4）診断

H5N1感染は、ウイルス分離、RT-PCR法により診断される。

インフルエンザA／H5N1は、通常のインフルエンザに比べ、鼻腔より咽頭で高頻度、高濃度に検出される[9]。咽頭におけるウイルス検出は、発症から2～15日間（中央値5.5日）程度に認められている。インフルエンザウイルス迅速診断キットによるH5N1感染の検出感度は、RT-PCRより低いとされており、タイの研究ではウイルス培養陽性例の36%がインフルエンザウイルス迅速キットで陽性であった。

5）症例検出とマネージメント

動物のH5N1感染が流行する国や地域すべての急性呼吸不全患者、特に患者が家禽に曝露されている場合には、ヒトのインフルエンザA（H5N1）感染の可能性を考慮すべきである[9]。

しかしながら、本症の初期症状が非特異的であること、他の原因による急性呼吸器感染症も多いことから、早期の症例検出は困難である。ウイルスRNAの検出は有用であるが、実験室診断には①ウイルス培養、②インフルエンザA（H5N1）RNAのPCR陽性、③蛍光抗体法陽性、④ペア血清によるH5抗原に対する少なくとも4倍以上の抗体上昇のうち、一つかそれ以上の陽性所見が必要である。

H5N1疑い症例、確定症例については、隔離下において観察、検査、治療を実施する。

6）予防ワクチン

最近になって、フランスで行われたインフルエンザA／Vietnam／1194／2004（H5N1）の不活化HAワクチンにアルミニウムアジュバントを併用したフェーズⅠスタディの結果と、中国で行われた同ウイルス株の不活化全粒子ワクチンにアルミアジュバントを併用したフェーズⅠスタディの結果が報告された[17][18]。アジュバントを併用したHAワクチン、不活化全粒子のいずれによっても、2回接種により高い安全性と免疫応答が認められている。

本邦においても、日本のワクチンメーカーによりアルミアジュバントを併用した不活化全粒子ワクチンが開発され、その免疫原性が確認されている。2007年10月、我が国においてもH5N1ワクチンが製造認可された。しかしながら、このワクチン株（Vietnam／1194／04）はClade 1に属し、最近インドネシアや中国で分離されているClade 2（subclade 1-3）ウイルスに対しては、効果が減弱することが予想される。

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Antimicrobial Susceptibility and Genetic Characteristics of *Streptococcus pneumoniae* Isolates Indicating Possible Nosocomial Transmission Routes in a Community Hospital in Japan[▽]

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A clinical study was designed to study *Streptococcus pneumoniae* isolates recovered from a community hospital in Japan from April 2001 to November 2002. A total of 73 isolates were defined as derived from inpatient, outpatient, and hospital staff groups. The MIC results showed that 20 strains (27.4%) were susceptible to penicillin G, 39 strains (53.4%) had intermediate resistance, and 14 strains (19.2%) had full resistance. Low susceptibility to macrolides was also detected: 32.9%, 32.9%, and 34.2% of all strains were resistant to erythromycin, clarithromycin, and azithromycin, respectively. Thirty strains (41%) were resistant to at least two different kinds of antibiotics. Nineteen disparate serotypes were detected besides two nontypeable strains, and the predominant serotypes were 19F and 23F. Pulsed-field gel electrophoresis (PFGE) pattern A was dominant in the serotype 19F group; this pattern was similar to that of the international clone Taiwan 19F. A total of 10 different patterns were detected in the 23F group and were distinguishable from those of the international clones Spain 23F and Taiwan 23F. Pattern b strains were identified in the same ward, and pattern d strains were found both in patients with nosocomial pneumococcal infections (NPI) and in outpatients. In conclusion, drug-resistant *S. pneumoniae* was spreading rapidly, especially isolates of the serotype 19F and 23F groups. PFGE data revealed interpatient transmission and suggested that there might be some association between NPI patient strains and outpatient strains.

Streptococcus pneumoniae is the most common pathogen of respiratory diseases, often leading to pneumonia, pharyngitis, sinusitis, otitis media, septicemia, and meningitis (1). Many studies have reported *S. pneumoniae* as one of the leading causes of community-acquired pneumonia (CAP) worldwide (2, 6, 19, 31, 32, 41) as well as in Japan (13). On the other hand, nosocomial pneumococcal infections (NPI) result in longer hospitalizations and higher mortality rates because of increased antimicrobial resistance induced after long-term antibiotic therapies (7, 23, 34, 35). *S. pneumoniae* also plays a very important role in nosocomial infections of the pulmonary tract and blood, which are more and more widely recognized (3, 8, 29, 34, 35). Age, previous hospitalization, and previous antimicrobial therapy have been thought to contribute to penicillin-resistant pneumococcal infection in nosocomial outbreaks (18, 27). Previous studies conducted to determine the antimicrobial susceptibilities of isolates from NPI found that a high percentage of pneumococcal pathogens had multidrug resistance, including resistance to new quinolones (4, 5, 28, 39). Masaki et al. reported a possible relationship between the pulsed-field gel electrophoresis (PFGE) patterns of *Moraxella catarrhalis* isolates from hospital- and community-acquired respiratory infections in a community hospital (20). Yet there still is not sufficient evidence to determine whether a similar pattern exists for *S. pneumoniae*.

