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Table 1. Characteristics of the five MRSA strains[a] isolated from food samples in this study

Origin	MLST/*spa*	SCC*mec*	*agr*	MIC (mg/L)									Amino acid change		Resistance genes detected
				OXA	FOX	TET	TOB	KAN	STR	ERY	CLI	CIP	LEV	GrlA	GyrA	
Pork	ST398/t011	V	I	8	16	128	0.25	2	256	>256	>128	0.125	0.125	NP	NP	*mecA*, *tet*(K), *tet*(M), *erm*(A), *erm*(C)
Veal	ST398/t1197	V	I	64	32	128	32	32	8	>256	>128	2	0.5	S80F	S84L	*mecA*, *tet*(K), *tet*(L), *tet*(M), *ant(4′)-Ia*, *aph(3′)*, *erm*(C), *msr*(A)
Chicken	ST125/t067	IVa	II	128	128	0.25	>256	128	8	0.25	0.06	64	4	S80F	S84L	*mecA*, *ant(4′)-Ia*, *aph(3′)*
Rabbit	ST125/t067	IVa	II	128	128	0.25	>256	128	8	>256	>128	64	4	S80F	S84L	*mecA*, *ant(4′)-Ia*, *aph(3′)*, *erm*(A), *erm*(C), *nrs*(A)
Wild boar	ST217/t032	IVa	I	32	32	0.125	0.25	2	8	0.25	0.06	16	4	S80F	S84L	*mecA*

OXA, oxacillin; FOX, cefoxitin; TET, tetracycline; TOB, tobramycin; KAN, kanamycin; STR, streptomycin; ERY, erythromycin; CLI, clindamycin; CIP, ciprofloxacin; LEV, levofloxacin; NP, not performed.

[a]The five strains were susceptible to the following seven antibiotics by the disc diffusion method: gentamicin; chloramphenicol; trimethoprim/sulfamethoxazole; vancomycin; teicoplanin; mupirocin; and fusidic acid.

Unveiling the role of MRSA ST398 as a zoonotic foodborne pathogen requires more research.

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Transparency declarations

None to declare.

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Molecular characterization of group B streptococci with reduced penicillin susceptibility recurrently isolated from a sacral decubitus ulcer

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Keywords: *Streptococcus agalactiae*, β-lactams, infection, PBP 2X alterations, MLST

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Sir,

Penicillin is the first-line antibiotic for group B streptococci (GBS) in disease therapy as well as in intrapartum chemoprophylaxis because resistance to this agent has so far not been reported among GBS clinical isolates. However, we have recently reported GBS isolates with reduced penicillin susceptibility (PRGBS), where altered penicillin-binding protein (PBP) 2X demonstrates a major contribution to the reduction of β-lactam susceptibility.[1] We have also shown a diversity of mutations in PBP genes among PRGBS, while those genes of the penicillin-susceptible strains were highly conserved, irrespective of their isolation dates.[2] Those PRGBS isolates have been mostly recovered from respiratory specimens. Here, we report a case of a lasting sacral decubitus ulcer from which PRGBS were isolated repeatedly, together with the molecular characteristics of the isolates.

A 58-year-old man underwent descending graft replacement of a Stanford type B dissecting aortic aneurysm at our medical centre. After the operation, the patient developed paraplegia, which was not improved after axillofemoral bypass surgery. He was then transferred to a special physical rehabilitation centre. During his prolonged rehabilitation stay, a severe decubitus ulcer developed in the sacral area, wound bleeding was seen and he was referred to our centre. He had not received antimicrobial treatment for more than 3 months except for a 1 day intravenous injection of piperacillin (2000 mg/day) due to the occurrence of a fever just 3 days before hospital admission. Bacterial culture of the specimen taken at the surface of the decubitus ulcer at his initial visit yielded heavy growth of GBS as well as small numbers of group G streptococci and *Proteus mirabilis*. GBS was still predominant together with group G streptococci, and a small number of *Staphylococcus aureus*, in a similar sample taken on the day of admission, which corresponded to 22 days after his initial visit. On day 6, operative treatment of the decubitus ulcer by debridement followed by a rotation flap was performed. After 4 day intravenous administration of cefazolin (2000 mg/day) starting on day 6, an agent used for conventional post-operative treatment in our medical centre, only *S. aureus* was recovered from post-operative fistula exudates.

The GBS serotype Ia isolates, which were detected at intervals, were PRGBS with a penicillin MIC of 0.25 mg/L determined by a broth microdilution method with a MicroScan MICroFAST panel type 3J system (Siemens Healthcare Diagnostics Inc., Tokyo, Japan) as described previously.[2] MICs of ampicillin, cefazolin and cefotaxime were 0.25, 2 and 1 mg/L, respectively (Table 1). PFGE patterns of ApaI-digested DNA[1] of these two isolates were identical. Multilocus sequence typing (MLST) was performed using specific primers as described previously.[3] Amplification of seven housekeeping genes, *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt*, by PCR was carried out using PrimeSTAR HS DNA polymerase (Takara Shuzo Co., Kyoto, Japan) with reaction conditions of one cycle of 98°C for 1 min, followed by 30 cycles of 98°C for 10 s, 55°C for 15 s and 72°C for 1 min, and finally one cycle of 72°C for 7 min. PCR products were purified and sequenced.[2] Allelic profile assignment and sequence type (ST) determinations were made using the GBS MLST databases (http://pubmlst.org/sagalactiae). Both PRGBS were found to have ST-1 with allelic profile 1121122, which has been a heterogeneous lineage comprising mainly serotype V and various other serotypes, and has been identified among carriage and invasive isolates.[4] Thus, those PRGBS were regarded as genetically identical by PFGE and MLST.

Table 1. MICs of antimicrobials for PRGBS isolates

Antimicrobial agent	MIC (mg/L)
Penicillin	0.25
Ampicillin	0.25
Cefazolin	2
Cefotaxime	1
Cefotiam	>4
Cefixime	>1
Cefepime	2
Cefozopran	1
Cefditoren	0.25
Meropenem	0.25
Clarithromycin	≤0.12
Erythromycin	≤0.12
Clindamycin	≤0.12
Levofloxacin	>8

The nucleotide and deduced amino acid sequences of PBP 2X from the two PRGBS isolates (GenBank accession number AB512415) were compared with those from the 2603V/R reference strain (ATCC BAA-611; GenBank accession number NC_004116). The PRGBS isolates shared amino acid substitution V405A, which has been found to be a key substitution, together with Q557E, responsible for penicillin non-susceptibility in PBP 2X.[1,2] In addition, the isolates shared three substitutions, G398A,[1] G329V and G429D, where the latter two were new substitutions for PRGBS. The G429D substitution, which corresponds to the G422D substitution in *Streptococcus pneumoniae*, may possibly be a compensatory mutation, as has been suggested for *S. pneumoniae*.[5]

In this case, PRGBS was consecutively and predominantly isolated from a sacral decubitus ulcer as a cause of mixed infection, and thus was found to be capable of surviving persistently at the site of infection, for >3 weeks. It remains unclear whether or not the results of *in vitro* penicillin non-susceptibility of PRGBS would predict the clinical significance of this kind of microbe in antimicrobial chemotherapy. Despite the relatively high MIC of cefazolin, 2 mg/L for this PRGBS, 4 day intravenous administration of cefazolin (2000 mg/day) seemed effective in this case, but accumulation of clinical data on an appropriate antimicrobial therapeutic strategy would be essential, especially in the cases of sepsis or meningitis in both neonates and elderly individuals.

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Transparency declarations

None to declare.

