First outbreak of methicillin-resistant *Staphylococcus aureus* USA300 harboring the Panton-Valentine leukocidin genes among Japanese health care workers and hospitalized patients

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This report describes the first outbreak of methicillin-resistant *Staphylococcus aureus* USA300 in a general hospital ward in Japan, involving 6 health care workers and 4 patients. This report emphasizes the need for health care personnel to be alert that methicillin-resistant *Staphylococcus aureus* harboring Panton-Valentine leukocidin gene poses a threat for both nosocomial and occupational infection.
Key Words: Community associated; methicillin-resistant *Staphylococcus aureus*; MRSA; USA300; nosocomial outbreak.
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Accumulating evidence indicates that community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA) isolates can readily produce outbreaks in hospitals, adding to the threat posed by these organisms.[1-3] CA-MRSA is genetically heterogeneous and includes a variety of clones such as the multilocus sequence (ST) 1 (USA400) and ST8 (USA300) types that emerged as major clones in the United States. The USA300 clone can replace preexisting methicillin-resistant *Staphylococcus aureus* (MRSA) clones, and it now represents the predominant CA-MRSA clone in the United States.[4,5] Until now, CA-MRSA infections reported in Japan have been sporadic, and most strains did not harbor the Panton-Valentine leukocidin (*pvl*) genes.[6]

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In September 2009, we were notified that a cluster of skin infections had broken out among health care workers (HCWs) and hospitalized patients in a general ward. We document herein the first outbreak of MRSA harboring *pvl* genes belonging to the USA300 clone in a health care setting in Japan.

METHODS

At the time of notification, 65 patients were being cared for by 96 HCWs in a general ward at the tertiary care, 1240-bed Kyoto University Hospital (Japan) where dermatologic disorders were quite prevalent. We were notified that a cluster of skin infections had developed among 4 HCWs (HCW 1-4), which appeared to be compatible with *S aureus* infection. Two weeks later, 1 patient (patient 4) developed a skin abscess in the left arm from which MRSA was isolated. The isolate was susceptible to erythromycin, clindamycin, and gentamicin. The antimicrobial susceptibility pattern was distinct from that of health care-associated MRSA (HA-MRSA) strains in Japan, which were usually multidrug resistant and of which minimum inhibitory concentration levels of β-lactams were high. Subsequently, skin abscesses developed on the legs of HCW 5 and HCW 6 and relapsed on the legs and chest of HCWs 1 and 5. Eventually, MRSA isolates were recovered from HCWs.

Table 1. Characteristics of patients with CA-MRSA infections and their treatment, Kyoto University Hospital, 2009

Case	Age, sex	Underlying disease	Onset of infection	Type of infection	Site of infection	Antimicrobial drug treatment	Drainage
Pt 1	60, F	Inflammatory bowel disease	3/29	CRBSI	Bloodstream	TEIC	None
			8/27	Pneumonia	Lung	LZD	None
Pt 2	65, F	Decubitas ulcer	8/22	Skin abscess	Thigh	GEN	Spontaneous
Pt 3	42, M	Polyarteritis nodosa	10/19	Folliculitis	Legs	ST	Spontaneous
Pt 4	14, F	Decubitus ulcer	11/2	Skin abscess	Arm	GEN	Surgical
HCW 1	31, F	None	9/19	Skin abscess	Arm	MINO	Surgical
			11/24	Skin abscess	Arm	CLI	None
HCW 2	27, F	None	9/21	Skin abscess	Arm	GEN	Surgical
HCW 3	27, F	None	9/21	Skin abscess	Leg	MINO	Surgical
HCW 4	25, F	None	9/21	Skin abscess	Leg	None	Surgical
HCW 5	27, F	None	11/20	Skin abscesses	Leg, chest	CLI	Spontaneous
			12/14	Folliculitis	Arm, finger	STMUP	None
HCW 6	31, M	None	11/24	Skin abscess	Thigh	CLI	Spontaneous

NOTE. Values in column 4 represent month and day.
CLI, clindamycin; *CRBSI*; catheter-related bloodstream infection; *F*, female; *GEN*, gentamicin (topical); *LZD*, linezolid; *M*, male; *MINO*, minocyclin; *MUP*, mupirocin (topical); *Pt*, patient; *ST*, trimethoprim-sulfamethoxazole; *TEIC*, teicoplanin.

Based on information derived from these cultures, we developed a case definition in which MRSA with a specific antibiogram was recovered from a clinical specimen. The case-defined antibiogram was susceptible to erythromycin, clindamycin, and gentamicin but resistant to levofloxacin and β-lactams with minimum inhibitory concentration levels below those of HA-MRSA.

We reviewed the antimicrobial susceptibility profiles of MRSA strains from all adult and pediatric hospitalized patients who were under care at Kyoto University Hospital during 2009 to detect any unidentified MRSA. HCWs were screened in the ward using nasal swabs to identify MRSA carriers.

Clinical specimens were inoculated onto mannitol salt agar plates and examined after 48 hours. Susceptibility testing proceeded according to the Clinical and Laboratory Standards Institute. The *mecA* gene, PVL determinants, and *arcA* gene on the arginine catabolite mobile element were detected, and SCC*mec* typing was performed by polymerase chain reaction.[7-9] The typing procedure included pulsed-field gel electrophoresis using the restriction enzyme *SmaI* as described.[5] Multilocus sequence typing (MLST) proceeded as described, and the nomenclature was specified as previously described (www.MLST.net).

Patients 1 to 3 who became infected with MRSA were newly identified based on the antimicrobial susceptibility profile described in the case definition (Table 1). A review of the medical records revealed that the first MRSA infection occurred in March 2009. Patient 1 developed catheter-related bloodstream infection followed by pneumonia and required intravenous anti-MRSA drug administration. Six of 9 skin and soft-tissue infections (skin abscesses, folliculitis) were treated with antibiotics, whereas 3 were cured by drainage alone. Patient 1 as well as HCW 1 and 5 developed recurrent infections. No case patient had a history of visiting abroad recently. All MRSA isolates recovered from the case patients contained SCC*mec* type IV, the *pvl* gene, and arginine catabolite mobile element-associated *arcA* gene. Pulsed-field gel electrophoresis-based findings identified all isolates as being identical to and indistinguishable from the USA300 clone. MLST defined all of them as ST8.

Excluding the isolates recovered from the case patients, 4 of 825 strains of MRSA isolates at our institution in 2009 had the same antimicrobial susceptibility profile as the outbreak strain. Those isolates were recovered from swab specimens, and the patients did not have a symptom of infection when the specimens were taken. Screening nasal swabs of HCWs did not identify any carriers of CA-MRSA USA300 other than the case patients.

DISCUSSION

To our knowledge, this is the first report to document an outbreak of health care-associated and -transmitted CA-MRSA USA300 in Japan. To date, outbreaks of PVL-positive CA-MRSA have been reported, especially in neonatal intensive care units and long-term care facilities in the United States and European countries.[1-3] However, no outbreaks of CA-MRSA in either community or nosocomial settings in Japan have been described. Only one sporadic infection with the USA300 clone in Japan has been documented.[10] We speculate that infected HCWs or unidentified PVL-positive MRSA carriers served as the source of infection for patients 1 to 4 because all of them became infected while in hospital. In addition, we considered that the causative agent was community associated because antibiograms of the outbreak strain were distinct from those of HA-MRSA strains. This was supported by a review

of the antibiotic susceptibility of MRSA strains isolated at our institution during 2009; the case-defined antibiogram occurred in only 0.5% of isolates.

The findings of this investigation have considerable public health implications. Although HA-MRSA remains a serious threat to hospitalized patients, the introduction of CA-MRSA strains into tertiary care hospitals like our hospital represents an especially serious challenge. Many of the infections caused by these strains have been reported to cause serious infections among healthy adults and can be severer and more life threatening to those who are highly immunocompromised. In this study, healthy HCWs suffered from skin abscesses, although most infections were mild and cured without parenteral anti-MRSA drugs. Considering that infection relapsed in some case patients, further investigations are needed to establish the management of PVL-positive MRSA carriers, especially when they are caregivers. Systematic studies involving health care settings are needed to reveal the transmission of such CA-MRSA isolates within the health care system. These would provide not only an accurate estimate of CA-MRSA prevalence but would help monitor the emergence of more resistant and/or virulent clones and help with therapeutic infection control and patient management policies.