Since pneumonia ranks as the fourth leading cause of death in Japan, it remains a very high priority to investigate the antimicrobial susceptibilities of *S. pneumoniae* isolates recovered from CAP or NPI and their genetic relatedness, in order to detect potential transmission routes. In the present study, antimicrobial susceptibilities were determined by examining MICs as well as the serotypes for all the isolates. Because PFGE has been reported as a suitable method for confirming interstrain genetic relatedness (16, 17, 21), it was used in this study to analyze and compare the molecular profiles of isolates from CAP and NPI patients.

MATERIALS AND METHODS

Setting. Tagami Hospital, a community hospital affiliated with Nagasaki University, is located in Nagasaki, Japan. There are 180 beds, 5 floors, and 9 wards in Tagami Hospital, including the outpatient department (first floor), 41 surgery beds (second floor, wards 2W and 2E), 53 internal medicine beds (third floor, wards 3W and 3E), and long-term-care wards with 86 beds (fourth floor, 4W and 4E, 42 beds; fifth floor, 5W and 5E, 43 beds).

Participants. Altogether, 51 patients and 5 hospital staff members were included in this research from April 2001 to November 2002 (19 months). Of 56 participants, 22 were female and 34 were male (ratio, 0.65), and the mean age was 67.6 years (range, 17 to 97). Patients' clinical records were reviewed. Forty-one patients (80.4%) had chronic obstructive pulmonary diseases, six patients were diagnosed with acute bronchitis, three patients were diagnosed with CAP, and only one patient had adenoiditis/pharyngitis. In this study, five hospital staff members took full responsibility for the medical treatment and had the highest frequency of contact with the recruited patients.

Nosocomial pneumococcal infection was defined according to the CDC rec-

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TABLE 1. Distribution of MICs for 73 strains of *S. pneumoniae*[a]

Antibiotic	No. of isolates for which the MIC (μg/ml) is:															
	≤0.004	0.008	0.016	0.032	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	≥128
PCG			6	7	7	2	13	7	17*	12	2					
CTRX			2	5	1	3	16	18	18	3*	4	3				
CDTR			2	4	2	15	15	16	12	1*	6					
IPM	1	2	12	9	6	19	16	7		1	*					
EM			2	24	12		3	8*	2	3	3	1		1	1	13
CAM		1	3	26	12		5	2*	4	4	1			1	2	12
AZM				6	21	10	4	4	3*	1	4	1	3	1		15
LVFX								7	33	26*	4	1	1	1		
VCM					1	5	24	40	3		*					

[a] Asterisks indicate the breakpoint for each antibiotic according to CLSI criteria.

ommendations (9): briefly, as pneumococcal infection that was demonstrated ≥72 h after admission, excluding those patients who were suspected of having pneumococcal diseases present or in incubation at admission. Pneumonia was diagnosed if there was an appearance of a new abnormal shadow and likely infiltration on a chest roentgenogram and if at least two of the following clinical and laboratory findings were present: fever (temperature, >37.8°C), cough, production of purulent sputum, dyspnea, and leukocytosis (leukocyte count, >10,000/ml).

Bacterial strains. A total of 73 isolates of pneumococci were recovered from 56 adult participants. The sources of the isolates were as follows: sputum (n = 62), blood (n = 1), bronchoalveolar lavage fluid (n = 1), nasal cavity (n = 1), and pharynx (n = 8). Culture plates were incubated overnight under a 5% CO_2 atmosphere, and optochin sensitivity and bile solubility tests were performed to confirm *S. pneumoniae*.

Serotyping and antimicrobial susceptibility test. Isolates were serotyped by capsular swelling (Quellung reaction) observed microscopically after suspension in pneumococcal typing antisera (Statens Serum Institut, Copenhagen, Denmark).