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Plasmid-mediated ArmA and RmtB 16S rRNA methylases in *Escherichia coli* isolated from chickens

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Keywords: aminoglycosides, amikacin, Southern hybridization, PFGE, genetic environment

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Sir,

High-level aminoglycoside resistance mediated by the production of 16S rRNA methylase has been increasingly reported among various Gram-negative pathogens. Six 16S rRNA methylase genes have been previously identified: *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA*.[1] However, in food animals, only two studies have described the presence of the *armA* and *rmtB* genes in pigs, respectively.[2,3] In China, aminoglycoside antibiotics are widely used for the prevention and control of *Escherichia coli* infections of chickens. However, it remained unknown if 16S rRNA methylase genes were present in *E. coli* isolated from chickens.

Between March and May 2008, a total of 120 individual *E. coli* strains were isolated from the livers ($n=154$) of diseased and dead chickens in four farms (Farms A–D) in Henan Province, China. Among them, 12 isolates exhibited high-level resistance to the aminoglycoside antibiotic amikacin (MICs >512 mg/L). These isolates were screened by PCR for the six known types of 16S rRNA methylase genes. The genes that produced positive results were further confirmed by DNA sequencing of the amplicons. In addition, phenotypic and genotypic tests for extended-spectrum β-lactamases in 16S rRNA methylase-positive isolates were performed according to CLSI recommendations and using PCR assays as previously described.[4,5] Overall, the *armA* and *rmtB* genes were detected in 3 and 9, respectively, of the 12 isolates that had high-level amikacin resistance. No positive amplicons were found to be present for the other four 16S rRNA methylase genes (Table 1). Multiplex PCR was performed to determine whether the 12 isolates were commensals (A and B1) or were associated with phylogroups exhibiting extraintestinal virulence (B2 and D).[6] Five of them were found to be associated with extraintestinal virulence (Table 1).

Genetic relationships of the *E. coli* isolates that produced 16S rRNA methylase were assessed by PFGE after digestion with XbaI. The PFGE patterns differentiated these samples ($n=12$) into seven major pulsotypes, indicating that both horizontal and vertical transfer could have played an important role in the dissemination of the 16S rRNA methylase genes (Table 1).

The 16S rRNA methylase resistance determinants from *armA*- or *rmtB*-positive isolates were transferred to *E. coli* J53Az^R and DH10B by conjugation and electroporation, respectively, to investigate whether the two determinants were localized on plasmids and whether transfer of these genes increased the resistance of the recipient *E. coli* to antimicrobials. Southern hybridization of digested plasmid DNA from the isolates and their transformants/transconjugants was performed with digoxigenin-labelled probes specific for *armA* and *rmtB*, respectively. As shown in Table 1, *armA* and *rmtB* genes could transfer among *armA*- and *rmtB*-positive isolates by conjugation and electroporation. Also, increases in MICs of multiple classes of antimicrobials were found for both the transconjugants and transformants, suggesting that co-transferred resistance to other antimicrobial agents could occur on these plasmids. Hybridization with the *rmtB*-specific probe indicated that *rmtB* genes from three isolates (isolates 63, 3 and 73) were localized on two differently sized digested fragments. Interestingly, hybridization with the *armA*-specific probe revealed that one isolate (isolate 14) contained two copies of the

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Accumulation of Plasmid-Mediated Fluoroquinolone Resistance Genes, *qepA* and *qnrS1*, in *Enterobacter aerogenes* Co-producing RmtB and Class A β-lactamase LAP-1

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Abstract. A new plasmid-mediated fluoroquinolone efflux pump gene, *qepA*, is known to be associated with the *rmtB* gene, which confers high-level resistance to aminoglycosides. We investigated the *qepA* gene in 573 AmpC-producing *Enterobacteriaceae* including one *Citrobacter freundii* known to harbor *rmtB*. Of them, two clonally unrelated *E. aerogenes* harbored *qepA*. Both isolates co-harbored *rmtB, qnrS1, qepA*, and bla_{LAP-1} on an IncFI type plasmid. The *qepA* was flanked by two copies of IS*26* containing IS*CR3C*, *tnpA, tnpR*, bla_{TEM}, and *rmtB*. The *qnrS1* and bla_{LAP-1} were located upstream of *qepA*. All the resistance determinants (*qepA, qnrS1, rmtB*, and bla_{LAP-1}) were co-transferred to *E. coli* J53 by filter mating from both isolates. Although the prevalence of *qepA* is currently low, considering the presence of IS*CR3C* and the possibility of co-selection and co-transferability of plasmids, more active surveillance for these multi-drug resistant bacteria and prudent use of antimicrobials are needed.

Keywords: *qepA, qnrS1, rmtB,* bla_{LAP-1}, IS*CR3C*, gene co-transferance

Introduction

Resistance to quinolones in bacteria belonging to the family *Enterobacteriaceae* most commonly arises stepwise as a result of chromosomal mutations in *gyrA* and/or *parC* genes encoding DNA gyrase and topoisomerase IV, respectively. Recently, plasmid-encoded quinolone resistance determinants such as *qnr* and *aac(6')-Ib-cr* have been shown to be distributed worldwide [1,2]. A new plasmid-mediated fluoroquinolone efflux pump, QepA, was identified in three *Escherichia coli* isolates from Japan [2,3] and Belgium [1]. In addition, the *qepA* gene was associated with the *rmtB* gene, which confers high-level resistance to aminoglycosides.

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We earlier detected *rmtB* in only one clinical isolate of *Citrobacter freundii* of the 413 AmpC-producing *Enterobacteriaceae* screened [4]. Therefore, we investigated the incidence of *qepA* among AmpC-producing *Enterobacteriaceae* including the strain previously reported to harbor *rmtB*.

Materials and Methods

A total of 572 consecutive, non-duplicate isolates, including *Enterobacter cloacae* (168), *Enterobacter aerogenes* (146), *C. freundii* (109), and *Serratia marcescens* (149), were collected from clinical specimens at 12 clinical laboratories in Korea between March and July 2006. One *C. freundii* isolated in 2003, which harbored *rmtB*, was included.

The MICs of ciprofloxacin and nalidixic acid were determined by the agar dilution method according to the CLSI (formerly NCCLS) guidelines [5]. For 223 isolates (69 *E. cloacae*, 28 *E. aerogenes*, 43 *C. freundii*, and 83 *S. marcescens*) showing ciprofloxacin MICs ≥0.25 μg/ml and/or nalidixic acid MICs ≥16 μg/ml, and the *C. freundii* isolate harboring *rmtB*, the presence of *qepA* was screened by PCR as described

previously [2]. A *qepA*-positive plasmid (pSTVqepA) [2] was used as a positive control.

Conjugation experiments were performed with the *qepA*-positive isolates as donors and azide-resistant *E. coli* J53 as a recipient strain by filter mating. Transconjugants were selected on LB agar plates supplemented with sodium azide (150 μg/ml) and ciprofloxacin (0.25 μg/ml). From the transconjugants, plasmid DNAs were extracted, and PCRs for *qepA, rmtB, qnrS1,* and $bla_{\text{LAP-1}}$ were performed using primers reported previously (Table 1). MICs for donors, transconjugants, and recipient were measured by agar dilution method in accordance with the CLSI guidelines [5]. The antimicrobials tested were ciprofloxacin, nalidixic acid, piperacillin, cefazolin, cefuroxime, ceftazidime, cefepime, and amikacin.

For the isolates harboring *qepA*, the known associated genes (IS*26*, $bla_{\text{TEM-1}}$, *rmtB*) [2], and other plasmid-mediated quinolone resistance genes (*qnrA, qnrB, qnrS1,* and *aac(6')-Ib-cr*) were also investigated with PCR as described previously [6,7]. From the plasmids recovered from the transconjugants, the sequences of DNAs adjacent to *qepA* and *qnrS1* were determined with a series of PCR primers (listed in Table 1) on a 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analyses and comparison with known sequences were performed with the BLAST program at the National Center for Biotechnology Information.

From the plasmid DNAs obtained from transconjugants, the sequences of DNA adjacent to *qepA* and *qnrS1* were determined with a series of outward-facing primers starting from both sides of the *qep* gene on plasmid DNA purified from transconjugants. The incompatibility group was determined by PCR-based replicon typing [8]. For determination of plasmid size, the plasmid DNAs from the transconjugants and the reference strain (*E. coli* NCTC50192) were subjected to electrophoresis on 0.7% agarose gel.