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ORIGINAL ARTICLE

Molecular characterization of *erm*(B)- and *mef*(E)-mediated erythromycin-resistant *Streptococcus pneumoniae* in China and complete DNA sequence of Tn*2010*

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Abstract

Aims: To characterize the *erm*(B)- and *mef*(E)-mediated erythromycin-resistant *Streptococcus pneumoniae* clinical isolates obtained from ten hospitals located different cities in China.

Methods and Results: Totally 83 *S. pneumoniae* were collected, and eighteen representative strains of 66 strains that exhibited erythromycin resistance were used for further characterization by antibiograms, serotyping, PFGE, MLST, DNA sequencing of the macrolide-resistance elements and mapping of the elements on the chromosome. Twelve isolates showed a high-level resistance to erythromycin, and six other isolates showed a low-level resistance to erythromycin. Thirteen isolates harboured a Tn*2010* transposon (26·4 kbp) encoding the *erm*(B), *tet*(M) and *mef*(E) genes and were classified into three types by Tn*2010* structures. The remaining five isolates harboured a Tn*6002* transposon (20·9 kbp) encoding the *erm*(B) and *tet*(M) genes and were classified into three types by Tn*6002* locations on the chromosome. Three of the Tn*6002* elements were located within the Tn*5252*-like element, implying that these composed a large mobile element. The MLST analyses showed that several clones had been disseminated and that the CC271 strains carrying the Tn*2010* element expressing the high-level resistance to erythromycin were predominant in China. Four new MLST strains, which were designated as ST3262, ST3263, ST3397 and ST3398 were also identified.

Conclusions: The erythromycin resistance determinant of *S. pneumoniae* that had been isolated in China was located in Tn*2010* or the Tn*6002* element and several clones had been disseminated, and the CC271 strains carrying the Tn*2010* element expressing the high-level resistance to erythromycin were predominant in China.

Significance and Impact of the Study: This is the first molecular analysis of erythromycin-resistant *Streptococcus pneumoniae* clinical isolates in China, and the first report of the complete nucleotide sequence of Tn*2010* (26 390 bp).

Introduction

Streptococcus pneumoniae is an important human pathogen associated with respiratory tract infections. Antibiotic treatment of these infections has become a growing problem because of the emergence of resistance to both penicillin and nonbeta-lactam antibiotics (Fuller *et al.* 2005; Daneman *et al.* 2006). During the last decade, a rapid increase in the resistance of *S. pneumoniae* to macrolides has been observed in China. Data from the China Bacterial Resistance Surveillance Study Group (BRSSG) and Ministry of Health National Antibacterial Resistance Investigation Net (MOHNARIN) showed that the rate of erythromycin resistance increased from 40% in 1999 to

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91·9% in 2008 (Li *et al.* 2001; Y. Li & Y. Lv, unpublished results).

Macrolide resistance in *S. pneumoniae* is mediated by two main mechanisms: target modification caused by a ribosomal methylase encoded mainly by the *erm*(B) gene is related to the MLS_B phenotype (resistance to macrolide-lincosamide-streptogram B) and an efflux pump system that is associated with the *mef* gene and related to the M phenotype and is resistant to 14- and 15-membered macrolides only. The Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin (PROTEKT) Surveillance Study has shown that the most common macrolide resistance mechanism is ribosomal methylation mediated by *erm*(B) (55·0% of erythromycin-resistant *S. pneumoniae*), except in Canada, Greece and the United States where the drug efflux mediated by *mef*(A) is predominant (Felmingham *et al.* 2007; Farrell *et al.* 2008). In addition, isolates carrying both resistance determinants are becoming increasingly common in several areas, such as some Asian countries, South Africa and the United States, since their isolation was first described in a South African study (Mcgee *et al.*, 2001; Farrell *et al.* 2005). These dual-gene isolates were mainly of serotype 19F or 19A, showed multi-drug resistance and were classed as clonal complex CC271. Recent studies have shown that both *erm*(B) and *mef* genetic elements have integrated into the Tn*916*-like transposon, which has been designated as Tn*2010* (Del Grosso *et al.* 2006, 2007).

Studies from China have shown that the MLS_B phenotype is dominant and 44·0–79·5% of erythromycin-resistant strains carry *erm*(B) as the sole resistance gene and that 17·8–44·1% had both *erm*(B) and *mef*(E) (Zhao *et al.* 2004; Yang *et al.* 2005; Wu *et al.* 2006). A detailed characterization of erythromycin-resistant *S. pneumoniae* has not been reported in China. The aim of this work was the genetic characterization of erythromycin-resistant *S. pneumoniae* and the genetic elements carrying *erm*(B) and/or *mef*(E).

Materials and methods

Bacterial strains and culture

Eighty-three *S. pneumoniae* clinical isolates were obtained from ten hospitals (i.e., hospital A, D, G, H, I, K, M, O, P and Q) in different cities throughout China. A, D, G, H, I, K, M, O, P and Q hospitals were located in Beijing, Shenyang, Nanjing, Shanghai, Hangzhou, Shenzhen, Changsha, Chengdu, Chongqing and Xian, respectively. These hospitals joined the Chinese Ministry of Health National Antibacterial Resistance Investigation Net (MOHNARIN) in the period 2004–2005 (Table 1). MOHNARIN is a National Surveillance Program aimed at providing geographically relevant data on the resistance trends of key pathogens and an alert mechanism for new emerging resistance threats. Representative isolates were selected from patients hospitalized in respiratory ward or intensive care unit (ICU). Each strain corresponded to one individual patient, and most of them were isolated from community-acquired infections. The number of strains obtained from A, D, G, H, I, K, M, O, P and Q hospitals was 4, 3, 4, 4, 4, 5, 5, 10, 31 and 13, respectively. Hospital P has isolated many strains from hospital-acquired infections; then, we obtained over-represented number of isolates from hospital P and included the isolates in this study. The 83 isolates were obtained from sputum (68 (82%)), nasopharynx (2 (2·4%)), blood (4 (5%)) and unrecorded sources. *S. pneumoniae* strains were grown in Brain Heart Infusion (BHI) broth (Difco, Detroit, MI) or on BHI agar plate at 37°C in the anaerobic jar of GasPak System (BBL, Fisher Scientific, Tokyo, Japan) using AnaeroPack-Anaero (Mitsubishi Gas Chemical Co., Tokyo, Japan), which is a disposable oxygen-absorbing and carbon dioxide-generating agent.

Antimicrobials and susceptibility test

The following antimicrobials were tested: penicillin, amoxicillin, ceftriaxone, cefotaxime, cefepime, imipenem, panipenem, erythromycin, clarithromycin, azithromycin, clindamycin, tetracycline, levofloxacin and vancomycin. All antimicrobials were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) of China. Broth dilution MICs (minimum inhibitary concentrations) were determined according to the recommended method of the Clinical and Laboratory Standards Institute (CLSI). The results were interpreted according to the CLSI criteria, using penicillin breakpoints in place before they were changed in 2008 (susceptible, $\leq$0·06 mg l^{-1}; intermediate, 0·12 to 1 mg l^{-1}; and resistant, $\geq$2 mg l^{-1}) (CLSI, 2006). *S. pneumoniae* ATCC49619 was used as the control strain.

Genomic DNA isolation

The genomic DNAs were prepared from *S. pneumoniae* using an ISOPLANT kit according to the manufacturer's instructions (Nippon Gene Co., Toyama, Japan).

Serotyping

Serotyping for *S. pneumoniae* was performed by PCR with primers specific for the genes responsible for the biosynthesis of type 1, 3, 4, 6B, 14, 18C, 19A, 19F and 23F and serogroups 6, 19, and 23 of the capsular polysaccharide (Brito *et al.* 2003; Lawrence *et al.* 2003).