MICs were determined by an agar dilution method according to CLSI (formerly NCCLS) guidelines (25). Serial twofold dilutions of each antibiotic (ranging from 0.008 to 128 μg/ml) were prepared. Mueller-Hinton agar supplemented with 5% horse blood was used as the culture medium. Approximately 0.01 ml (10^5 CFU/ml) of a bacterial suspension of each isolate was inoculated onto antibiotic-containing agar and incubated overnight at 37°C. The MIC of each antibiotic was defined as the lowest concentration that prevented visible bacterial growth. All isolates were tested for susceptibility to the following nine antibiotics: penicillin G (PCG) (Meiji Seika Kaisha, Tokyo, Japan), ceftriaxone (CTRX) (Chugai Pharmaceutical Co., Tokyo, Japan), cefditoren (CDTR) (Meiji Seika Kaisha), imipenem (IPM) (Banyu Pharmaceutical Co., Tokyo, Japan), erythromycin (EM) (Dainippon Pharmaceutical Co., Osaka, Japan), clarithromycin (CAM) (Taisho Pharmaceutical Co., Tokyo, Japan), azithromycin (AZM) (Pfizer Japan Inc., Tokyo, Japan), levofloxacin (LVFX) (Daiichi Pharmaceutical Co., Tokyo, Japan), and vancomycin (VCM) (Shionogi Co., Osaka, Japan). Penicillin-susceptible *S. pneumoniae* (PSSP) was defined as strains for which the penicillin MICs were <0.125; penicillin-intermediate *S. pneumoniae* (PISP) and penicillin-resistant *S. pneumoniae* (PRSP) were defined by MICs of ≥0.125 and ≤1 and by MICs of ≥2, respectively.

PFGE. PFGE was performed on all 73 strains. The Spanish multidrug-resistant serotype 23F clone (ATCC 700669), the Taiwan multidrug-resistant serotype 19F clone (ATCC 700905), and the Taiwan multidrug-resistant serotype 23F clone (ATCC 700906) were used as reference standards. Strains were grown overnight in brain heart infusion broth, and PFGE of SmaI chromosomal digests was performed as described previously (42). DNA banding patterns were interpreted according to the criteria of Tenover et al. (37): a difference of more than three bands in the profile was needed to distinguish between PFGE types.

RESULTS

Antimicrobial susceptibility test. The MICs for the 73 strains are shown in Table 1. According to the CLSI breakpoint criteria, 20 strains (27.4%) appeared susceptible to PCG (MIC, ≤0.063) (PSSP), 39 strains (53.4%) showed intermediate resistance (MIC, 0.125 to 1) (PISP), and 14 strains (19.2%) showed full resistance (MIC, ≥2) (PRSP). Low susceptibility to macrolides was also detected—32.9%, 32.9%, and 34.2% of all strains were resistant to EM, CAM, and AZM, respectively—while the strains showed relatively high susceptibility to all the cephems. Surprisingly, all isolates were fully susceptible to IPM and VCM. In addition, as many as 7 (9.6%) strains were resistant to LVFX.

Serotyping. Aside from two nontypeable strains, 19 disparate serotypes were detected (Fig. 1), mainly serotypes 3 (4.1%), 6A (6.8%), 23A (6.8%), 6B (8.2%), and the predominant serotypes 19F (10 strains [13.7%]) and 23F (25 strains [34.2%]). Within the inpatient group, 17 of 23 strains (73.9%) belonged to serotypes 19F and 23F, compared to 38.1% for the outpatient group ($P < 0.05$ by the chi-square test). Additionally, only two strains of 19F, but no 23F strains, were detected in the staff group. Furthermore, of 73 strains, 52 (71.2%) were

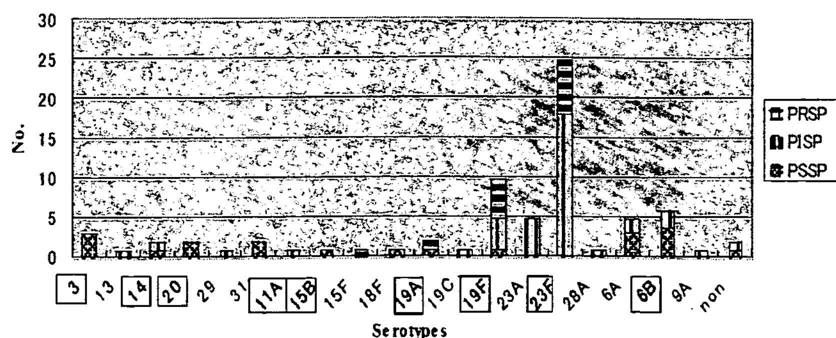

FIG. 1. Serotype distribution plotted against penicillin susceptibility. Boxed serotypes were covered by PS23.