Results and Discussion

The *qepA* gene was detected in two *E. aerogenes* isolates (YS10 and YS11) from two separate patients in one hospital. The two isolates had been isolated from bile and sputum, respectively. To investigate the clonality of the isolates, PFGE analysis was carried out according to the manufacturer's protocol (Bio-Rad, Inc., Hercules, CA, USA), and the two isolates were found not to be related according to the criteria of Tenover et al [9] (data not shown). Both isolates harbored *qepA, qnrS1, rmtB,* $bla_{\text{TEM-1}}$, $bla_{\text{LAP-1}}$, and *aac(6')-Ib* [2]. Sequence analysis revealed that *qepA* was flanked by two copies of IS*26* containing IS*CR3C* recently described by Cattoir et al [10], *tnpA, tnpR,* bla_{TEM}, and *rmtB*, as was observed in *E. coli* C316 (AB103506) [2].

The *qnrS1* and $bla_{\text{LAP-1}}$ were located upstream of *qepA*, separated by 12,806-bp and 15,060-bp, respectively, that included truncated *Tn2*, IS*26*, and CS12 fimbrial gene cluster of *E. coli,* which was also identified at downstream of *qnrS1* in *K. pneumoniae* NK745 [11] and *Salmonella enterica* serovar Infantis [12] (Fig. 1). By filter mating, all the above resistance determinants except *aac(6')-Ib* were transferred to *E. coli* J53 and were carried on a IncFI type plasmid of about 120-kb. The MICs of ciprofloxacin, nalidixic acid, amikacin, and β-lactams for donors, transconjugants, and *E. coli* J53 are summarized in Table 2.

QepA, the most recently identified plasmid-mediated fluoroquinolone efflux pump, showed considerable similarity in amino acid sequence to the MFS type efflux pumps of *Actinomycetales (Nocardia farcinica, Streptomyces globisporus,* and *Streptomyces clavuligerus*) [2] and soil bacteria, and this might be related to the agricultural use of fluoroquinolones, as well as their use in humans. The finding that *qepA* is usually linked with *rmtB*, which confers multiple resistance to aminoglycosides, reminds us that aminoglycosides are derived from *Actinomycetales* (*Streptomyces* spp and *Micromonospora* spp) and that there is a potential for co-selection of the QepA determinant by the use of aminoglycosides. In addition, the fact that *rmtB*-harboring *C. freundii* did not contain *qepA* indicates that *rmtB* is not necessarily associated with *qepA*. This finding supports the hypothesis that the uptake of *qepA* from soil bacteria by *rmtB*-producing *E. coli,* which is frequently isolated from pigs exposed to aminoglycosides, with further dissemination via food, may be the way in which this new resistance trait evolved [13].

Both of the *E. aerogenes* isolates characterized in this study harbored *aac(6')-Ib* in addition to *qnrS1*. Although the gene was not mutated into *aac(6')-Ib-cr* in these isolates, this could happen during persistent exposure to fluoroquinolones and reminds us of a recent Korean report of a *K. pneumoniae* isolate harboring both *qnrS1* and *aac(6')-Ib-cr* [14]. Accumulation of *qnr, aac(6')-Ib-cr,* and *qepA* in bacterial cells is more likely a result of plasmid conjugation or natural transformation.

In this study, the presence of *qepA* was first identified in *E. aerogenes* isolates and was linked with the high-level aminoglycoside resistance gene, *rmtB*, as reported previously in *E. coli* [1]. It is also

Table 1. Primers used for PCR and sequencing.

Target and Primer	Sequence (5'-3')	Reference
Genes surrounding *qepA*		
IS26-1	5' - TTA CAT TTC AAA AAC TCT GC - 3'	[17]
IS26-2	5' - ATG AAC CCA TTC AAA GGC CGG - 3'	
tnpR-F	5' - CGA CAC TGC CGA TAT GAT CC - 3'	This study
tnpR-R	5' - CGG GCA ATA CTG AGC TGA TG - 3'	
TEM-F	5'- ATA AAA TTC TTG AAG ACG AAA-3'	[18]
TEM-R	5'- GAC AGT TAC CAA TGC TTA ATC -3'	
rmtB-F	5'- CCC AAA CAG ACC GTA GAG GC -3'	[19]
rmtB-R	5'- CTC AAA CTC GGC GGG CAA GC -3'	
SP-1	5'- ATG CTG GCG ACG ATC CGC AC -3'	This study
SP-2	5'- CGG CGA AGG CAG CTA CGG CT -3'	
qepA - F	5' - AGC AGC GCG CTG AAT CCA - 3'	[1]
qepA - R	5' - CGA ACC CAG TGG ACA TAA - 3'	
new qepA - F	5' - GCA GGT CCA GCA GCG GGT AG - 3'	[2]
new qepA - R	5' - CTT CCT GCC CGA GTA TCG TG - 3'	
SP-3	5'- CAC ATC AGG GGG CAC GGT CG -3'	This study
Genes surrounding *qnrS1*		
LAP-F	5' - CAA TAC AAA GCA CAG AAG ACC - 3'	[10]
LAP-R	5' - CCG ATC CCT GCA ATA TGC TC - 3'	
LS-1	5' - TGC TCT CTT ACG GTT GCG AT - 3'	This study
LS-2	5' - GTC GTA CAC CGC AGG AAA AA - 3'	
qnrS-F	5'- ACG ACA TTC GTC AAC TGC AA -3'	[7]
qnrS-R	5'- TAA ATT GGC ACC CTG TAG GC -3'	
qS14-1	5' - ATA GGG GTA CTA CGC CGG AA - 3'	This study
qS14-2	5' - AGG CTC ATG ACC CAC CTT TC - 3'	
seal4	5'- GGT CTC CCC TGA TTT CTG GA - 3'	This study
YdaA	5'- AGC CAG TTT GAA CGA GCC TT - 3'	This study

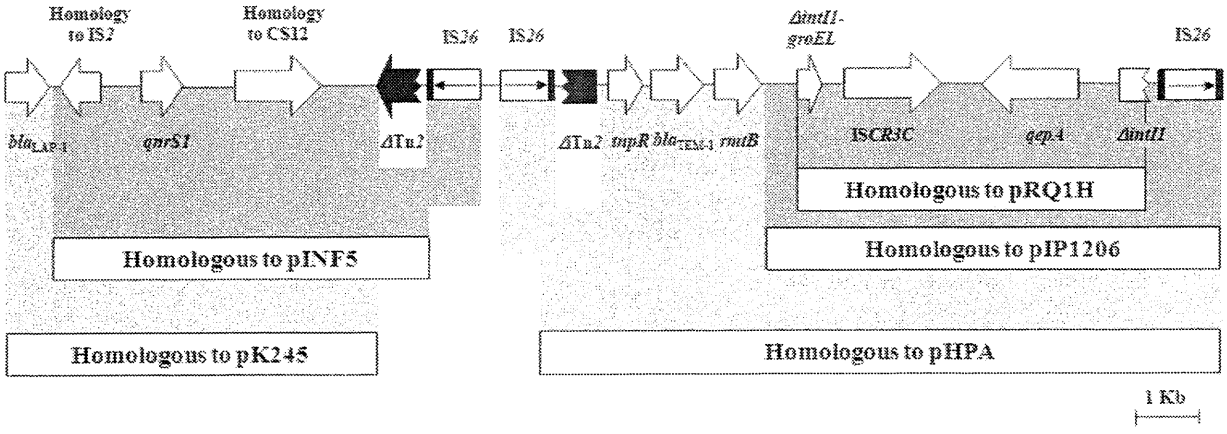

Fig. 1. Genetic environments of the *qepA*, *qnrS1*, and bla_{LAP-1} genes and comparison with other plasmids harbouring *qepA* or *qnrS1*. A distance scale is presented below the map. The reading frames are shown as arrows with the arrowhead indicating the direction of transcription. Plasmid pK245 is from *K. pneumoniae* [11], plasmid pINF5 is from *S. enterica* serotype Infantis [12], and plasmids pRQ1H, pIP1206 and pHPA are from *E. coli* [1,2,20].