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Table 1 Chinese clinical isolated erythromycin-resistant *Streptococcus pneumoniae* and characteristics

Strain	Hospital	City	Age	Date of isolation	Source	Diagnosis/underlying disease*	HAI/CAI†	Antibiotic(s)used before isolation‡	Serotype	PFGE§	ST	Genetic element¶	P/G**
05D058	D	Shenyang	37	2005/01/06	Sputum	Pneumonia	CAI	None	19A	a3	320	Tn*2010*	A
05M016	M	Changsha	3	2004/10/29	Blood	Leukemia	CAI	None	19F	a1	271	Tn*2010*	A
05O173	O	Chengdu	<1	2005/02/27	Sputum	Bronchitis	CAI	None	19F	a2	271	Tn*2010*	A
05P107	P	Chongqing	73	2004/12/14	Sputum	Pneumonia/diabetes	CAI	None	19F	a1	271	Tn*2010*	A
05P117	P	Chongqing	29	2004/12/20	Sputum	Pneumonia/postoperative	HAI	PEN	19F	a1	271	Tn*2010*	A
05P128	P	Chongqing	24	2005/01/04	Sputum	Pneumonia/Guillain–Barre Syndrome	HAI	CLI + LVX	19F	a1	271	Tn*2010*	A
05P286	P	Chongqing	58	2005/05/16	Sputum	Pneumonia/postoperative	HAI	PEN + MEZ/SBT	19F	a1	271	Tn*2010*	A
05P288	P	Chongqing	56	2005/05/24	Sputum	Pneumonia	CAI	None	19F	a1	271	Tn*2010*	A
05P294	P	Chongqing	56	2005/05/29	Sputum	Bronchitis/cardiopathy	HAI	None	19F	a1	271	Tn*2010*	A
05P311	P	Chongqing	75	2005/06/27	Sputum	Pneumonia/COPC	HAI	None	19F	a1	271	Tn*2010*	A
05P343	P	Chongqing	36	2005/08/23	Sputum	Pneumonia/postoperative	HAI	PEN	19F	a1	271	Tn*2010*	A
05O035	O	Chengdu	4	2004/10/18	Sputum	Bronchitis	CAI	None	19F	a4	236	Tn*2010*	B
05P006	P	Chongqing	44	2004/09/18	Sputum	Pneumonia	CAI	None	NT††	e	3398	Tn*2010*	C
05O071	O	Chengdu	4	2004/12/14	Sputum	Bronchitis	CAI	A/C + PEN	NT††	d	3397	Tn*6002*	D
05K022	K	Shenzhen	22	2004/11/03	Sputum	Pneumonia	CAI	None	23F	b	342	Tn*6002*‡‡	E
05Q056	Q	Xian	50	2004/12/02	Sputum	Pneumonia/COPC	HAI	AZM + LVX	23F	b	342	Tn*6002*‡‡	E
05P141	P	Chongqing	65	2005/01/12	Sputum	Bronchitis/COPD	CAI	None	6B	f	3263	Tn*6002*‡‡	E
05O070	O	Chengdu	1	2004/12/13	Eyes secretion	Conjunctivitis	CAI	None	6B	c	3262	Tn*6002*	F

*COPD, chronic obstructive pulmonary disease.

†HAI, hospital-acquired infection; CAI, community-acquired infection.

‡Drug abbreviations: A/C, amoxicillin–clavulanic acid; PEN, penicillin; CLI, clindamycin; MEZ, mezlocillin; SBT, sulbactam; AZM, azithromycin; LVX, levofloxacin.

§PFGE: Types designated with a lower-case letter and related clones designated with the same letter followed by a number as described in text.

¶Tn*2010* element encoded *erm*(B), *mef*(E) and *tet*(M). Tn*6002* element encoded *erm*(B) and *tet*(M).

**P/G: phenotype/genotype, combination types according to different phenotype and genotype of mobile genetic element (Table 3, Fig. 2).

††NT: nontypeable.

‡‡Tn*6002* element was inserted into downstream of *orf28* in Tn*5252* (Ayoubi *et al.* 1991).

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PFGE

Streptococcus pneumoniae DNA embedded in an agarose block was prepared using a modification of the method of Lefevre *et al.* (1993). The guidelines proposed by Tenover *et al.* were basically used for the interpretation of PFGE results in this study. With these guidelines, strains that differed by between one and six bands were considered to be related clones, and banding pattern difference of three fragments could have occurred as a result of a

Table 2 Oligonucleotide primer pairs used

Gene	Primer Designation	Primer Sequence (5′-3′)	Product size (bp)	Reference
Resistance and transposons related genes				
erm(A)	ermA0	TCT AAA AAG CAT GTA AAG GAA	472	Sutcliffe *et al.* (1996)
	ermA2	CAG AAT CTA CAT TAG GCT TAG GG		Trallero *et al.* (2007)
erm(B)	ermB1	GAA AAG GTA CTC AAC CAA ATA	639	Sutcliffe *et al.* (1996)
	ermB2	AGT AAC GGT ACT TAA ATT GTT TAC		
mef	mef1	AGT ATC ATT AAT CAC TAG TGC	345	Sutcliffe *et al.* (1996)
	mef2	TTC TTC TGG TAC TAA AAG TGG		
tet(M)	tetM1	GAA CTC GAA CAA GAG GAA AGC	740	Brenciani *et al.* (2007)
	tetM2	ATG GAA GCC CAG AAA GGA T		
aphA3	aphA1	TAA AAG ATA CGG AAG GAA TGT CTC	824	this study
	aphA2	TCG ACC GGA CGC AGA AGG CAA TGT		Caillaud *et al.* (1987)
int	int-for	GCG TGA TTG TAT CTC ACT	1046	Brenciani *et al.* (2007)
	int-rev	GAC GCT CCT GTT GCT TCT		
xis	xis-for	AAG CAG ACT GAG ATT CCT A	139	Brenciani *et al.* (2007)
	xis-rev	GCG TCC AAT GTA TCT ATA A		
tndX	tndX-for	ATG ATG GGT TGG ACA AAG A	610	Brenciani *et al.* (2007)
	tndX-rev	CTT TGC TCG ATA GGC TCT A		
tnpR	tnpR-for	CCA AGG AGC TAA AGA GGT CCC	1528	Brenciani *et al.* (2007)
	tnpR-rev	GTC CCG AGT CCC ATG GAA GC		
tnpA	tnpA-for	GCT TCC ATG GGA CTC GGG AC	2115	Brenciani *et al.* (2007)
	tnpA-rev	GCT CCC AAT TAA TAG GAG A		
Transposons mapping				
orf24	TN6-rev	CCA TCA AAC ATT CAT TCA GC	3356	Brenciani *et al.* (2007)
orf20	J13	GGT TTT GTG GTT AGT TTT		
orf20	J12	CCC ATT GAA GAC GCA GAA GT	4841/7689*	Brenciani *et al.* (2007)
orf15	J15-rev	AAA GGA AGC CGA TAG GAT AAA		
orf15	J15	TTT ATC CTA TCG GCT TCC TTT	3956	Brenciani *et al.* (2007)
tet(M)	O7	CGG TAG TTT TT CTG CAT CAA C		
orf12	L27	CCT ATG GTT ATG CAT AAA AAT CCC	1424	this study
tet(M)	tetM2	ATG GAA GCC CAG AAA GGA T		Brenciani *et al.* (2007)
tet(M)	tetM1	GAA CTC GAA CAA GAG GAA AGC	3835/9344†	Brenciani *et al.* (2007)
xis	xis-rev	GCG TCC AAT GTA TCT ATA A		
tet(M)	tetM1	GAA CTC GAA CAA GAG GAA AGC	4002	Brenciani *et al.* (2007)
mel	OM21	GGC AAA ATC ACT GAG TAT TGG		Del Grosso *et al.* (2004)
mel	msrA2	TTA ATT TCC GCA CCG ACT A	3621	Amezaga *et al.* (2002)
orf9	SG3	GAA TCT TTA GCC AGC GGT ATC		Del Grosso *et al.* (2004)
mef(E)	OM18	TGC TTG CCC TGC CCA TAT TG	3550	Del Grosso *et al.* (2004)
xis-rev	xis-rev	GCG TCC AAT GTA TCT ATA A		Brenciani *et al.* (2007)
Inverse PCR				
orf24	TN1	ATA AAG TGT GAT AAG TCC AG		This study
orf24	L7	GTA GAA GCT AAA GAT GGT AAA CTT		This study
int	TN4	AGG CTT TAC GAG CAT TTA AG		This study
int	N1	GCA GAA ATC AGT AGA ATT GCC C		This study

*The former product size was expected according to the reported sequence of Tn*916* (accession no.EFU09422), with the later according to the reported sequence of Tn*6002* (accession no AY898750).