TABLE 2. Characteristics of multidrug-resistant strains and details of serotype 19F and 23F strains

Strain	Serotype	Resistance[a] to the following antibiotic:								
		PCG	CTRX	CDTR	IPM	EM	CAM	AZM	LVFX	VCM
PSSP (n = 8)										
S-1	14					O	O	O		
S-2	3					O	O	O		
S-3	Nontypeable					O		O		
S-4	19F					O	O	O		
S-5	6B					O	O	O		
S-6	6B					O	O	O		
S-7	6B					O	O	O		
S-8	3					O	O	O	O	
PISP (n = 11)										
I-1	23F					O	O	O		
I-2	19F					O	O	O		
I-3	23A					O	O	O	O	
I-4	23A					O	O	O		
I-5	Nontypeable					O	O	O		
I-6	23F					O		O		
I-7	6B					O	O	O		
I-8	23F					O	O			
I-9	23F		O	O				O		
I-10	23F		O	O					O	
I-11	19F					O	O	O		
PRSP (n = 11)										
R-1	19F	O				O	O	O		
R-2	23F	O	O	O						
R-3	23F	O	O	O						
R-4	23F	O	O	O						
R-5	23F	O				O	O			
R-6	19F	O				O	O	O		
R-7	23F	O				O	O	O		
R-8	23F	O						O		
R-9	19F	O							O	
R-10	15F	O	O	O		O	O	O	O	
R-11	19F	O				O	O	O		

[a] Designated by "O".

found to be covered by a 23-valent polysaccharide vaccine (PS23).

Characteristics of multidrug-resistant strains. Thirty strains (41%) were found to be resistant to at least two different antibiotics examined in the present study, in which strains were described as PSSP, PISP, or PRSP according to their susceptibilities to penicillin (Table 2). Macrolides elicited the highest resistance: 83.3% of strains were resistant to EM, CAM, or AZM, or to all three. Seven strains (23.3%) appeared to be simultaneously resistant to penicillin and macrolides. One

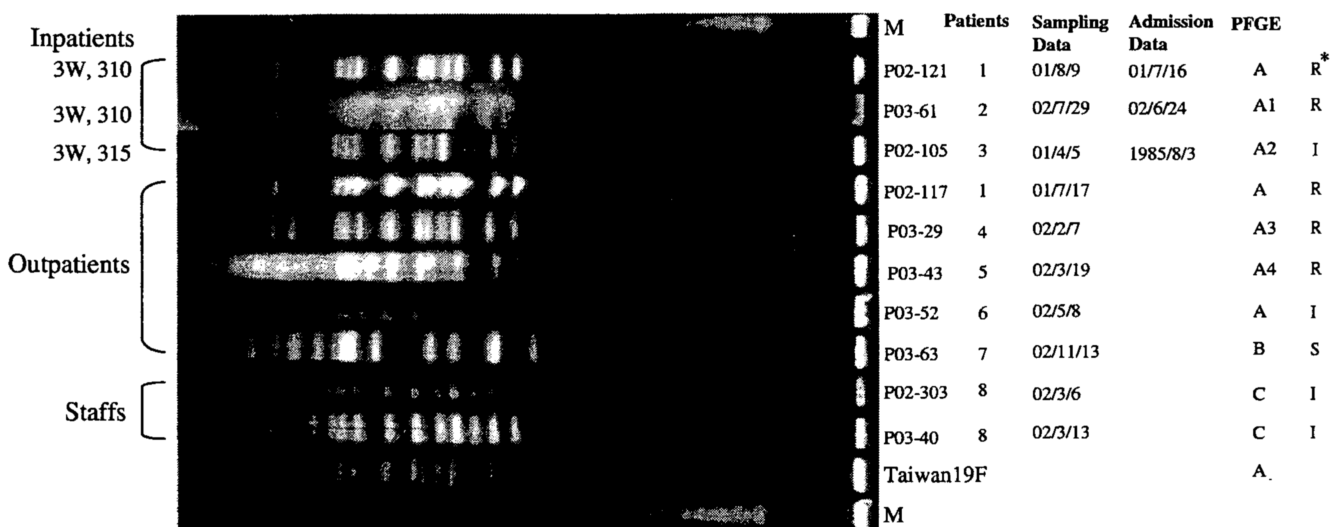

FIG. 2. PFGE results for SmaI-digested DNA from serotype 19F strains and a representative of the multidrug-resistant clone Taiwan 19F. M, molecular size marker; I, PISP; R, PRSP. Asterisk indicates PCG susceptibility.