Table 2. MICs of antimicrobial agents for donors, transconjugants, and the recipient.

Antimicrobials (breakpoints for susceptibility/resistance)	YS10	Transconjugate from YS10	YS11	Transconjugate from YS11	*E. coli* J53
Ciprofloxacin (1/4)	4	2	16	1	0.015
Nalidixic acid (16/32)	16	8	64	16	4
Piperacillin (16/128)	256	32	256	32	1
Cefazolin (8/32)	≥512	2	≥512	2	1
Cefuroxime (8/32)	128	8	64	8	4
Ceftazidime (8/32)	4	0.25	2	0.25	0.25
Cefepime (8/32)	0.125	0.06	0.06	0.06	0.03
Amikacin (16/64)	≥1,024	≥1,024	≥1,024	≥1,024	0.5

noteworthy that another quinolone resistance determinant, *qnrS1*, was again linked with a class A β-lactamase, bla_{LAP-1} in both strains. Moreover, all of them were co-transferred by conjugation. Liu et al [15] recently reported that 58.3% (28/48) of the *rmtB*-positive *E. coli* isolates harbored *qepA* gene, indicating a strong linkage between *qepA* and *rmtB*. In addition, IncF type plasmid is the second most frequent type of plasmid in *rmtB*-producing *Enterobacteriaceae* in Korea [16].

To our knowledge, this is the first report of *qepA* in *Enterobacteriaceae* other than *E. coli*, suggesting a probable intergeneric gene transfer. In a sampling from a wide geographic area in Korea (12 hospitals and 572 bacterial isolates), we found that there is currently a low prevalence (0.3%) of the plasmid-mediated fluoroquinolone efflux gene, *qepA* in *E. aerogenes*. While this is a fortunate circumstance for the moment, considering the effect of IS*CR3C*, strong linkage between *qepA* and *rmtB*, and the frequency of IncF type plasmid in *rmtB* producers, these organisms could become a greater nosocomial infection problem with appropriate foci of selective pressure. Continued build up of resistance in clinically relevant Gram-negative bacilli would pose serious challenges in our increasingly complex hospital system. It is important to continue to promote prudent and limited use of antibiotics in all economic areas, including the health care system and the agricultural industry. Furthermore, it is important to have public health support to continue active surveillance of clinical *Enterobacteriaceae* isolates for plasmid-mediated fluoroquinolone resistance genes and the various other resistance determinants.

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RESEARCH LETTER

A novel insertion sequence, IS*1642*, of *Mycobacterium avium*, which forms long direct repeats of variable length

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Abstract

A new insertion sequence (IS), IS*1642*, was identified in a *Mycobacterium avium* strain isolated from a human patient. IS*1642* had a size of 1642 bp and contained a single ORF encoding a probable transposase of 503 amino acid residues homologous (79% identity) to that of IS*1549* found in *Mycobacterium smegmatis*. The IS*1642* included imperfect inverted repeats (5′-cctgacttttatca-3′, 5′-tgataaaagtcggg-3′) on its ends, and was flanked by direct repeats of variable length ranging from 5 to 161 bp. It was suggested that the IS*1642* was widely distributed in many *M. avium* strains of human patients, and the Southern blot profile of IS*1642* was very diverse among the strains examined. The transposition event of IS*1642* was observed by *in vitro* repeated passages, showing that the IS*1642* is actually a transposable element. In light of these characteristics, IS*1642* could be a new useful marker when genotyping with high discrimination is required.

Introduction

The insertion sequence (IS) on a bacterial genome facilitates gene rearrangements, which could contribute to the evolution of the organism (Mahillon & Chandler, 1998). ISs are widely distributed in most microorganisms, including the *Mycobacterium* species. For example, genome sequence analysis revealed that the *Mycobacterium avium* ssp. *paratuberculosis* strain K-10 contained 19 kinds of ISs, with 58 total copies in the genome (Li *et al.*, 2005). The genome of *Mycobacterium tuberculosis* H37Rv contained 56 loci with homology to ISs (Gordon *et al.*, 1999).

Bacterial ISs usually contain one or several ORFs, which encode enzymes such as transposases that catalyze the movement within the genome. ISs typically contain short terminal inverted repeat sequences (IRs), ranging from 10 to 40 bp. Upon insertion into the host genome, ISs are flanked on either side by short directly repeated sequences (DRs). The length of a direct repeat, which is usually a fixed characteristic of each IS, generally ranges between 2 and 14 bp (Mahillon & Chandler, 1998).

ISs are used as markers in restriction fragment length polymorphism studies for species typing and for molecular epidemiological purposes [e.g. IS*6110* in *M. tuberculosis* (Otal *et al.*, 1991; Small & van Embden, 1994) and IS*1245* in *M. avium* (Guerrero *et al.*, 1995; Pestel-caron & Arbeit, 1998; Ritacco *et al.*, 1998; van Soolingen *et al.*, 1998; Motiwala *et al.*, 2006)]. When isolates from different sources have few distinguishing phenotypic characteristics, the use of ISs as probes could enable powerful discrimination. On the other hand, because of their mobility, IS could be potential useful markers for identifying substrains or tracking the genetic drift (Hernandez Perez *et al.*, 1994; Laurent *et al.*, 2002).

In this study, we identified a novel IS, designated IS*1642*, which was flanked by unusually long, variable-length direct repeats, in *M. avium* clinical isolates. Here, we present molecular and genetic characterizations of this IS.

Materials and methods

Bacterial strains and growth conditions

Mycobacterium avium clinical strains isolated from human patients were kindly provided by Dr K. Ogawa of NHO Higashi Nagoya National Hospital and Dr Matsumoto of the

Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Japan. These strains were identified as the *Mycobacterium avium/intracellulare* complex by the *Mycobacterium* identification kit (Kyokuto Pharmaceutical Industrial Co. Ltd), and further identified *as M. avium* by PCR (Nishimori *et al.*, 1995). *Mycobacterium avium* ssp. *paratuberculosis* K-10 (BAA968), two *M. avium* strains (25291 and 15769), and two *M. intracellulare* strains (13950 and 25225) were obtained from the American Type Culture Collection (ATCC). Five *M. intracellulare* strains, *Mycobacterium marinum*, *Mycobacterium szulgai*, *Mycobacterium simiae*, *Mycobacterium fortuitum*, and *Mycobacterium abscessus*, and *Mycobacterium bovis* BCG Japanese strain were from our laboratory. The laboratory strain *M. tuberculosis* H37Rv was also included for analysis. These strains were cultured on Middlebrook 7H10 supplemented with 10% (v/v) OADC enrichment (BD) at 37 °C.

DNA manipulations

Mycobacterial genomic DNA was isolated as described (Pelicic *et al.*, 1997), with a minor modification. Bacterial cells were harvested and suspended in 1 mL of acetone. The cells were pelleted by centrifugation (10 min at 5000 *g*). The pellet was resuspended in 500 μL solution I (25% sucrose/50 mM Tris · HCl, pH 8.0/50 mM EDTA/500 mg mL^{-1} lysozyme) and incubated overnight at 37 °C. Then 500 μL of solution II (100 mM, Tris · HCl, pH 8.0/1% SDS/400 μg mL^{-1} proteinase K) was added, and the samples were incubated for 5 h at 55 °C. Genomic DNA was extracted from the lysate using the bacteria genomicPrep Kit (GE Healthcare).

