308, and one *E. coli* MRY05-309 (Table1). Bacterial strains were grown in Luria-Bertani (LB) broth or LB agar plates (Becton Dickinson Diagnostic Systems, Sparks, Md.) supplemented with appropriate antimicrobial agents.

Antimicrobial agents susceptibility test

MIC of each antimicrobial agent was measured by the agar dilution method in accordance with the protocol recommended by the CLSI in document

Table 1. Bacterial strains used in this study

Bacterial species and strain	Characteristics	Source or reference
K. pneumoniae strains		
MRY05-304, MRY05-305, MRY05-306, and MRY05-307[a]	Clinical isolates; strains resistant to amikacin, gentamicin, and cefotaxime isolated in Yamanashi, Japan	16
K. oxytoca MRY05-308[a]	Clinical isolate; a strain resistant to amikacin, gentamicin, and cefotaxime isolated in Yamanashi, Japan	16
E. coli MRY05-309[a]	Clinical isolate; a strain resistant to amikacin, gentamicin, and cefotaxime isolated in Yamanashi, Japan	16
E. coli CSH2	*met*B F^- nalidixic acid^r rifampin^r	T. Sawai, Chiba University
E. coli DH10B	F^- *mcrA* Δ(*mrr*$^-$ *hsd*RMA$^-$ *mcr*BC) ϕ80lacZ ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G	Invitrogen

[a]*K. pneumoniae* MRY05-304, *K. pneumoniae* MRY05-305, *K. pneumoniae* MRY05-306, *K. pneumoniae* MRY05-307, *K. oxytoca* MRY05-308 and *E. coli* MRY05-309 correspond to the strains *K. pneumoniae* 01-140, *K. pneumoniae* 01-142, *K. pneumoniae* 03-252, *K. pneumoniae* 03-518, *K. oxytoca* 01-141, and *E. coli* 01-139, respectively, which have been described in the previous study (16).

M7-A8[18]. Antimicrobial agents were obtained from the following sources: amikacin, Bristol Pharmaceuticals K.K., Tokyo, Japan; arbekacin, kanamycin, and streptomycin, Meiji Seika Kaisha Ltd., Tokyo, Japan; gentamicin and sisomicin, Schering-Plough K.K., Osaka, Japan; isepamicin, Asahi Kasei Corporation, Tokyo, Japan; tobramycin, Shionogi & Co., Ltd., Osaka, Japan; neomycin, Nippon Kayaku Co., Ltd., Tokyo, Japan; cefotaxime, Aventis Pharma, Ltd., Tokyo, Japan; ceftazidime, GlaxoSmithKline K.K., Tokyo, Japan; imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; and ciprofloxacin, Bayer Yakuhin, Ltd., Tokyo Japan.

Detection of genes for 16S rRNA methylsae by multiplex PCR analysis

Primers used for PCR amplification of the 16S rRNA methylase genes were followed by Doi *et al*[19]. The supernatant of centrifuged bacterial suspension after boiling for 10 minutes was used as the DNA template for PCR. Initial denaturation at 94°C for 2 minutes was followed by 30 cycles of amplification with 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1.5 minutes. The final extension step was 72°C for 5 minutes. PCR products were analyzed with a 2% agarose gel electrophoresis followed by ethidium bromide staining.

Detection of genes for β-lactamase by PCR analysis

PCR primers for detection of bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$-type, $bla_{CTX-M-2}$-type, and $bla_{CTX-M-9}$-type genes and amplification conditions were designed in accordance with the instructions of Nagano *et al.*[20]. The DNA template prepared for PCR analysis was also used in the multiplex PCR to detect 16S rRNA methylase genes. PCR products were analyzed with a 2% agarose gel electrophoresis followed by ethidium bromide staining, and all PCR amplicons derived from CTX-M-type β-lactamase genes were subjected to direct sequencing analyses. DNA sequences were determined on both strands using BigDye Terminator Cycle Sequencing Ready Reaction kits and an ABI 3100 DNA sequence analyzer (Applied Biosystems, Foster City, Calif.). The nucleotide sequence was analyzed by the BLAST homology search system [http://blast.ncbi.nlm.nih.gov/Blast.cgi].