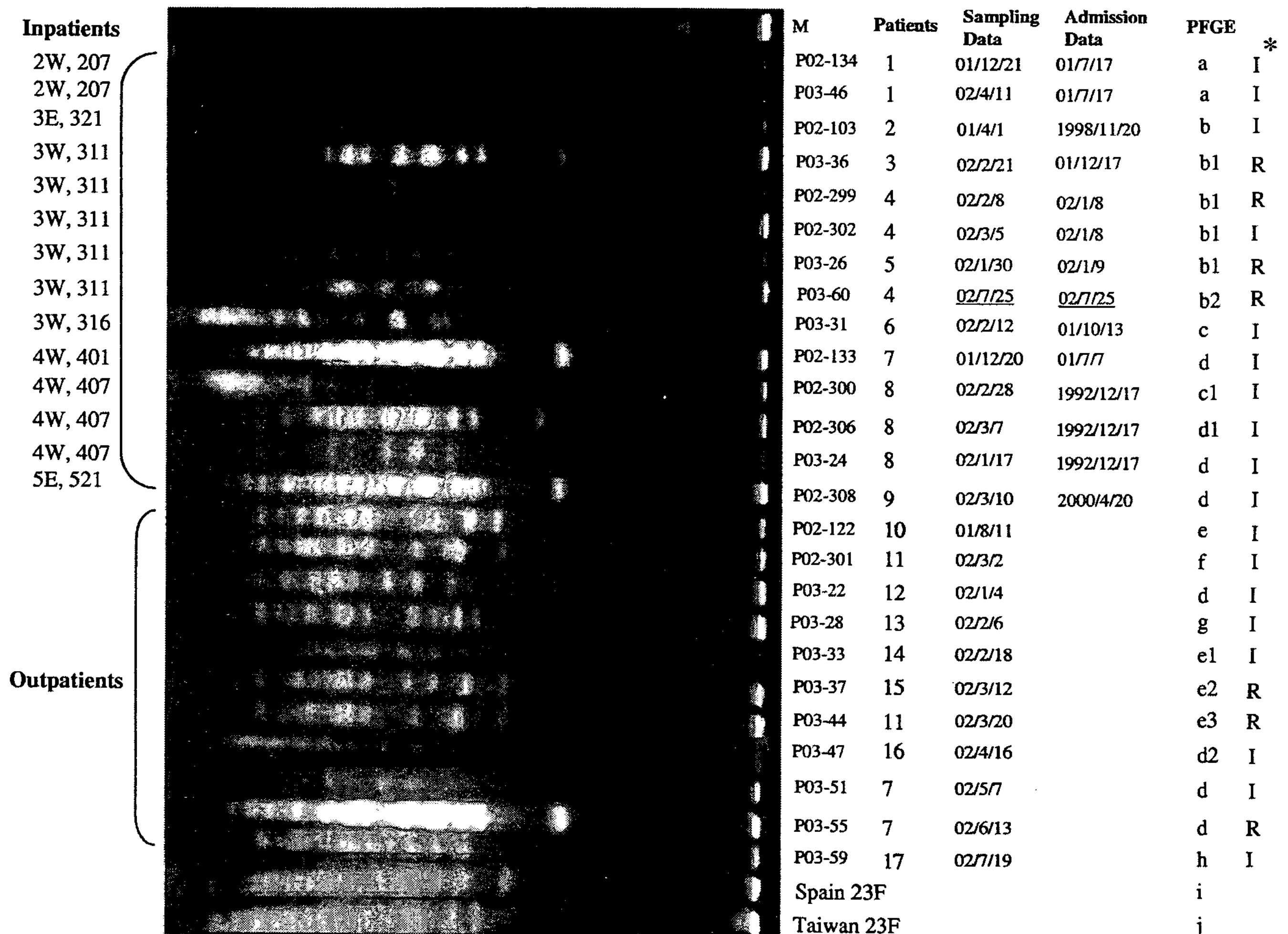

FIG. 3. PFGE results for SmaI-digested DNA from serotype 23F strains and the multidrug-resistant clones Spain 23F and Taiwan 23F. M, molecular size marker; I, PISP; R, PRSP. Asterisk indicates PCG susceptibility.

serotype15F strain was uniquely resistant to penicillin, cephems, and macrolides as well as fluoroquinolone, indicating a very wide spectrum of antimicrobial resistance. Seven and 11 strains in the serotype 19F and 23F groups, respectively, were multidrug resistant. In the serotype 19F group, PSSP and PISP strains showed the same pattern of resistance to all the macrolides, while PRSP strains expressed variance. Although the serotype 23F group showed results similar to those seen with the 19F group, five strains were proven to be resistant to cephems, whereas no such resistance has been found in the serotype 19F group yet (Table 2).

Molecular characteristics by PFGE. PFGE was performed on all 73 strains, and the DNA patterns showed tremendous diversity (data not shown). In the serotype 19F group, three DNA patterns were detected among 10 strains (Fig. 2). Seven strains showed pattern A, which was similar to the pattern of the internationally spreading Taiwan 19F clone; two strains had pattern C; and only one strain showed pattern B. Three of the pattern A strains were from inpatients who were regarded as NPI patients and resided in different wards, and the others were from outpatients. The strains with pattern C were both from the same member of the hospital staff. The pattern B strain, isolated from an outpatient, was the only PSSP strain in the serotype 19F group.