PCR and nucleotide sequencing

The primers used in this study are listed in Table 1. PCRs were performed with Phusion high-fidelity DNA polymerase (New England Biolabs). Thermal cycling conditions comprised preincubation at 98 °C for 30 s, followed by 30 cycles at 98 °C for 10 s, at 68 or 55 °C for 30 s, and at 72 °C for 2 min, and a final extension at 72 °C for 10 min. For examination of direct repeats, genome DNA was digested by PvuII or NotI, and self-ligated. The direct repeat region was amplified by PCR with the primer set IS1642-DR1 and IS1642-DR2 (Table 1), which match the ends of IS*1642* and face the outwards. Two amplified products were obtained, and the nucleotide sequences were determined by sequencing. For sequencing, PCR products were purified using the GFX PCR purification kit (GE Healthcare). Sequencing was carried out using the Big Dye DNA sequencing kit (Applied Biosystems) and the ABI Prism 3130XL Genetic Analyzer. Nucleotide sequences and deduced amino acid sequences were analyzed by GENETYX-MAC software, version 14.0.1 (Genetyx Co., Tokyo, Japan).

Table 1. List of primers used in this study

Primer	Sequence (5′–3′)
IS1642-1F	TTGTGTAGGGCTGTGACCTG
IS1642-1R	ACGTAGGCTGTGGATGTTTG
IS1642-2F	TGACCTGTGTCTTCGGTTG
IS1642-2R	TGTGCTTGCCGGCTTGGATAG
IS1642-3F	TCATCGAACCGACCAGCAAG
IS1642-3R	CACCACATCAGGTAAAAACG
IS1642-DR1	CTACCGTACCGTCACTATCC
IS1642-DR2	TCTTCACCGTGCGCACATAG
PIS-2	CGGGATCCCCGCCATTTCACCTGAAACC
PIS-3	CCCAAGCTTGCGAACAAAAAATCGACGCC

Southern blot analysis

Genome DNA was digested with restriction endonuclease PvuII (New England Biolabs), which had no recognized sites in the IS*1642* sequence. The digested DNA samples were electrophoresed on a 0.8% agarose gel. DNA fragments were transferred onto a nylon membrane and hybridized with a digoxigenin-labelled probe prepared using the PCR DIG Probe Synthesis Kit (Roche) with primer sets IS1642-3F and IS1642-3R designed to amplify a 515-bp portion of IS*1642*. Hybridized bands were visualized by chemiluminescence detection (Labeling and Detection Starter Kit II, Roche).

Promoter activity analysis

The entire IS*1642* region was amplified by PCR with the primer set PIS-2 and PIS-3 (Table 1), and cloned upstream of a promoterless green fluorescent protein (GFP) gene on a cloning vector pVV16 (a kind gift from Dr Vissa). The plasmids were transformed into *Mycobacterium smegmatis* mc^2 155, and the expression of these genes was assessed by measuring the fluorescence of GFP with a fluorescence plate reader (Perkin Elmer, Wallac 1420 ARVO MX).

Nucleotide sequence accession numbers

AB453386 is the GenBank accession number for the nucleotide sequence of the entire region of insertion element IS*1642*. The GenBank accession number for the nucleotide sequence of the region containing insertion element IS*1642* and the 161-bp direct repeats is AB453387.

Results

Identification of new IS, IS*1642*

A new IS was identified in an *M. avium* strain isolated from a human patient. The IS was found 673 bp upstream of the start codon of the gene corresponding to the MAP0076 gene of the *M. avium* ssp. *paratuberculosis* K-10 genome. This

sequence contained a nucleotide sequence of 1642 bp, with a GC content of 64%, which approximates those of the mycobacterial genomes (62–70%) (Wayne & Kubica, 1986). The sequence contained a single ORF coding for a protein of 503 amino acids and 14-bp imperfect inverted repeats (5′-cctgacttttatca-3′, 5′-tgataaaagtcggg-3′) at its ends. The complete nucleotide sequence of the IS and the deduced amino acid sequence is shown in Fig. 1a. Database searches revealed that this IS was 79% identical to that of IS*1549* of *M. smegmatis* (accession number AF006614) (Plikaytis *et al.*, 1998) at the amino acid sequence level. Hence, we assigned IS*1642* to the newly identified IS.

IS*1549* is an insertion element that was reported to be distantly related to the IS*4* family (Plikaytis *et al.*, 1998). Transposases of the IS*4* family typically contain conserved regions, N3 and C1, but IS*1549* lacks the N3 region (Plikaytis *et al.*, 1998). Analysis of alignment indicated that IS*1642* also contained the C1 region, but not the N3 region. The C1 signature sequence of the IS*4* family is Y-(X_2)-R-(X_3)-E-(X_6)-K (Rezsöhazy *et al.*, 1993), and the corresponding IS*1642* sequence was Y-(X_2)-L-(X_3)-E-(X_6)-K. It was reported that IS*1549* possesses unique N2 and N3 regions. These sequences were found in IS*1642* as well (Fig. 1a). While the N2 signature sequence was D-(X_2)-T-(X)-YFE-(X_{10})-G-(X)-SK (Alexander *et al.*, 2003), the corresponding IS*1642* sequence was D-(X_2)-T-(X)-HFE-(X_{10})-G-(X)-SK. The N3 signature sequence was AD-(X)-G-(X_5)-N (Alexander *et al.*, 2003), and the corresponding IS*1642* sequence was identical.

Identification of direct repeats

IS*1642* identified upstream of the MAP0076 gene was flanked by direct repeats of 161 bp, which was a duplication of the nucleotide sequence of the region upstream of the MAP0076 gene. Compared with the usual direct repeats of general ISs, this direct repeat is extremely long. Because Southern blot analysis indicated that the *M. avium* strain contained multiple copies of IS*1642* (Fig. 2, lane 1), we sought to determine the length of the direct repeats of IS*1642* of other copies. Two direct repeats were successfully identified by the method described in Materials and methods. The length of the direct repeats was 5 and 59 bp,