†The former product size was expected according to the reported sequence of Tn*916* (accession no.EFU09422), with the later according to the reported sequence of Tn*2009* (accession no AF376746).

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single genetic event. Types were designated with a lowercase letter, and related clones were designated with the same letter based on the visual comparison of patterns. The followed number showed the differences of one to three restriction fragments that were likely because of a single genetic event (Singh *et al.* 2006).

MLST analyses

Internal fragments of the *aroE, gdh, gki, recp, spi, xpt* and *ddl* genes, which are the seven housekeeping genes of *S. pneumoniae*, were amplified by PCR from chromosomal DNA using the primer pairs described in the MLST database (http://spneumoniae.mlst.net) (Enright and Spratt 1998). The allele numbers of the seven housekeeping loci and the resulting sequence types were also obtained from the MLST website.

Detection of resistance genes

Detection of the macrolide-resistant genes *erm*(A), *erm*(B) and *mef*, the tetracycline-resistant gene *tet*(M), the kanamycin-resistant gene *aphA3* and the *int* (integrase), *xis* (excisase) and *tndX* (resolvase) genes of the Tn*916* transposon family, and the *tnpA* (transposase) and *tnpR* (resolvase) genes related to the Tn*917* transposon was carried out by PCR using the primers described in Table 2 (Sutcliffe *et al.* 1996; Brenciani *et al.* 2007; Trallero *et al.* 2007). DNA sequence analysis for each of the determinants, and *orf20* and P0 was also performed as necessary (Warburton *et al.* 2007). The drug-resistant determinants within the Tn*6002* or Tn*2010* elements are located in the order *erm*(B) and *tet*(M), and *erm*(B), *tet*(M) and *mef*(E), respectively. To determine a linkage between the *erm*(B) and *tet*(M) pair, and the *tet*(M) and *mef* pair, respectively, six primer combinations were used to analyze the reciprocal orientation of the three genes, and the PCR products were analyzed.

Structure of the composite elements and detection of chromosomal insertion sites

Based on the results of PCR of transposon-related genes and the linkage between resistance genes, the structure of the resistance gene-carrying elements was examined by a series of PCRs with the primers designed by Brenciani *et al.* (2007) and Del Grosso *et al.* (2004) (Table 2). The sizes of the fragments obtained by PCR were compared with those reported previously (Del Grosso *et al.* 2006; Brenciani *et al.* 2007). One strain chosen as representative for phenotype/genotype A and the transposon was sequenced completely. Complete DNA sequence analysis of the Tn*2010*-like element was performed in the representative strain 05P294.

orf24 and *int* are located at each end of the transposable element of the Tn*916* derivatives (Flannagan *et al.* 1994). The DNA regions flanking both ends of the transposable elements were investigated using inverse PCR (iPCR) assays. Chromosomal DNA was digested with NdeI, and the fragments were ligated. PCR were performed with primer pairs for *orf24* end and the *int* end, respectively (Table 2). The PCR products were purified and sequenced and submitted to DNA sequencing analysis. If one of the terminal junctions of the element could not be obtained by iPCR, it was obtained by direct PCR amplification of the region spanning the end of the elements and the chromosomal DNA downstream/upstream of the insertion site based on the published genome database for the *S. pneumoniae* R6 strain (Hoskins *et al.* 2001). A serial of reverse primers were designed on the basis of the genomic sequence at an approximately 1·5-kb interval from the insertion point to approximately 15 kb downstream or upstream.

Nucleotide sequence accession number

The complete nucleotide sequence of transposon Tn*2010* of strain 05P294 was assigned GenBank accession no. AB426620. The partial DNA sequences of Tn*2010* from strains 05D058, 05O035 and 05P006 containing the *erm*(B) elements and its junctions into Tn*916* were assigned GenBank accession nos. AB426621, AB426622 and AB426623, respectively. The partial DNA sequences for Tn*2010* from the three strains containing the mega elements and its junctions into Tn*916* were assigned GenBank accession nos. AB426624, AB426625 and AB426626, respectively.

Results and discussion

Drug susceptibilities of the isolates

Of the 83 isolates, 66 (80%) exhibited resistance to the macrolides examined (i.e., erythromycin, clarithromycin, azithromycin and clindamycin). Erythromycin resistance level (MIC mg l^{-1}) ranged between 1 and >256 mg l^{-1}, and 36 (43%) strains exhibited a high level of erythromycin resistance of >256 mg l^{-1}, and six (7%) strains exhibited low level of erythromycin resistance between 1 and 8 mg l^{-1}. Susceptibility (MIC) to erythromycin by the 17 erythromycin-sensitive strains was ≤0·25 mg l^{-1}. MICs of penicillin for the 83 isolates were distributed between 0·016 and 4 mg l^{-1}, and 33 (40%) of 83 isolates exhibited penicillin resistance (i.e., MIC > 0·016 mg l^{-1}) according to the previous criteria of CLSI when this surveillance study was performed. The criteria was changed in 2008, and the susceptibility breakpoint for nonmeningitis

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isolates is >8 mg L^{-1} now. These data indicated a high incidence of erythromycin-resistant strains, which is consistent with the results of other reports (Zhao *et al.* 2004; Yang *et al.* 2005). Of the 83 clinical isolates, a total of eighteen erythromycin-resistant strains from six hospitals were selected as representative strains for further analysis of their erythromycin-resistant determinants (Table 1). We focused on these eighteen isolates including six isolates showing low-level resistance to erythromycin (MICs were between 1 and 8 mg l^{-1}) and twelve showing high-level resistance to erythromycin (>256 mg l^{-1}) and intermediate resistance (or resistance) to penicillin according to the previous criteria (MICs were between 1 and 2 mg l^{-1}). The reason was that macrolide and penicillin resistances are significant in the clinical setting and that the low-level erythromycin-resistant *S. pneumoniae* isolate was not common in China and little was known about the strains.

Phenotypic and genotypic characterization of isolates

Of the 18 strains, twelve were highly resistant to erythromycin and clindamycin (MIC ≥ 128 mg l^{-1}) and showed intermediate resistance or full resistance to penicillin (MIC ≥ 1 mg l^{-1}) and tetracycline (MIC 4–16 mg l^{-1}). Among the other six low-level erythromycin-resistant strains (MIC 1–8 mg l^{-1}), five were susceptible to penicillin (MIC ≤ 0·25 mg l^{-1}), and one susceptible to both penicillin and clindamycin (Table 3). All tested strains were susceptible to vancomycin, and two strains were resistant to levofloxacin.

erm(B) and *tet*(M) were identified in all 18 isolates, but *erm*(A) and *aphA3* were not identified in any isolate (Table 3). *erm*(A) confer the low-level resistance to macrolides in *S. pneumoniae* and is found in other countries (Sutcliffe *et al.* 1996; Trallero *et al.* 2007). *aphA3*-encoding aminoglycoside resistance is encoded on the pneumococcal conjugative transposon Tn*1545* that belongs to the Tn*916* family and spread in pneumococci (Caillaud *et al.* 1987). Of the 18 isolates, thirteen strains carried *mef*(E) in addition to *erm*(B) and *tet*(M). In these 13 strains, eleven strains showed high resistance to erythromycin, and belonged to serotype 19, and the other two strains had low resistance to erythromycin (Table 1). One of the two strains belonged to serotype 19, and the other strain was nontypeable by PCR. Of the five strains that had *erm*(B) and *tet*(M), but did not carry *mef*(E), one strain showed high resistance to erythromycin, and four strains showed low resistance to erythromycin, two of these four strains belonged to serotype 23F and the other two belonged to serotype 6B.

PFGE analysis showed that of the thirteen strains carrying both *erm*(B) and *mef*(E), twelve showed similar profiles: with nine isolates having an identical profile (typed as a1), which were isolated from hospitals P and M, and the other three isolates differed by one or two bands, indicating that the strains were related (i.e., type a2, a3 and a4) (Table 1, data not shown) (Tenover *et al.* 1995). These results indicated that the 'type a' strains were clonally spread in China. Strain 05P006 also carried both *erm*(B) and *mef*(E), but showed a different pattern. Five strains shared four different patterns (type b, c, d and f) (Table 1). Two 'type b' strains (serotype 23F), 05K022 and 05Q056, were isolated from hospitals K and Q, respectively.