In the serotype 23F group, including predominant DNA patterns b and d, a total of 10 different patterns were detected (Fig. 3). All six pattern b strains were from inpatients residing in the same ward except for one patient, and three of these strains were isolated from the same patient on different dates. Furthermore, except for P03-60, all these strains belonged to NPI patients. As many as eight strains showed pattern d; of these, four were from inpatients defined as having NPI and the others were from outpatients. Strains P02-306 and P03-24 were isolated from the same inpatient, while P03-51 and P03-55 were from the same outpatient. The internationally spreading clones Spain 23F and Taiwan 23F each showed a unique pattern that had no relatedness to the patterns of any of the 23F clinical isolates in this study.

DISCUSSION

Drug-resistant *S. pneumoniae* has spread widely in the world since the 1970s, with the increased resistance expanding to a wider spectrum of antibiotics (1, 12, 15, 22, 33). In this study,

we found that 72.6% and 83.3% of all strains were not susceptible to penicillin and macrolides, respectively, numbers that were very close to the findings of previous studies (14, 30). One possible cause of the increase in drug resistance is long-term antimicrobial therapy (15). To date it has been thought that the priority for clinicians is to choose the appropriate chemotherapy for patients, usually with underlying diseases. The strains in this study showed the most susceptibility to IPM and VCM, a finding consistent with the results of a previous national survey in Japan (30). These findings suggested that the application of IPM and VCM might sometimes be effective in empirical therapy of pneumococcal infection with the prescription of β-lactam antibiotics.

We also found that the multidrug-resistant strains were relatively concentrated in serotypes 3, 14, 6B, 19F, 23A, and 23F. This suggested that strains of these serotypes may acquire drug resistance more easily than strains of other serotypes. Fortunately, these serotypes were covered by PS23, except for the serotype 23A strain. The vaccine coverage rate was 71.2% in this study, which indicated that preventive use of pneumococcal vaccine would be an effective measure to reduce morbidity and mortality in cases of pneumococcal infection, especially in the elderly.

As is well known, the *pbp1a*, *pbp1b*, *pbp2x*, *pbp2a*, *pbp2b*, and *pbp3* genes encode penicillin-binding proteins (PBPs), and altered *pbp1a*, *pbp2x*, and *pbp2b* genes play a very important role in inducing penicillin resistance in *S. pneumoniae* by decreasing the affinity for β-lactam antibiotics (10, 24, 40). On the other hand, it has been reported that the *erm*(B) gene, which encodes the 23S rRNA methylase, and the *mef*(A) gene, which is assumed to encode the efflux pump system, are the predominant mechanisms of macrolide resistance (36, 38). No analyses of these genes as an alternative application of PFGE for detecting the transmission route were performed in this study. Although in the field of molecular epidemiology, the incidence of mutation in these genes often gives us a hint for explaining the interlinks in the genetic transmission of a pathogen, which is useful for explaining the modification and spread of antibiotic resistance characteristics, PFGE is thought to be one of the most discriminating fingerprinting methods for verifying interstrain genetic relatedness, and in comparison to other frequently used methods such as amplified fragment length polymorphism or multilocus sequence typing, PFGE uniquely analyzes the whole chromosomal DNA and is comparatively cost-effective (11, 26).

The PFGE patterns of all 73 strains showed significant diversity based on their serotypes (data not shown). Since serotype 19F and 23F strains constituted the majority, molecular analysis focused on such strains. We found genetic relatedness for most of the serotype 19F strains isolated from either NPI patients or outpatients, and the pattern was undistinguishable from that of the Taiwan 19F clone. Our previous national survey had already demonstrated that the Taiwan19F clone was spreading widely in Japan (30), which suggested that the causative pathogen spreading between NPI patients and outpatients might have originated from the same parent strain. A previous study indicated that there was some association between hospital- and community-acquired respiratory infections caused by *Moraxella catarrhalis* (20). In this study, we detected pattern A in the serotype 19F group in both NPI patients and outpatients, as well as pattern d in the serotype 23F group, indicating that some association existed between NPI patients and outpatients even in pneumococcal infection.

There was an NPI outbreak caused by pattern b strains in ward 3W, 311, which proved that the pathogen can spread from patient to patient under crowded conditions. Although strains P03-31 and P02-300 and strains P02-306 and P02-308 were isolated from NPI patients on separate floors, they expressed the same PFGE patterns, c and d, respectively. Why did strains isolated from different backgrounds express similar molecular characteristics? One possible reason is that the pathogen may be spread by patients or hospital staff members. We found some evidence to support the assumption of interpatient transmission. It was a pity that we failed to prove direct transmission between hospital staff members and patients in this study. Since PFGE is the suitable fingerprinting method, it appears to be necessary to use a larger sample size and a more accurate research design in a future study.

In conclusion, the prevalence of drug-resistant *S. pneumoniae* was still high, and the predominant serotypes were 19F and 23F. PFGE data demonstrated that the Taiwan 19F clone was spreading widely and that the Spain 23F and Taiwan 23F clones were not spreading in Japan, but some unique clones of 23F were. The data also suggested that there was some association between isolates from NPI patients and those from outpatients. Finally, we demonstrated possible NPI transmission from patient to patient due to crowded conditions. Further work should be done to expose the possible transmission route between hospital staff members and patients.