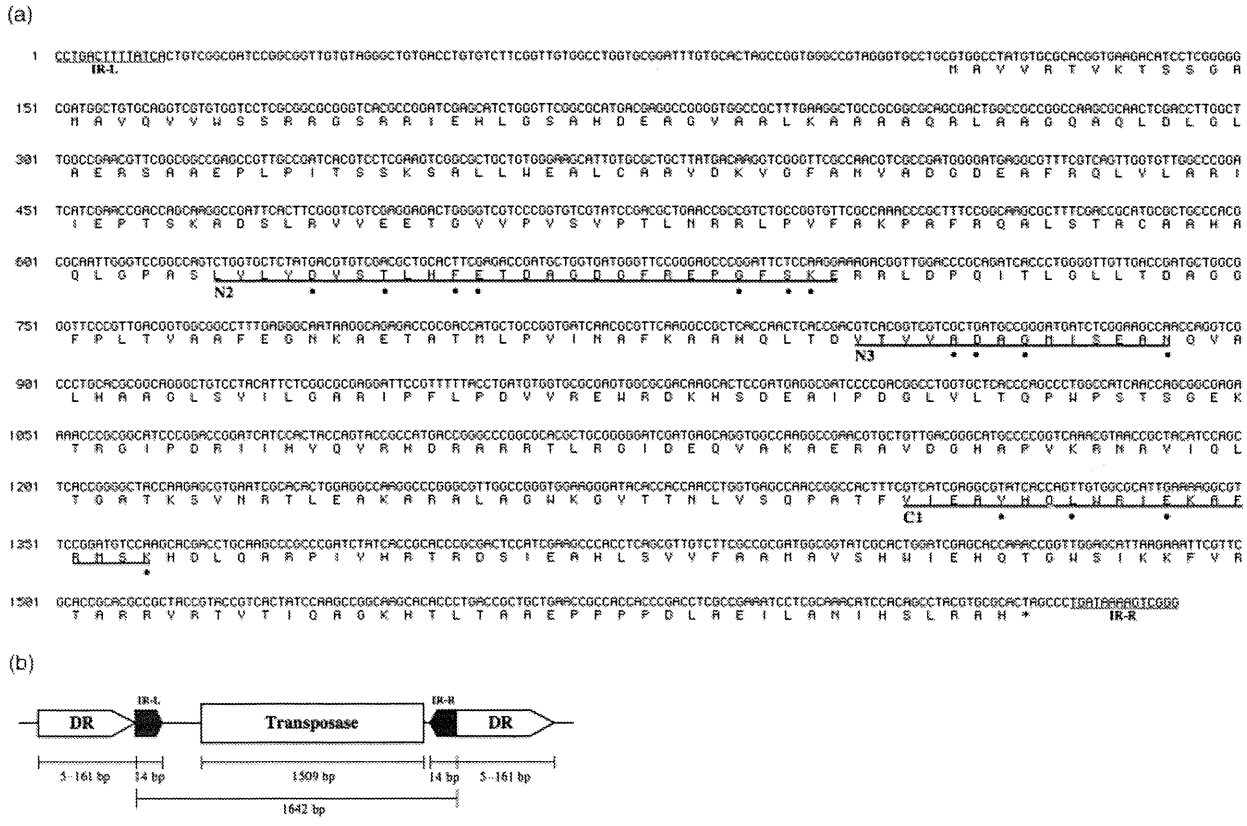

Fig. 1. Nucleotide sequence and structure of IS*1642*. (a) Nucleotide sequence of IS*1642* and deduced amino acid sequence of ORF. Inverted repeats, designated IR-R and IR-L, are underlined. Conserved regions, N2, N3, and C1, are underlined in bold. Dots below amino acids indicate the conserved signature sequences of these regions. (b) Schematic representation of the IS*1642* and the DR. The IR and the DR, and the beginning and end of the ORF encoding a putative transposase are shown.

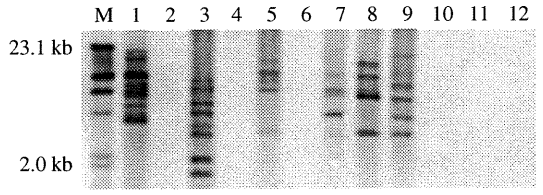

Fig. 2. Southern blot profile of IS*1642* of *Mycobacterium avium* strains. M, molecular size marker, lambda HindIII. lanes 1–9, human *M. avium* isolates; lane 10, *M. avium* ssp. *paratuberculosis* K-10; lane 11, *M. avium* ssp. *avium* ATCC25291; lane 12, *M. avium* ssp. *avium* ATCC15769. Numbers on the left indicate the sizes of the DNA marker.

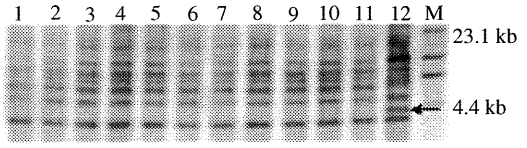

Fig. 3. Change in the Southern blot profile of IS*1642* of the human *Mycobacterium avium* isolate by *in vitro* repeated passages. lane 1, original *M. avium* cells; lanes 2–11, *M. avium* cells subcultured once from 10 individual colonies from the original culture plate; lane 12, *M. avium* cells passaged 10 times from the original culture plate. M, molecular size marker, lambda HindIII. The additional band that appeared after the repeated passage is indicated by the arrow. Numbers on the right indicate the sizes of the DNA marker.

respectively. The IS with the 5 bp direct repeat was found to be within the coding region of MAP2026 gene. The sequence flanking the 59 bp direct repeat sequence did not match with any gene in the database. We sought to find a conserved sequence at the insertion sites, but sequence specificity was not obvious among these three target sites.

Distribution of IS*1642*

The distribution of IS*1642* among *M. avium* strains was examined by Southern blot analysis with the following strains: *M. avium* ssp. *paratuberculosis* K-10, *M. avium* ssp. *avium* ATCC25291, *M. avium* ssp. *avium* ATCC15769, and nine *M. avium* strains, isolated from different patients at NHO Higashi Nagoya National Hospital. The DNA bands were found in only some of the *M. avium* strains isolated from human patients, but not in *M. avium* ssp. *paratuberculosis* K-10, *M. avium* ssp. *avium* ATCC25291, and *M. avium* ssp. *avium* ATCC15769 (Fig. 2). The Southern blot profile indicated that the band patterns of IS*1642* were very diverse and the copies were multiplied. *Mycobacterium intracellulare* ATCC13950, *M. intracellulare* ATCC25225, five *M. intracellulare* clinical isolates, and seven other mycobacterial species were negative for IS*1642* on the Southern analysis profile (data not shown). The prevalence of IS*1642* among *M. avium* strains was further examined with eight strains isolated from human patients at the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases by PCR with the three sets of specific primers (primers IS1642-1F and IS1642-1R, IS1642-2F and IS1642-2R, IS1642-3F, and IS1642-3R) (Table 1). All strains were positive for IS*1642* (data not shown), suggesting that IS*1642* is widely distributed among *M. avium* clinical strains.

Mobility of IS*1642*

Because the Southern blot profile of IS*1642* was very different among strains, we explored whether or not the IS*1642* exhibits frequent mobility by examining changes in the Southern blot pattern during repeated passages *in vitro*. One *M. avium* clinical strain was streaked on a 7H10 agar plate, and passaged 10 times. A single colony was used for inoculation at each passage. After the passages, genome DNA was extracted. For reference, 10 colonies were individually subcultured once from the initial culture plate and genome DNA samples were extracted. The genome DNA samples were subjected to Southern blot analysis. While the original cells and the cells of 10 colonies with a single subculture showed identical patterns, the passaged cells contained an additional band (Fig. 3), indicating that transposition of the IS occurred during the *in vitro* repeated culture.

Discussion

In this study, we discovered a new IS, IS*1642*, in *M. avium* clinical strains isolated from human patients. Our results showed that multiple copies of IS*1642* are present in several *M. avium* strains. The deduced amino acid sequence of the ORF of IS*1642* was highly homologous to the transposase of *M. smegmatis* IS*1549* (Plikaytis *et al.*, 1998). IS*1549* is an insertion element whose transposases exhibited homology to that of IS*1623* and IS*1634*, and it was suggested that these transposases represent an emerging group in the IS*4* family because they exhibit a characteristic lack of the typical conserved N3 region of IS*4* family and yet possess unique N2 and N3 motifs (Vilei *et al.*, 1999; Alexander *et al.*, 2003). A notable feature of IS*1549* and IS*1634* is that they are flanked by unusual long direct repeats that may vary in length (Plikaytis *et al.*, 1998; Vilei *et al.*, 1999). The lengths of the direct repeats of these ISs range up to 500 bp, in contrast to most usual ISs flanked by short direct repeats of 2–14 bp. IS*1642* found in this study also exhibited these characteristics. IS*1642* contained the C1 region and unique N2 and N3 motifs, but not the N3 region of the IS*4* family, suggesting that IS*1642* belongs to this new group. IS*1642* was flanked by direct repeats of variable lengths ranging up to 161 bp. Considering that IS*1642* was homologous to the Iss, which form variable length direct repeats, it would be likely that the direct repeat sequences found in this study were actually created by IS*1642*. The actual range of lengths

of direct repeats may be larger as reported in other ISs. Because the homology level of the entire region of the amino acid sequence was relatively low among ISs of this group (e.g. 38% identity between IS*1549* and IS*1634*), the conserved structures of the amino acid sequence might play an important role in the formation of the characteristic long direct repeats of variable lengths. There was no conserved sequence at the insertion sites among the three target sites, suggesting that the insertion events by IS*1642* take place randomly on the genome.

To date, the advantage of formation of long, variable-length direct repeats is not well elucidated. While insertion with a long target duplication may decrease the likelihood of destroying essential genes at the target sites on the host genome, it would be expected that an insert flanked by long direct repeats could be easily removed by homologous recombination between the repeats. Nobusato *et al.* (2000) reported insertions with long target duplications in the restriction and modification enzyme genes of *Helicobacter pylori* strains. They considered that the long duplication may control the copy number of the genes, thus keeping expression of the genes at an appropriate level. Further studies are required to elucidate the significance of the long, variable-length direct repeats.