MLST analysis was performed. Of the 13 strains carrying both erythromycin-resistant genes, 10 belonged to ST271, 1 belonged to ST320 and 1 belonged to ST236. These are included in the clonal complex CC271, which is common in erythromycin-resistant *S. pneumoniae* (Ko and Song 2004; Del Grosso *et al.* 2007). In strain 05P006, *xpt* and *ddl* belonged to new alleles, and the concatenation was designated as ST3398 (Table 1). Among the five strains carrying the single erythromycin-resistant gene (*ermB*), two (i.e., 05K022 and 05Q056) belonged to ST342, which is included in CC271. In strain 05O071, *gdh* and *ddl* of the seven housekeeping genes belonged to new alleles and the strain was designated as ST3397. In strain 05O070 and 05P141, new concatenations were found and were designated as ST3262 and ST3263, respectively.

The twelve strains that were isolated from four hospitals (D, M, O and P), belonged to serotype 19F except one strain of 19A, clonal complex 271, and were clonally related by PFGE analysis (Farrell *et al.* 2005, 2008). These strains were identical to one of the internationally spread multidrug-resistant pneumococcal clone Taiwan^{19F}-14 (Del Grosso *et al.* 2007). Taiwan^{19F}-14 belongs to clonal complex CC271 and is resistant to penicillin, erythromycin and tetracycline, carries Tn*2010* element encoded *erm(B)*, *mef(E)* and *tet(M)*.

Composite elements and insertion sites

The results of sequence analysis of the region carrying the drug-resistant genes showed that the erythromycin-resistant genes were carried on the Tn*2010* or Tn*6002* transposons (Table 1). Tn*6002* (20·9 kbp) is a result of the insertion of the *erm*(B)-containing DNA fragment (2·8 kb) into Tn*916* (18 kb), and Tn*2010* (26·4 kb) is the result of the insertion of mega (macrolide efflux genetic element) (5·5 kbp), which includes the *mef*(E)-*msr*(D) operon encoding macrolide resistance, into *orf6* of Tn*916* in Tn*6002* (Fig. 1). The *erm*(B) element, which is composed of P0, P1, *erm*(B), P3 and P4, lies just before nucleotide T of the stop codon TAA of ORF20 of Tn*916*, which is located upstream of P0 in both Tn*2010* and

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Table 3 Drug susceptibilities and the drug resistance gene or transposon-related gene of *Streptococcus pneumoniae* strains

Drug susceptibilities (MIC mg l^{-1})													Drug resistance gene or transposon-related gene									
Strain	ERY	CLI	CLR	AZM	TET	LVX	VAN	PEN	AMX	A/C	CXM	CTR	*erm*(A)	*erm*(B)	*mef*(E)	*tet*(M)	*aphA3*	*int*	*xis*	*tndX*	*tnpA*	*tnpR*
05D058	>256	>256	256	>256	4	1	1	2	4	2	4	0·5	–	+	+	+	–	+	+	–	–	–
05M016	>256	>256	>256	>256	8	1	1	2	4	4	8	2	–	+	+	+	–	+	+	–	–	–
05O173	>256	>256	>256	>256	8	1	1	1	1	1	2	0·5	–	+	+	+	–	+	+	–	–	–
05P107	>256	>256	>256	>256	8	1	1	2	8	4	8	2	–	+	+	+	–	+	+	–	–	–
05P117	>256	>256	>256	>256	8	16	1	2	8	4	8	2	–	+	+	+	–	+	+	–	–	–
05P128	>256	>256	>256	>256	8	8	1	1	4	2	8	2	–	+	+	+	–	+	+	–	–	–
05P286	>256	>256	>256	>256	8	1	1	1	4	4	8	1	–	+	+	+	–	+	+	–	–	–
05P288	>256	>256	>256	>256	8	1	1	1	4	4	8	2	–	+	+	+	–	+	+	–	–	–
05P294	>256	256	256	>256	16	1	1	2	4	2	8	1	–	+	+	+	–	+	+	–	–	–
05P311	256	256	128	>256	8	1	1	2	2	2	8	1	–	+	+	+	–	+	+	–	–	–
05P343	>256	>256	256	>256	8	1	0·5	1	4	2	8	2	–	+	+	+	–	+	+	–	–	–
05O035	1	8	2	16	2	1	0·5	0·25	0·5	0·25	0·5	0·125	–	+	+	+	–	+	+	–	–	–
05P006	1	0·125	2	64	8	1	1	0·016	0·016	0·016	0·016	0·016	–	+	+	+	–	+	+	–	–	–
05O071	>256	>256	>256	>256	8	1	1	1	2	1	2	0·5	–	+	–	+	–	+	+	–	–	–
05K022	8	32	16	128	8	1	1	0·062	0·016	0·016	0·125	0·031	–	+	–	+	–	+	+	–	–	–
05Q056	8	256	16	128	16	1	1	0·062	0·031	0·031	0·062	0·031	–	+	–	+	–	+	+	–	–	–
05P141	8	8	8	64	2	1	1	0·031	0·016	0·062	0·062	0·016	–	+	–	+	–	+	+	–	–	–
05O070	8	256	8	32	2	1	1	0·016	0·016	0·016	0·016	0·008	–	+	–	+	–	+	+	–	–	–

ERY, erythromycin; CLI, clindamycin; CLR, clarithromycin; AZM, azithromycin; TET, tetracycline; LVX, levofloxacin; VAN, vancomycin; PEN, penicillin; AMX, amoxicillin; A/C, amoxicillin–clavulanic acid; CXM, cefuroxime; CTR, ceftriaxone.

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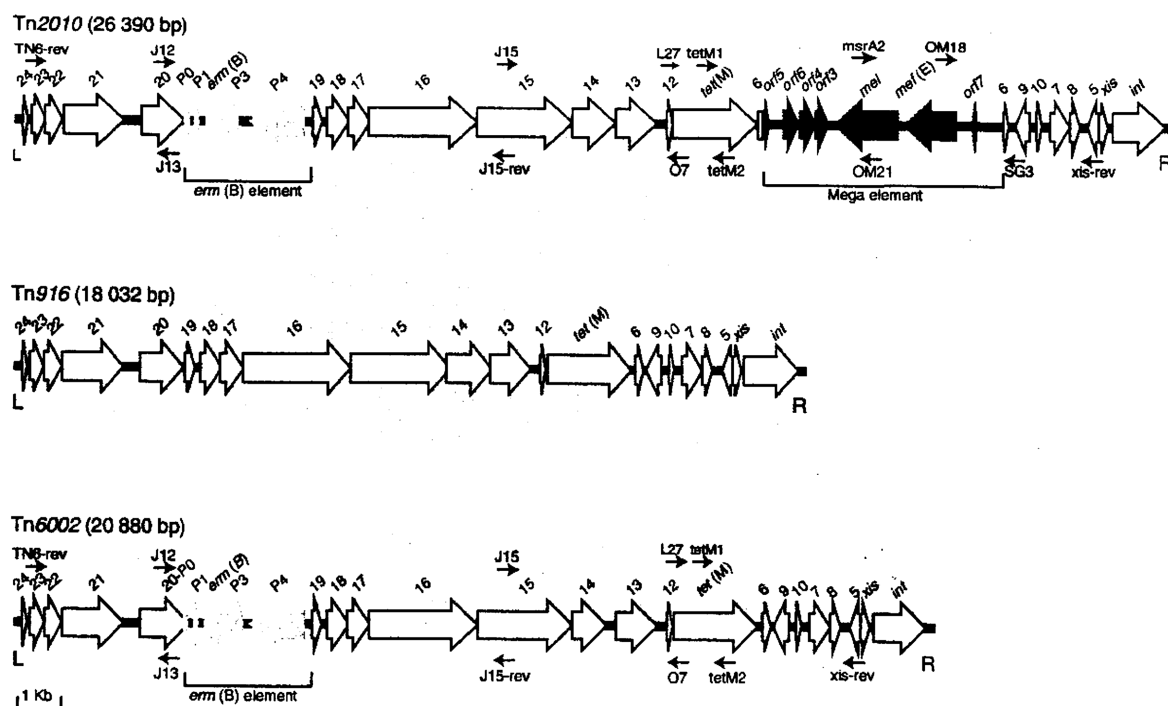

Figure 1 Comparative representation of the ORF maps of Tn*916* and Tn*916* family transposons with Tn*2010*, carrying *erm*(B) and mega element and Tn*6002*, carrying only the *erm*(B) element. The black bar represents Tn*916*, and the white arrows indicate the locations of the relevant ORFs. The light-grey and dark-grey arrows correspond to *erm*(B) and mega elements, respectively. The black arrowheads above and below ORFs show the positions and directions of the primers used to map the transposons.