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Two hydrophobic segments of the RTN1 family determine the ER localization and retention

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Abstract

Reticulon (RTN) proteins are localized to the endoplasmic reticulum (ER), and are related to intracellular membrane trafficking, apoptosis, inhibiting axonal regeneration, and Alzheimer's disease. The RTN proteins are produced without an N-terminal signal peptide. Their C-terminal domain contains two long hydrophobic segments. We analyzed the ER localization signal of human RTN1-A. Mutant proteins lacking the first (39 residues) or second (36 residues) hydrophobic segment showed ER localization. On the other hand, the mutant lacking both hydrophobic segments was cytosolic. Enhanced green fluorescent protein (EGFP) tagged with the first or second hydrophobic segment of RTN1-A was localized to the ER. These results suggest that each hydrophobic segment determines the ER localization. In addition, EGFP tagged with the truncated form of the first hydrophobic segment exhibited the localization to the Golgi rather than the ER. This suggests that the length of the hydrophobic segment contributes to the ER retention of RTN1-A.
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Keywords: Reticulon; RTN1-A; Localization; Endoplasmic reticulum; ER retention

Reticulon (RTN) family genes and RTN-like genes are widely distributed in eukaryotes [1]. Four reticulon family genes have been identified in human [2–5]. The RTN family proteins localize to the endoplasmic reticulum (ER) predominantly [6]. Their N-terminal regions are highly variable, and the variety is seemed to contribute to interaction with various proteins involved in apoptosis, intracellular membrane trafficking, and Alzheimer's disease [7–11]. RTN4-B/Nogo-B (formerly called RTN-XS) interacts with Bcl-XL and Bcl-2 to reduce their anti-apoptotic activity [7]. The RTN1 family proteins interact with AP50 and SNARE proteins, and regulate endocytosis and exocytosis [9,10]. Furthermore, interaction of four RTN families with BACE1 decreases the production of amyloid-β [11]. The RTN protein is synthesized without an N-terminal signal peptide. The ER localization signal of the RTN protein has not been identified. The RTN proteins share a C-terminal reticulon homology domain (RHD) containing two long hydrophobic segments, HS1 and HS2. The RTN1 family consists of RTN1-A, B, and C. The RHD of the RTN1 family consists of 188 amino acid residues and is critical for association with the ER membrane [6]. HS1 and HS2 consists of 39 and 36 amino acid residues, respectively, and are unusually long as the transmembrane domain. Recently, a unique membrane topology of RTN4-C has been reported [12]. HS1 of RTN4-C forms a hairpin structure in the membrane, indicating the cytosolic orientation of a loop region (66 amino acid residues), which lies between HS1 and HS2. This is coincident with that the RTN proteins are not glycosylated though many RTN proteins have a N-linked glycosylation motif (N-*X*-S/T) in the loop region. Because only N-linked glycosylation sites located in the ER lumen are glycosylated [13], N-linked glycosylation has been applied to topology assays of the membrane proteins [14,15]. The topology

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of HS2 remains uncertain. The long stretch and the unique topology of HS1 and HS2 might have relation to the ER localization of the RTN protein.

In this study we analyzed the ER localization of human RTN1-A in CV-1 cells. Our results suggest that both HS1 and HS2 of the RTN1 family determine the ER localization, and that the length of the hydrophobic segment contributes to the ER retention.

Materials and methods

Construction of expression plasmids. The cDNAs for deletion mutants of human RTN1-A (GenBank Accession No., NM_021136) were constructed by polymerase-chain reaction (PCR) using Ex *Taq* DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) and a cDNA for RTN1-A [9] as a template. Each PCR product was digested with *Bam*HI and *Sal*I, and inserted into pCMV-Tag2C (Stratagene, La Jolla, CA). The full-length cDNA for ST6Gal I sialyltransferase (GenBank Accession No., NM_173216) was obtained by PCR using a human lymph node MATCHMAKER cDNA library in pACT2 (Clontech, Palo Alto, CA) and primers 5′-ATAAAGCTTGCCACCATGATTCACACCAACCTGA AGAAAAAG-3′ and 5′-ATAGGATCCCGGCAGTGAATGGTCCG GAAGCCAG-3′. The PCR product was digested with *Hin*dIII and *Bam*HI, and introduced to pEGFP-N1 (Clontech), which had been opened with the same enzymes. The cDNAs for HS1 (amino acids 600–638) and HS2 (amino acids 705–740) with flanking amino acid residues (WRDIK and RIYKS for HS1; VDSLK and KHQAQ for HS2) were synthesized using primers corresponding to the desired regions, digested with *Eco*RI and *Kpn*I, and introduced to pEGFP-C3 (Clontech), respectively. The cDNA for SALH, a fused hydrophobic segment consisting of the signal-anchor (amino acids 5–26) of ST6Gal I sialyltransferase and the latter half of HS1 (amino acids 619–638) with following amino acid residues (RIYKS), was synthesized, digested with *Eco*RI and *Kpn*I, and introduced to pEGFP-C3.