Conjugation experiments, preparation of plasmids, and replicon typing by PCR

Conjugal transfer of plasmid from six aminoglycoside-resistant strains to a recipient *E. coli* CSH-2 (Table 1) was performed by broth mating method. Transconjugants were selected on LB agar plates containing arbekacin (25 μg/ml), rifampin (100 μg/ml) and nalidixic acid (50 μg/ml). Plasmid DNA preparation was carried out by two methods using Qiagen plasmid midi kit (Qiagen K.K., Tokyo, Japan) or the method of Kado and Liu[21]. Plasmid replicon typing by PCR was followed by Carattoli *et al.*[22].

Digestion of plasmid DNA with restriction enzymes and Southern hybridization analysis

Transformation of *E. coli* DH10B with the plasmid DNA prepared from each parental strain was performed using standard electroporation techniques. Transformants were selected on LB agar plates containing arbekacin (25 μg/ml). Plasmids were digested with EcoRI (New England Biolabs, Beverly, Mass.), and were electrophoresed through 1.0% agarose gel and transferred to nylon membrane (Bio-Rad Laboratories, Hercules, CA.). Hybridization analysis was performed by the PCR DIG detection system (Roche Diagnostics, Tokyo, Japan) using digoxigenin-labeled *rmtB* probes.

PFGE analysis

Genomic DNA preparations from four *K. pneumoniae* strains MRY05-304, MRY05-305, MRY05-306, and MRY05-307, were digested

with XbaI (New England Biolabs). PFGE was performed with the CHEF-Mapper system (BioRad Laboratories) at a constant voltage of 6 V/cm with 12- to 40-seconds pulses for 24 hour. Banding patterns of four strains were compared and interpreted by the criteria proposed by Tenover *et al*[23]. The similarity of fingerprinting pattern was calculated by the unweight pair group method using arithmetic averages by Bio Image Advances Quantifier Version 4.1.0.0. (R. M. Luton, Inc.

Table 2. Results of antimicrobial susceptibility test

Species and strain	MIC (μ g/ml)												
	KAN	TOB	AMK	ABK	GEN	SISO	ISP	STR	NEO	CAZ	CTX	IPM	CIP
K. pneumoniae MRY05-304	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	256	4	4	>64	≤ 0.5	≤ 0.5
K. pneumoniae MRY05-305	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	256	1	2	>64	≤ 0.5	≤ 0.5
K. pneumoniae MRY05-306	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	512	4	16	>64	≤ 0.5	≤ 0.5
K. pneumoniae MRY05-307	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	256	4	4	>64	≤ 0.5	≤ 0.5
K. oxytoca MRY05-308	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	256	4	4	>64	≤ 0.5	≤ 0.5
E. coli MRY05-309	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	256	4	8	>64	≤ 0.5	≤ 0.5

Abbreviations: KAN, kanamycin; TOB, tobramycin; AMK, amikacin; ABK, arbekacin; GEN, gentamicin; SISO, sisomicin; ISP, isepamicin; STR, streptomycin; NEO, neomycin; CAZ, ceftazidime; CTX, cefotaxime; IPM, imipenem; CIP, ciprofloxacin.