Tn*6002* (Cochetti *et al.* 2007, 2008; Del Grosso *et al.* 2007). According to the previous reports, the noncoding region between the left end of the *erm*(B) element and the ATG start codon of P0 of the open reading frame (ORF) upstream of the *erm*(B) element span a region of 49 bp in case of Tn*2010* and 48 bp in the case of Tn*6002*, respectively (Fig. 1). The ATG start codon of P0 of the upstream ORF of the *erm*(B) element is located between nucleotides 50 and 52 nucleotide from left end of *erm*(B) element in the case of Tn*2010* and from the corresponding nucleotides 49–51 in the case of Tn*6002*. In Tn*6002*, nucleotide 'A' at the position 43 in the left end of the *erm*(B) element is deleted from Tn*2010*. The insertion of the *erm*(B) element into Tn*916* has resulted in the ORF20 coding sequence of Tn*916* being extended by 19 amino acids in Tn*2010*, and 71 amino acids in Tn*6002*, beyond original C-terminal amino acid of ORF20. The translation stop codon of the extended ORF20 of Tn*6002* is shared with that of P0 of the *erm*(B) element. The complete nucleotide sequence of Tn*6002* is found within these two transposons (Warburton *et al.* 2007). Thus, we compared the sequence of the Tn*2010*-like and Tn*6002*-like elements with that of Tn*6002*, with the exception of the *mega* element region.

Thirteen strains carrying the Tn*2010* element were divided into three groups (A to C) according to the genetic structure of the Tn*2010* element (Table 3, Fig. 2). Eleven type A strains, which were isolated from four different hospitals, belonged to serotype 19F and sequence type ST271. With the exception of one strain designated 05D058, which showed serotype 19A and ST320, this data implied that these strains were clonally related by PFGE analysis, and carried Tn*2010* elements that are identical to the prototype Tn*2010*. Like the *erm*(B) element of Tn*6002*, the Tn*2010* element of another two strains (i.e., 05O035 classed as type B, and 05P006 classed as type C) of the 13 strains had an adenine deletion at the noncoding region seven nucleotides upstream from the P0 open reading frame of the *erm*(B) element of Tn*2010* (Del Grosso *et al.* 2007; Warburton *et al.* 2007). The five strains of Tn*6002* except 05O071 strain of type D and the two strains of Tn*2010*, which had an adenine deletion at the noncoding region seven nucleotides upstream from the P0 open reading frame, showed the low-level resistance to macrolides. These implied that an adenine deletion associated to the low-level resistance to erythromycin. On the other hand, the data that 05P071-carrying Tn*6002* element (type D) had the deletion and still

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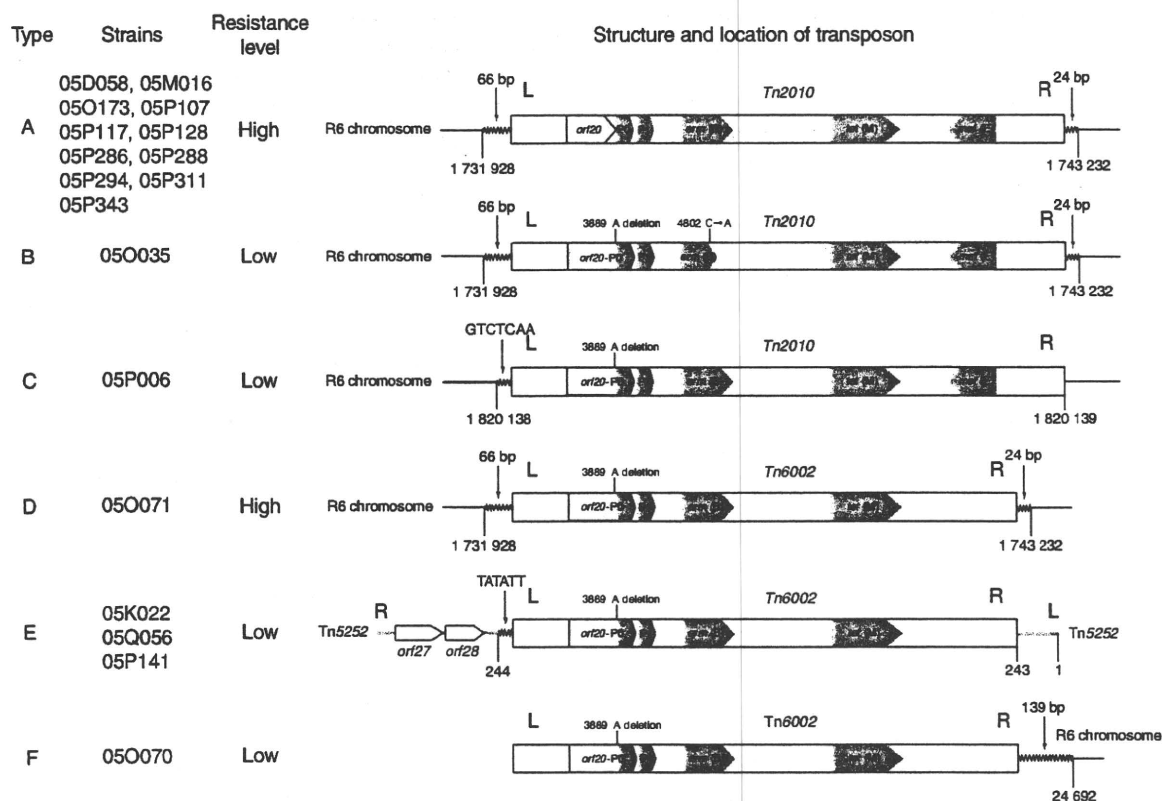

Figure 2 Junctions of Tn*2010* and Tn*6002* with the chromosome or other transposon in the 18 strains tested. 'Type' designated A to F refers to combination types based on the strains' phenotype and genotype of mobile genetic elements (Table 1). Resistance level indicates the erythromycin resistance level, high means MIC values ≥ 128 mg l^{-1} and low means MIC values between 1 to 8 mg l^{-1}. Boxes represent transposons identified in the study and the dark-grey arrows indicate the main resistance genes carrying by the transposons. Light-grey arrows indicate *orf20* of the transposons. One nucleotide 'A' deletion at 3889 position caused the gene fusion between *orf20* and P0, and resultant ORF was designated as *orf20*-P0. The black bar represents the *S. pneumoniae* R6 chromosome, and the light-grey bar represents the transposon Tn*5252*. Thin white arrows indicate the ORFs encoded on Tn*5252*. The numbers under the chromosome and transposons are the positions of the first nucleotides upstream and downstream of the inserts. The zigzag lines indicate exogenous sequences showing no homology with other bacterial sequences. In type F, only the right end of Tn*6002* could be identified, it was not possible to identify the left end.

showed the high-level resistance, also suggested that one adenine nucleotide deletion in *erm*(B) element did not simply relate to the phenotype of low-level resistance to erythromycin. The erythromycin resistance level would be affected by the gene expression, the host factor(s) (i.e. ribosomal RNAs), structure of *erm*(B) or the presence of another resistance determinant. The DNA sequence analysis of each *erm*(B) element of the isolates showed that the type B strain 05O035 had one nucleotide substitution in the *erm*(B) coding sequence (TGC converted to TGA), which resulted in a premature stop codon at amino acid number 161 in the 246 amino acid chain of the wild-type *erm*(B) (Fig. 2) (Tomich *et al.* 1980). These data implied that the low-level resistance to macrolide of strain 05O035 might be because of the nucleotide substitution. To elucidate the relations between the phenotypes and the genotypes in the other isolates, further molecular analyses are needed.