Our results suggested that IS*1642* is widely distributed among *M. avium* clinical strains. In addition, the experiment of repeated passage suggested that IS*1642* is indeed capable of frequent transposition within the genome. This is consistent with the observation that the Southern blot profile was very divergent among the strains tested. Considering this polymorphism, it would be rather inappropriate to use the Southern blot pattern of IS*1642* as a genetic typing tool for classification of different clinical strains. Alternatively, genotyping by IS*1642* could be useful to confirm the clonality of strains because it would enable high-precision discrimination.

IS elements reportedly often carry an outward-directed promoter sequence, bringing about a constitutive expression of downstream genes at insertion sites (Safi *et al.*, 2004; Soto *et al.*, 2004). IS*1549* was found to show promoter activity (Plikaytis *et al.*, 1998). Because IS*1642* was homologous with IS*1549*, we examined IS*1642* for promoter activity. However, the expression of GFP was at an undetectable level. IS*1642* would have no or very low outward-directed promoter activity, or the promoter sequence could be formed as a hybrid of the IS sequence and genome sequence at insertion sites, thus turning on the expression of otherwise silent genes on the genome, as reported by Szeverényi *et al.* (1996). It might also be possible that promoters of IS1642 could not be recognized by *M. smegmatis*.

IS*1642* would be involved in the gene rearrangements on the genome, thus contributing to evolution of the organism, like many other ISs. Although *M. avium* is an opportunistic pathogen, treatment is often very difficult once the infection is established, despite long-term administration of antibiotics. It may be possible that IS*1642*, which has a relatively high mobility, serves to facilitate establishment of chronic infection by adapting the phenotype including the pathogenicity and drug resistance of the organism for conditions at the focus of the infection *in vivo*.

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Antimicrobial Ointments and Methicillin-Resistant *Staphylococcus aureus* USA300

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We tested 259 methicillin-resistant *Staphylococcus aureus* isolates and 2 USA300 ATCC type strains for susceptibility to bacitracin and neomycin contained in over-the-counter antibacterial ointments. Resistance to both bacitracin and neomycin was found only in USA300. The use of over-the counter antimicrobial drugs may select for the USA300 clone.

Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is rapidly spreading worldwide. MRSA USA300 is a clone of increasing public health concern among rapidly disseminating CA-MRSA strains in the United States (*1*). MRSA USA300 is designated as sequence type (ST) 8 by multilocus sequence typing (MLST) and possesses staphylococcal cassette chromosome *mec* (SCC*mec*) type IVa. Although the rapid dissemination of the USA300 clone may occur because of a high virulence level that arises from the production of Panton-Valentine leukocidin (PVL) or an existing arginine catabolic mobile element (ACME) (*2*), there is no conclusive evidence to support this hypothesis (*3*). Furthermore, the hypothesis cannot account for the rapid dissemination of MRSA in countries where USA300 clones are not the dominant clones (most European countries, South Korea, and Japan) (*4–6*). In most European countries, the dominant CA-MRSA clone is the European clone (ST80, SCC*mec* type IV, PVL positive and ACME negative) (*1,4*). In South Korea, only 1 isolate was a USA300 clone among 138 MRSA isolates collected from patients with bacteremia and soft tissue infection (*5*). In Japan, the MRSA USA300 clone is rare (*6*).

In many cases, soft tissue infection acquired in communities was treated by using over–the-counter (OTC) drugs called triple-antibiotic ointment (TAO), e.g., Neosporin (polymyxin B [PL-B] sulfate, 5,000 units/g; bacitracin, 400 units/g; and neomycin, 3.5 mg/g) and Polysporin triple ointment (PL-B sulfate, 10,000 units/g; bacitracin, 500 units/g; and gramicidin 0.25 mg/g). These ointments contain antimicrobial drugs at concentrations far exceeding their MICs among *S. aureus* strains (16–32 μg/mL [equivalent to 124–248 unit/mL] for PL-B, <1–64 units/mL for bacitracin, and <1–>128 μg/mL for neomycin) (*7,8*). It is hypothesized that CA-MRSA cases in the United States were under the selective pressure of TAOs.

In this study, we tested the susceptibilities of MRSA isolates, including the USA300 clone, to the antimicrobial drugs in TAOs. We also considered the possible role of TAOs in spreading the USA300 clone.

The Study

We selected 222 MRSA isolates that were not classified as the New York/Japan (NY/JP) clone on the basis of the absence of SCC*mec kdpC* (*9*). In addition, 37 NY/JP clone-like isolates were used. A total of 259 MRSA isolates were tested in our study. Of these 259 isolates, 227 were collected during 2004–2010 at Nagoya Medical Center, and 32 isolates were collected in 2006–2009 at Kyoto University Hospital, including 9 USA300 outbreak isolates (*6*). Details of isolates used in this study are shown in Table 1. ATCC BAA1556 (USA300 FPR3757) (American Type Culture Collection, Manassas, VA, USA) and ATCC BAA1717 (USA300-HOU-MR TCH1516) strains were also used in our study. Susceptibilities to bacitracin and neomycin were tested by the Kirby-Bauer disk diffusion method (Becton Dickinson, Franklin Lakes, NJ, USA). MICs of bacitracin, neomycin, and PL-B for USA300 strains were determined by the agar dilution method according to the Clinical and Laboratory Standard Institute M07-A8 guidelines (*10*). To observe interaction among these 3 antimicrobial drugs, a double-disk synergy test was performed with modification by using ATCC BAA1717 (*11*).

SCC*mec* were determined according to the method of Hisata et al. (*12*). Isolates possessing both PVL and *arcA* (*13*) were analyzed by pulsed-field gel electrophoresis as described in our previous study (*9*). Moreover, USA300 isolates were genotyped by MLST (www.mlst.net) and staphylococcal protein A (*spa*) typing (www.spaserver.ridom.de).

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Table 1. Source of methicillin-resistant *Staphylococcus aureus* isolates, Japan, 2004–2010

Source	Outpatients		Inpatients		Health care workers
	No. community-acquired infections*	No. hospital-acquired infections	No. community-acquired infections*	No. hospital-acquired infections	
Skin and soft tissue	23	7	4	23	0
Bloodstream	0	2	1	19	0
Respiratory tract	0	2	1	17	0
Urinary tract	0	2	2	3	0
Ear	8	0	0	1	0
Eye	3	0	0	3	0
Others	1	1	2	3	0
Carriage	14	10	3	29	0
Screening	50	0	2	20	3
Total	99	24	15	118	3

*Community-acquired infections were determined on the basis of patients' histories according to Centers for Disease Control and Prevention (Atlanta, GA, USA) guidelines (www.cdc.gov/mrsa/diagnosis/index.html).

Nineteen of the 259 isolates harbored both the PVL and the *arcA* gene. Of these 19 isolates, 18 had been collected from Kyoto University Hospital and 1 from Nagoya Medical Center (Table 2). All 19 PVL- and ACME-positive isolates were determined to be ST8 by MLST. These isolates showed USA300 PFGE patterns identical to ATCC BAA1556 and were of SCC*mec* type IVa. SCC*mec* elements of other isolates were determined as type I (n = 4), IIa (n = 37), IIb (n = 52), II untypeable (n = 14), IV (n = 104), and V (n = 9). The SCC*mec* element of the remaining 20 isolates could not be identified.

The 18 USA300 isolates collected from Kyoto University Hospital showed the same *spa* type (t008). However, the 1 USA300 isolate collected from Nagoya Medical Center was of *spa* t190.