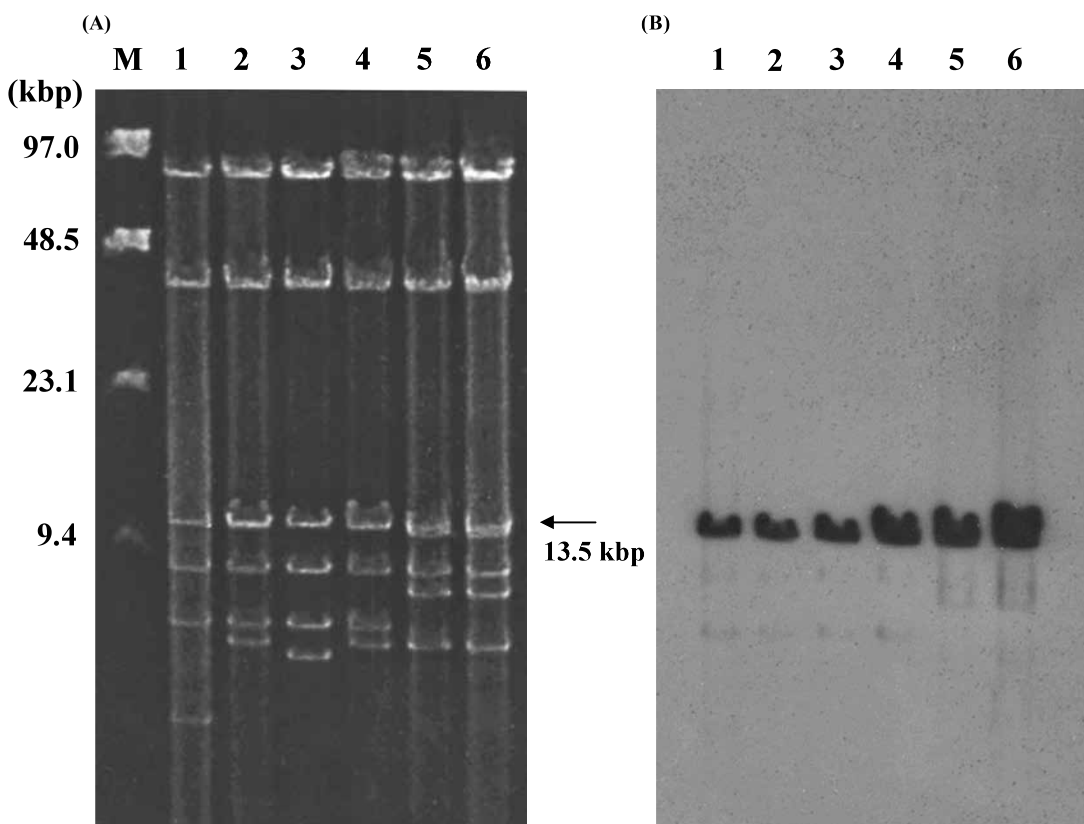

Fig. 1A. EcoRI restriction fragment patterns of *rmtB*-carrying plasmids. M, low range PFG marker (New England Biolabs); lanes 1 to 6, transconjugants of *E. coli* CSH2 that harbor *rmtB* plasmid isolated from *E. coli* MRY05-309, *K. pneumoniae* MRY05-304, *K. oxytoca* MRY05-308, *K. pneumoniae* MRY05-305, *K. pneumoniae* MRY05-306, and *K. pneumoniae* MRY05-307.

Fig. 1B. Southern hybridization by *rmtB* probe. The *rmtB* probe was hybridized to the 13.5-kbp EcoRI restriction fragment (arrow) of Fig 2A.

Jackson, Mich.).

RESULTS

Susceptibilities to antimicrobial agents

The MICs of aminoglycosides for all six strains tested were higher than 1,024 μg/ml, while those of streptomycin and neomycin ranged from 256 to 512 μg/ml and from 1 to 4 μg/ml, respectively. MICs of cefotaxime for all six strains were higher than 64 μg/ml, but those of ceftazidime ranged from 2 to 16 μg/ml. All six strains were susceptible to imipenem (MIC, ≤ 0.5 μg/ml) (Table 2).

Multiplex PCR amplification of 16S rRNA methylase genes

A DNA amplicon approximately 200 bp in size was generally observed among all six strains by the multiplex PCR for identification of 16S rRNA methylase genes, and this finding suggested the presence of *rmtB* among these strains. All *E. coli* DH10B or *E. coli* CSH2 transformants showed the same results by the same multiplex PCR analysis, and this finding suggested that the *rmtB* would be mediated by plasmids.