Analysis of the regions flanking from the transposons revealed that all Tn*2010* elements of type A and type B were inserted between 1 731 928 and 1 743 232 bp within the same insertion site as that of the prototype Tn*2010*, where the *orf24* end of Tn*2010* was inserted at the 3′ end of *spr1764* of the *S. pneumoniae* R6 genome and the *int* end of Tn*2010* was inserted between *spr1774* and *spr1775* of the *S. pneumoniae* strain R6, resulting in a deletion of approximately 11 300 bp (Fig. 2) (Hoskins *et al.* 2001; Del Grosso *et al.* 2007). The twelve strains with type A and type B Tn*2010* insertions, which were identical to one of the internationally spread multidrug-resistant pneumococcal clone Taiwan^{19F}-14 (clonal complex CC271). The Tn*2010* element of Taiwan^{19F}-14 strain

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Development of an immunochromatographic assay for the rapid detection of AAC(6′)-Iae-producing multidrug-resistant *Pseudomonas aeruginosa*

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Objectives: To develop an easy-to-use method for the rapid detection of antibiotic-resistant bacteria. Here, a new immunochromatographic assay specific for aminoglycoside 6′-*N*-acetyltransferase AAC(6′)-Iae was designed. AAC(6′)-Iae is a significant marker molecule for multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates in Japan.

Methods: Monoclonal antibodies specific for AAC(6′)-Iae were used to construct the assay. The assessment of the assay was performed using 116 *P. aeruginosa* clinical isolates obtained from hospitals in the Kanto area of Japan where little was known about AAC(6′)-Iae producers. PCR analyses of the *aac(6′)-Iae* and class 1 integron, antimicrobial susceptibility testing and PFGE analysis were performed to characterize positive strains.

Results: The detection limit of the assay was 1.0×10^5 cfu. Of 116 clinical isolates, 60 were positive for AAC(6′)-Iae using the assay. The results of assessment with clinical isolates were fully consistent with those of *aac(6′)-Iae* PCR analyses, showing no false positives or negatives. All positive strains detected by the assay showed MDR phenotypes that were resistant to several classes of antibiotic. PFGE analysis showed that 59 of 60 positive strains tightly clustered, and these included clonal expansions.

Conclusions: The developed assay is an easy-to-use and reliable detection method for AAC(6′)-Iae-producing MDR *P. aeruginosa*. This approach may be applicable for screening and investigation of antibiotic-resistant bacteria as an alternative to PCR analysis.

Keywords: aminoglycoside 6′-*N*-acetyltransferase, molecular epidemiology, rapid diagnosis

Introduction

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* often cause nosocomial outbreaks, and result in life-threatening infections in compromised patients. Hence, the rapid detection of such bacteria is crucial to early infection control to prevent nosocomial infection.

Patterns of bacterial antibiotic resistance are becoming more complex with multiple mechanisms.[1] In *P. aeruginosa* isolates, antibiotic resistance is often due to the production of exogenous enzymes including antibiotic-modifying or -degrading enzymes. Most of these enzyme genes are found in class 1 integrons,[2] and they complicate bacterial antibiotic resistance. Integrons can be transmitted between bacteria via plasmids and transposons; integron carriers therefore often lead to nosocomial outbreaks.

Previously, we identified an aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iae*, from MDR *P. aeruginosa* IMCJ2.S1.[3] Subsequent studies have revealed that *aac(6′)-Iae*-carrying MDR *P. aeruginosa* were isolated in Miyagi, Tokyo and Hiroshima.[3–5] Given that all identified *aac(6′)-Iae* have been linked with bla_{IMP} and *aadA1* in integron In113, AAC(6′)-Iae (responsible for amikacin resistance) might be a significant marker molecule for MDR *P. aeruginosa* in Japan. Two diagnostic methods were developed to detect these strains:[3] a loop-mediated isothermal amplification (LAMP) system using *aac(6′)-Iae*-specific primers; and an agglutination method using anti-AAC(6′)-Iae polyclonal antibody (pAb). Although these methods are highly sensitive, LAMP was time consuming and required specialized tools and well-trained medical technicians. The agglutination assay was found to cause some problems such as inter-rater errors in routine work with many samples.

In this study, we designed a new rapid detection method for AAC(6′)-Iae-producing *P. aeruginosa* using an

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immunochromatographic assay. Here, we report its effectiveness in clinical screening and molecular epidemiology as an alternative to PCR analysis.

Materials and methods

Bacterial strains

P. aeruginosa IMCJ2.S1 was used as positive control strain of *aac(6′)-Iae*. *P. aeruginosa* IMCJ798 carrying *aac(6′)-Iaf*,[6] *P. aeruginosa* IMCJ509 carrying *aac(6′)-Ib* and *Acinetobacter baumannii* A260 carrying *aac(6′)-Iad*[7] were used in the specificity tests of the assay.

Purification of recombinant proteins

aac(6′)-Iae, *-Iaf*, *-Ib* and *-Iad* were amplified with their specific primers from IMCJ2.S1, IMCJ798, IMCJ509 and A260, respectively [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Cloning and protein purification were performed as described previously.[6]

Preparation of monoclonal antibodies

Anti-AAC(6′)-Iae monoclonal antibodies (MAbs) were prepared as described previously.[8] His-AAC(6′)-Iae [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)] was used for immunization and screening of hybridomas by ELISA. The animal experiments were approved by the Ethics Committee for Animal Experiments at the Research Institute of the International Medical Center of Japan.

Assembly of the assay

The assay was assembled as described previously.[8] The composition and principle of the assay are described in Figure S2 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)].

Analytical sensitivity testing of the assay

In the tests using bacteria, 100 μL serial 2-fold dilutions of overnight culture were suspended with 900 μL of extraction buffer (20 mM Tris-HCl, pH 7.5, 1.0% Triton X-100). Then, 100 μL aliquots were applied onto the sample area. In parallel, equivalent bacterial dilutions were also spread onto Mueller–Hinton (MH) agar plates to determine the cfu. The line intensity was quantified using QuantityOne software (Bio-Rad). The mean intensity of triplicate measurements at each point was plotted. The detection limit (*y*-axis) was defined as the intensity greater than the sum of the average values and 3× standard deviation of the endpoint values in the linear standard curve. The cfu (*x*-axis) corresponding to the theoretical detection limit was calculated by the equation in Figure 1(c).

Assessment of the assay

To assess the assay, 116 *P. aeruginosa* clinical isolates were obtained from 13 hospitals located in the Kanto area of Japan where little was known about AAC(6′)-Iae producers. These isolates were associated with nosocomial infections from 2004 to 2009; they include 14 strains from a Tokyo hospital (hospital A in Figure 2) in our previous work.[4] In the assessment, colonies on MH agar were directly picked up with a swab, and were suspended in a soft test tube containing extraction buffer. After lysing cells physically and chemically, four drops of lysate were dropped onto the assay. The results were determined by visual inspection 10 min after applying the samples.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed by the broth micro-dilution method recommended by the CLSI.[9] In this study, MDR *P. aeruginosa* was defined as showing resistance to imipenem (MIC ≥ 16 mg/L), amikacin (MIC ≥ 32 mg/L) and ofloxacin (MIC ≥ 4 mg/L) based on the criteria of the Ministry of Health, Labor, and Welfare of Japan.[10]

PFGE

PFGE assays were performed as described previously.[3]

PCR amplification and DNA sequencing

The *aac(6′)-Iae* and class 1 integrons were amplified with the specific primer sets (Table S1). All amplicons were sequenced to identify their contents with primers listed in Table S1.

Results

Development of immunochromatography specific for AAC(6′)-Iae

We obtained three MAbs with high reactivity to the recombinant His-AAC(6′)-Iae from *P. aeruginosa* IMCJ2.S1. Western blotting analysis and ELISA using the subtype AAC(6′)-I proteins of AAC(6′)-Iaf, -Ib and -Iad identified in clinical isolates in Japan showed that two MAbs, 1H7 and 3F12, specifically recognized AAC(6′)-Iae (Figure S1). Thus, these MAbs were utilized for the assembly of the immunochromatography assay (Figure S2). The assembled assay worked in the preliminary test using 0.5 μg of His-AAC(6′)-Iae prepared in PBS (Figure 1a).

Sensitivity testing using IMCJ2.S1 indicated that a clear line appeared with $>1.2\times10^5$ cfu of bacteria (Figure 1b), whereas reference lines appeared in all cases. The intensity of the test line was correlated with the number of bacteria in the range 5.8×10^4 to 4.6×10^5 cfu, indicating a high degree of linearity ($r^2=0.9766$) (Figure 1c). The theoretical detection limit for bacteria was 1.0×10^5 cfu per test.