ATCC BAA1717 and 9 USA300 isolates collected during 2007–2009 at Kyoto University Hospital were resistant to both bacitracin and neomycin. The USA300 isolate detected at Nagoya Medical Center in 2004 was bacitracin resistant and neomycin susceptible. The other 9 USA300 isolates and ATCC BAA1556 were susceptible to both drugs (Table 2). Highlander et al. (*14*) found that the bacitracin- and aminoglycoside-resistant genes were located on pUSA300-HOU-MR, a plasmid typically observed in the USA300 strain TCH1516. The resistance to bacitracin and neomycin may depend on the presence of the plasmid and may be absent in some USA300 clones.

On the other hand, nearly all MRSA isolates that were determined to be a type other than USA300 were susceptible to bacitracin. One isolate was determined to have intermediate resistance to bacitracin. Also, 11 (4.5%) of the 240 MRSA isolates not deemed to be USA300 were resistant to neomycin, while 132 (55%) demonstrated intermediate resistance (Table 2). A study performed in the 1990s reported that most MRSA strains were susceptible to bacitracin, and many were resistant to neomycin (*8*). Our findings were consistent with the previous study.

MICs of bacitracin, neomycin, and PL-B were 400 units/mL, 128 μg/mL, and 400 units/mL, respectively, among most USA300 isolates with resistance to both bacitracin and neomycin (Table 2). The concentrations of neomycin and PL-B in the TAOs were ≈10 to 30× higher than the MICs of both drugs. In addition, neomycin and PL-B were observed to be weakly synergistic (Figure). However, Bearden et al. reported that despite containing antimicrobial drugs at concentrations far exceeding their MICs among MRSA, PL-B and neomycin ointment, or PL-B and gramicidin ointment exhibited deficient bactericidal activity in time-kill assays (*15*). Bacitracin may thus be required for sufficient bactericidal activity. Acquiring resistance to bacitracin and neomycin may be essential for survival under the selective pressure of TAOs. If so, bacitracin resistance should be considered a key characteristic of the USA300 clone.

Table 2. Bacitracin and neomycin susceptibility of MRSA USA300 and other MRSA isolates*

Bacitracin/neomycin	MRSA USA300 (MICs of bacitracin, neomycin, and polymyxin B)†			Other MRSA
	Kyoto University Hospital	Nagoya Medical Center	ATCC type strains	
R/R	9 (400, 128, 200–400)	0	BAA1717 (400, 128, 400)	0
R/S	0	1 (400, 0.25, 400)		0
S/R	0	0		11
I/S	0	0		1
S/I	0	0		132
S/S	9 (6.25–12.5, 0.25, 400)	0	BAA1556 (6.25, 0.25, 400)	96
Total	18	1	2	240

*MRSA, methicillin-resistant *Staphylococcus aureus*; ATCC, American Type Culture Collection (Manassas, VA, USA); R, resistant; S, susceptible; I, intermediate resistance.
†MICs are expressed as units/mL for bacitracin and polymyxin B and in μg/mL for neomycin.

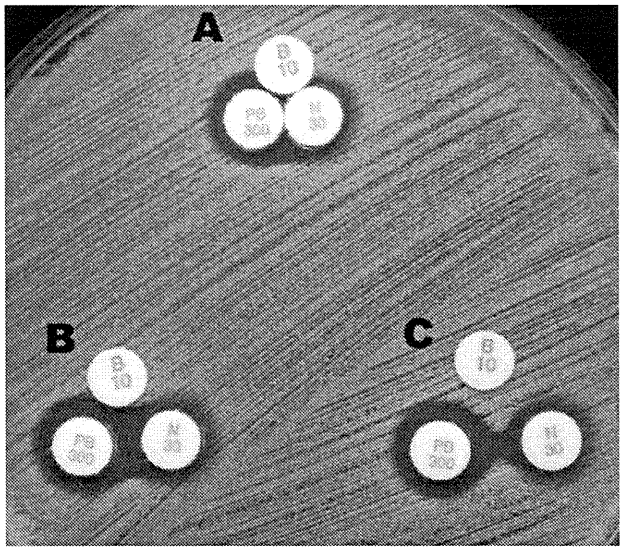

Figure. Double-disk synergy test with 3 disks, bacitracin (B10 disk), neomycin (N30 disk), or polymyxin B (PL-B, PB300 disk) was performed with USA300 strain ATCC BAA1717. Disks were placed at 6 mm (A), 9 mm (B), and 11 mm (C) distance from disk centers. Neomycin and PL-B were found to be weakly synergistic.

TAOs containing bacitracin, neomycin, and PL-B are widely used in the United States; thus, bacitracin- and neomycin-resistant strains may be selected by the selective pressure of the TAOs. Although bacitracin and neomycin ointments are also available as OTC drugs in Japan, use of the ointments is not widespread. As a result, the selective pressure that leads to bacitracin and neomycin resistance is weak in Japan.

Conclusions

The emergence of MRSA USA300 depends partly on the virulence of MRSA USA300, but it may be influenced by usage of OTC drugs. In each country, susceptibilities of MRSA USA300 to bacitracin and neomycin should be thoroughly investigated, and relationships between the dissemination of MRSA USA300 and the usage of OTC drugs should be clarified. Such an investigation will provide valuable information regarding the emergence of organisms resistant to OTC topical antibiotics and likely a warning against the indiscriminate use of antimicrobial drugs. Further studies are required to validate these findings.

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Close cooperation between infectious disease physicians and attending physicians can result in better management and outcome for patients with *Staphylococcus aureus* bacteraemia

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Abstract

Staphylococcus aureus bacteraemia (SAB) is a serious infection that demands prompt clinical attention for good outcome. To assess the impact of intervention by infectious diseases physicians (IDPs) in cases with SAB, a retrospective cohort study of patients with SAB was performed in a 1240-bed, university hospital in Japan, with the aim of comparing the management and outcome of patients during the initial and the latter half of the intervention period,. Three hundred and forty-six patients with SAB during the 7-year period, from 2002 to 2008, were included, and 194 patients in the initial half of the period (from 2002 to 2005) were compared with 152 patients in the later period (from 2006 to 2008). There was no significant difference between the two groups with respect to patient's clinical background, although more patients in the later period were receiving immunosuppressive treatment. The proportion of methicillin resistant *S. aureus* was lower during the later period (56.2% vs. 43.3%; p 0.02). Echocardiography was used more frequently (37.1% vs. 64.5%; p < 0.001). Infective endocarditis and metastatic infections were diagnosed more frequently (10.8% vs. 20.4%; p 0.01). Follow-up blood cultures were obtained more regularly (52.1% vs. 73.7%; p <0.001) and therapy was more frequently administered for at least 14 days (47.4% vs. 82.2%; p <0.001). The 30-day mortality improved during the intervention period (25.8% vs. 16.4%; p 0.04). The total number of blood cultures received by the laboratory increased annually and the total number of consultations increased by approximately 1.6-fold compared to 2002. Proactive intervention by IDPs raised awareness of optimal management of bacteraemia and improved the adherence to the standards of care, which subsequently resulted in an improvement in the outcome.

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Introduction

Infectious diseases are major causes of morbidity and mortality and contribute to increased healthcare costs. Clinical intervention by a multidisciplinary infection control team including infectious disease physicians (IDPs) reduces hospitalization duration and treatment costs for infected patients [1–3]. Although some studies suggest that intervention by IDPs can improve the quality of management of infectious disease [4–7], there is little evidence available to confirm that such intervention improves survival.

Antibiotics are prescribed by attending physicians (rather than by specialist IDPs) in Japan, as they are in most European countries. In addition, few hospitals have infectious diseases departments and IDPs are not routinely consulted about patients with bloodstream infection (BSI). In 2002, we started a hospital-wide, active clinical intervention by IDPs for the treatment of all bacteraemic patients at our hospital and found that mandatory intervention to treat candida BSI improves prognosis [8]. In addition to mandatory intervention for bacteraemia, IDP consultations are initiated by request from an attending physician or by an IDP when laboratory findings and the results of therapeutic drug monitor-