Typing of β-lactamase gene by sequence analysis

All six parental strains and transformants were positive for $bla_{\text{CTX-M-9}}$-type and bla_{TEM} by PCR. Moreover, sequence analysis of each $bla_{\text{CTX-M-9}}$-type PCR product revealed that these strains harbored $bla_{\text{CTX-M-14}}$ instead of $bla_{\text{CTX-M-9}}$; and $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-9}}$ are indistinguishable by the PCR primers and condition employed in this study.

Conjugation experiments and restriction profiles of plasmids

All six plasmids carrying both *rmtB* and $bla_{\text{CTX-M-14}}$ were successfully transferred to *E. coli* CSH2 from parent strains by conjugation at a frequency of 10^{-6} to 10^{-7} cells per recipient cell. The results of the plasmid replicon typing show all six plasmids are classified as IncA/C. The restriction patterns of plasmids with EcoRI are shown in Fig. 1A. One-to four-band differences were observed among six plasmids, although they were prepared from three different bacterial species including *K. pneumoniae*, *E. coli* and *K. oxytoca*. More precisely, the plasmids prepared from four *K. pneumoniae* strains gave two slightly different restriction patterns, i.e., at most

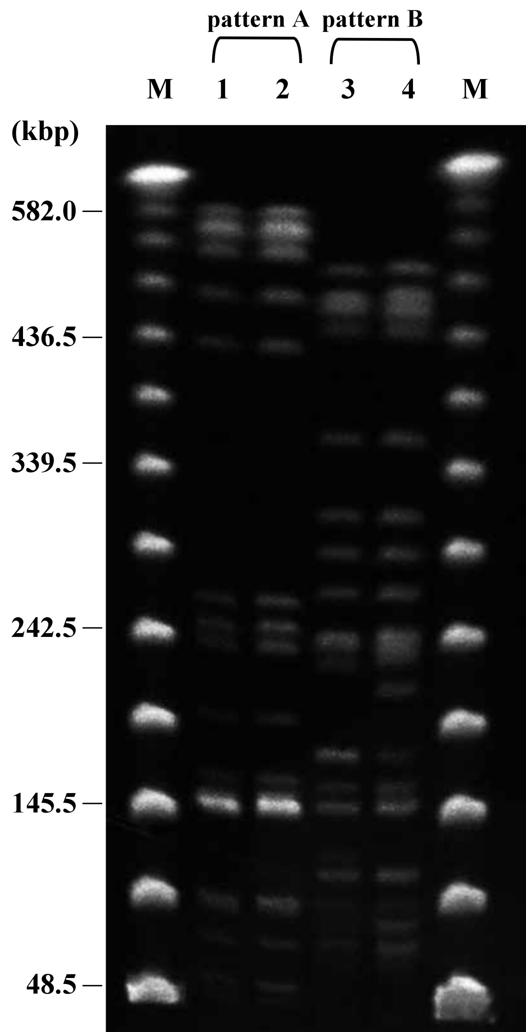

Fig. 2. PFGE fingerprinting patterns of XbaI-digested total DNA preparations from 4 *K. pneumoniae* isolates. M, lambda ladder PFG marker (New England Biolabs) as a molecular size marker. Lanes 1 to 4, *K. pneumoniae* MRY05-304, *K. pneumoniae* MRY05-305, *K. pneumoniae* MRY05-306, and *K. pneumoniae* MRY05-307.

two-band disparities. Although 4 different profiles were observed in the restriction patterns among the six plasmids, the *rmtB* probes hybridized to the same 13.5-kbp EcoRI restriction fragment in each plasmid by Southern hybridization analysis (Fig. 1B).

RFLP of total DNA preparations from four K. pneumoniae strains

By PFGE analysis, the fingerprinting profiles of 4 *K. pneumoniae* strains were classified into two patterns, A and B (Fig. 2). The similarity between the two patterns, A and B, was calculated to be 60%. *K. pneumoniae* MRY05-304 and MRY05-305 demonstrated pattern A, and strains MRY05-306 and MRY05-307 showed pattern B. The banding patterns of strains MRY05-304 and MRY05-305 showed two-band difference. On the other hand, those of strains MRY05-306 and MRY05-307 demonstrated a three-band difference by PFGE analysis, and the former strain was isolated six months earlier than the latter strain in the same hospital.