The assay was also evaluated using strains carrying the subtype *aac* gene (Figure 1d) and the subtype proteins of AAC(6′)-Iaf, -Ib and -Iad (Figure 1e). Test lines did not appear with any proteins or bacteria, whereas reference lines appeared in all cases.

Assessment of the assay using clinical isolates

The assessment of the assay using 116 *P. aeruginosa* clinical isolates revealed that 60 (52%) of the 116 isolates were positive. These results were fully consistent with those of *aac(6′)-Iae* PCR analyses, indicating that the developed assay has no false positives or negatives. As for the source of positive strains, isolates from urine were the most frequent, followed by those from sputum.

Characterization of detected AAC(6′)-Iae-positive isolates

Antimicrobial susceptibility testing indicated that all positive strains showed an MDR phenotype. In addition to imipenem, amikacin and ofloxacin, effective increases in MICs of piperacillin,

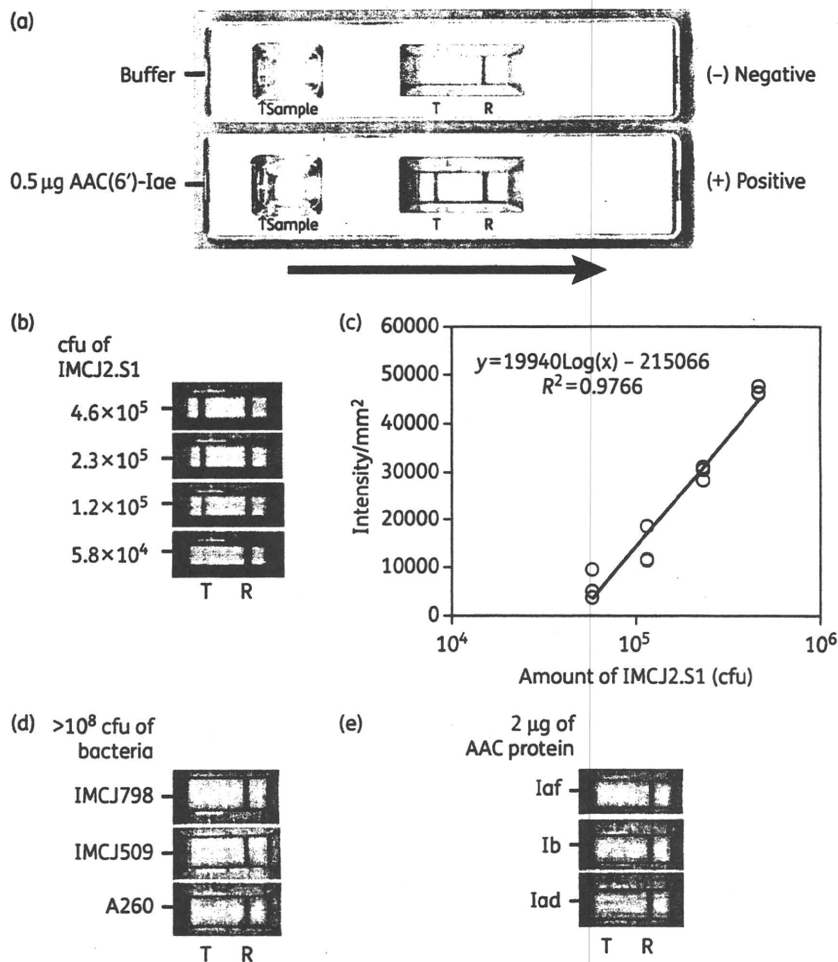

Figure 1. (a) Immunochromatography using MAbs 1H7 and 3F12. In negative cases, a single line appears at the position of the reference line (R) only. In positive cases, another line also appears at the position of the test line (T) in addition to the reference line. The arrow indicates the direction of lateral flow. (b) Sensitivity testing using positive control strain IMCJ2.S1. (c) Relationship between IMCJ2.S1 amount and the intensity of each test line. (d) Specificity testing using an excess amount of bacteria carrying the subtype *aac* gene. (e) Specificity testing using an excess amount of the subtype AAC(6′) proteins.

piperacillin/tazobactam, ceftazidime, aztreonam and meropenem were observed for most positive strains. In contrast, arbekacin, gentamicin and polymyxin B showed relative antibiotic potency towards positive strains.

PFGE assay of the tested 116 isolates revealed that the genetic lineages of AAC(6′)-Iae-positive strains were relatively similar to the IMCJ2.S1 strain previously isolated in Miyagi and outbreak-associated strains in a Tokyo hospital (hospital A, Figure 2).

The genetic environments of *aac(6′)-Iae* were determined by PCR and DNA sequencing. Forty-eight (80%) of 60 strains carried bla_{IMP-1}, *aac(6′)-Iae* and *aadA1* in their integron; these were identical to In113 in IMCJ2.S1. In the other 12 (20%) positive strains, bla_{IMP-1} was replaced with bla_{IMP-10} due to substitution of guanine by thymine at position 145 in bla_{IMP-1}. However, the 59 bp element of the bla_{IMP-10} cassette was identical to bla_{IMP-1} in In113.

All positive strains showed serotype O:11.

Discussion

Several immunochromatographic assays have been developed to identify various infectious agents such as influenza virus.[8] Most of these target their secretory proteins and cellular components. In this study, we designed an immunochromatographic assay to detect AAC(6′)-Iae-producing MDR *P. aeruginosa*. To our knowledge, this is the first report of immunochromatography using antibodies specific for a molecule that confers antibiotic resistance to bacteria. Immunological diagnosis can utilize antibodies against antigens of interest. Therefore, this approach could serve as a model for other molecules involved in antibiotic resistance.

The analytical sensitivity of the assay was 10^5 cfu/test when bacterial lysate of the positive control strain IMCJ2.S1 was used. This is a sufficient detection limit, because the colony counts of the used samples ranged from 10^8 to 10^9/test when the nearly equal amounts of colonies were analysed to determine cfu (data not shown). Most AAC(6′)-Iae-positive strains

Figure 2. PFGE assay of 116 *P. aeruginosa* clinical isolates used in the assessment of the developed immunochromatography assay. The asterisk indicates the *P. aeruginosa* IMCJ2.S1 strain previously found in Miyagi. Areas enclosed within squares indicate the positive isolates detected.

detected were isolated from urine and sputum. Further work is needed to evaluate the assay using such clinical specimens. Additionally, considering that antibiotic resistance genes can be transmitted between different kinds of Gram-negative bacteria via plasmids and transposons, the developed assay might also allow the detection of AAC(6′)-Iae in other Gram-negative species involved in nosocomial infections.

AAC(6′)-Iae producers were found to be newly detected from hospitals in five prefectures of Gunma, Saitama, Kanagawa, Chiba and Ibaraki, following the previous reports of *P. aeruginosa* carrying *aac(6′)-Iae* in Miyagi, Tokyo and Hiroshima.[3–5] These AAC(6′)-Iae producers showed a similar genetic background; some of them were spread clonally. But it must also be noted that PFGE patterns of some negative strains were similar to those of positive strains (Figure 2). These observations suggest that strains with similar genetic backgrounds acquired resistance via a small mobile element. As such, the analysis of class 1 integrons indicated that all positive strains carried an In113 or In113-derived integron. However, the mode of transmission of In113 is still unknown. Further analysis is required to examine whether In113 is plasmid encoded.

All positive isolates showed MDR phenotypes. These data strongly demonstrate that AAC(6′)-Iae plays a crucial role as a marker molecule for MDR *P. aeruginosa* in Japan. But not all MDR *P. aeruginosa* isolates could be detected using the present assay. Actually, 21 (37%) of 56 negative strains showed MDR phenotypes; the number was coincident with amikacin resistance (data not shown). Furthermore, 16 (76%) of 21 negative strains were positive for both *aac(6′)-Ib* and bla_{IMP-1}; these strains were found to have caused the clonal nosocomial infection in hospital M (Figure 2). Immunochromatography can adopt multiple test lines in the current assay. Further work is in progress to design an immunochromatography assay targeting AAC(6′)-Ib and metallo-β-lactamase IMP.

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Transparency declarations

None to declare.

Supplementary data

Table S1, Figure S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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