DISCUSSION

In the present study, a probable patient to patient nosocomial transmission of RmtB and CTX-M-14 co-producing strains and horizontal transmission of *rmtB* and $bla_{\text{CTX-M-14}}$ harboring IncA/C type plasmids in Japanese hospital during 2001 and 2003 was recognized by PFGE typing and plasmid analysis. Although three different bacterial species were found to harbor the *rmtB*, restriction patterns of six plasmids prepared from six strains were considerably similar, and *rmtB* probes hybridized to the same 13.5-kbp EcoRI restriction fragments. These observations imply that the *rmtB*-carrying plasmids originated from a progenitor plasmid which also encoded $bla_{\text{CTX-M-14}}$. *rmtB* is also associated with other resistance genes such as plasmid-mediated fluoroquinolone-resistant gene, *qepA*[24], but we could not find *qepA* by PCR[25] (data not shown). Given the fact that the strains isolated in Taiwan[26, 27] and Korea[28] also harbor both *rmtB* and $bla_{\text{CTX-M-14}}$, the plasmids carrying both *rmtB* and $bla_{\text{CTX-M-14}}$ would somewhere have emerged before 2001 and then been disseminated to different geographical areas, very possibly Asian countries. The plasmids would then have further been transferred horizontally among various gram-negative bacilli, causing nosocomial infections in separate medical facilities in Asian countries. These findings may predict further global transmission of RmtB-producing gram-negative nosocomial bacilli which also produce CTX-M-type enzymes.

The size of plasmids carrying *rmtB* and $bla_{\text{CTX-M-14}}$ was estimated to be more than 100 kbp. These plasmids were classified as IncA/C by PCR typing. IncA/C palsmids identified in various *Enterobacteriaceae*[28, 29] and other gram-negative bacteria[30, 31] were related to multidrug resistance. In Korea, *K. pneumonia*- and *E. coli*- harboring IncA/C plasmid carrying *rmtB* and $bla_{\text{CTX-M-14}}$ were isolated between 1995 and 2005[28]. In our study, three different bacterial species harboring *rmtB* and $bla_{\text{CTX-M-14}}$ were found in the same hospital in Japan: four *K. pneumonia*, one *K. oxytoca*, and one *E. coli.*

Further, plasmid-mediated dissemination of *rmtB* accompanied by widely distributing $bla_{\text{CTX-M-14}}$ among pathogenic gram-negative bacilli may indeed become a grave clinical problem in the near future, because aminoglycosides tend to be prescribed together with other classes of antimicrobial agents including broad-spectrum β-lactams.

PFGE analysis of four *K. pneumoniae* strains suggested that these four strains independently isolated from four patients were separated into two different clusters (Fig. 2). The similarity of the two clusters was calculated to be about 60%. This suggested that two different genetic lineages of *K. pneumoniae* strains with a very similar IncA/C plasmid had been spread nosocomially in a hospital,

and then separately transmitted from patient to patient. As shown in Figure 2, pattern A and pattern B had two and three difference of banding pattern which demonstrated these two groups were clonal strains, respectively[23]. Pattern B demonstrated a three-band difference and the former strain was isolated six months earlier than the latter one. This finding implied that some rearrangements may well have occurred in the plasmid or chromosome of these two strains over a period of six months.

In conclusion, we found a probable nosocomial transmission of *rmtB*-harboring gram-negative bacterial species belonging to the family *Enterobacteriaceae*. Indeed, isolation of 16S rRNA methylase-producing gram-negative bacteria among clinical strains is still very rare at present[17], but the possible risk of further plasmid-mediated horizontal transfer of 16S rRNA methylase genes into strains possessing resistance to both fluoroquinolones and broad-spectrum β-lactams, including oxyimino-cephalosporins, cephamycins and carbapenems, should be carefully monitored to prevent future global proliferation of gram-negative pathogenic bacteria that have acquired a very wide range of antimicrobial resistance.

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CONFLICT OF INTEREST

None to declare.

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