Transfection of CV-1 cells and fluorescence microscopy. CV-1 cells were cultured in 35-mm dishes in 2 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2 air. Transfection was performed using Lipofectamine (Invitrogen, Carlsbad, CA) and 1 μg of each plasmid for 6 h. Transfection reagents were removed and the cells were incubated in fresh medium for 18 h. The cells expressing the FLAG-tagged proteins were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 min. The cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and further incubated in blocking solution (PBS containing 0.2% gelatin and 0.1% Triton X-100) for 30 min. The cells were treated with 2 μg/ml anti-FLAG M2 monoclonal IgG (Sigma, St. Louis, MO) in blocking solution for 1 h. After washing with blocking solution three times, the cells were incubated with 5 μg/ml Alexa488-labeled anti-mouse IgG (Molecular Probes, Eugene, OR) in blocking solution for 1 h. The cells were washed with blocking solution three times and then with PBS three times. Fluorescence images were obtained using a microscope (Zeiss, Jena, Germany). The cells expressing EGFP constructs were fixed as described above, washed with PBS, and subjected to fluorescence microscopy.

Results and discussion

Two hydrophobic segments of RTN1-A determine the ER localization

To characterize the ER localization of human RTN1-A, FLAG epitope-tagged constructs including mutants, in which HS1 (amino acids 600–638 of RTN1-A) and/or HS2 (amino acids 705–740) in the RHD (Fig. 1A) were deleted as shown in Fig. 1B, were constructed. These constructs were expressed in CV-1 cells and the localization was examined by immunocytochemistry. FLAG-tagged RTN1-A, a control for ER localization [9], ΔHS1, and ΔHS2 were localized to the ER, whereas ΔHS1-2 showed cytosolic localization (Fig. 1C). These results suggest that at least one hydrophobic segment is required for the ER localization of RTN1-A and that there is no other region involved in association with the ER membrane. Next we examined whether the hydrophobic segments could direct a soluble protein to the ER. We constructed EGFP tagged with HS1 or HS2 in its C-terminal extension (Fig. 2A). EGFP showed the fluorescent signal in the nucleus and the cytoplasm. On the other hand, EGFP-HS1 and EGFP-HS2 exhibited the ER localization (Fig. 2B). Taken together, these results suggest that both HS1 and HS2 determine the ER localization.

The length of the hydrophobic segment is a determinant for ER localization

HS1 (39 residues) and HS2 (36 residues) are unusually long as the transmembrane segment (~20 residues). This promoted us to examine whether the length of the hydrophobic segment is critical for the ER localization of RTN1-A. We constructed EGFP tagged with the former (FH; amino acids 600–619 of RTN1-A) or latter (LH; amino acids 619–638) half of HS1 (Fig. 3A). As shown in Fig. 3B, EGFP-FH and EGFP-LH accumulated in the perinuclear region with a minor presence in the ER (Fig. 3B). The perinuclear region seemed to be the Golgi complex. To confirm the Golgi localization of EGFP-FH and EGFP-LH, CV-1 cells expressing the constructs were treated with nocodazole, which induces microtubule depolymerization and the fragmentation and dispersion of the Golgi. ST6Gal I fused to EGFP (ST6Gal I-EGFP), which had been shown to localize to the Golgi [16], was used as a control for the Golgi localization. In the presence of nocodazole, the accumulation of the perinuclear fluorescence of the constructs disappeared, and punctate structures dispersed throughout the cytoplasm were observed (Fig. 3C). These results suggest that the truncated form of HS1 in EGFP-FH or EGFP-LH could not retain the construct in the ER, and therefore, the construct is transported to the Golgi. EGFP-SALH, an EGFP construct tagged with a fused hydrophobic segment consisting of the signal-anchor (amino acids 5–26) of ST6Gal I sialyltransferase and the latter half of HS1, showed the ER localization (Fig. 3A and B). There were few punctate structures observed in the nocodazole-treated cells expressing EGFP-SALH (Fig. 3C). The fused hydrophobic segment consists of uncharged 37 residues, and its length is the middle of HS1 (39 residues) and HS2 (36 residues). Taken together, these results suggest that the length of the hydrophobic segment of RTN1-A determine the ER localization and retention.

In the present study, we analyzed the ER localization of RTN1-A. Our results provide evidence that two hydrophobic segments, HS1 and HS2, determine the ER localization

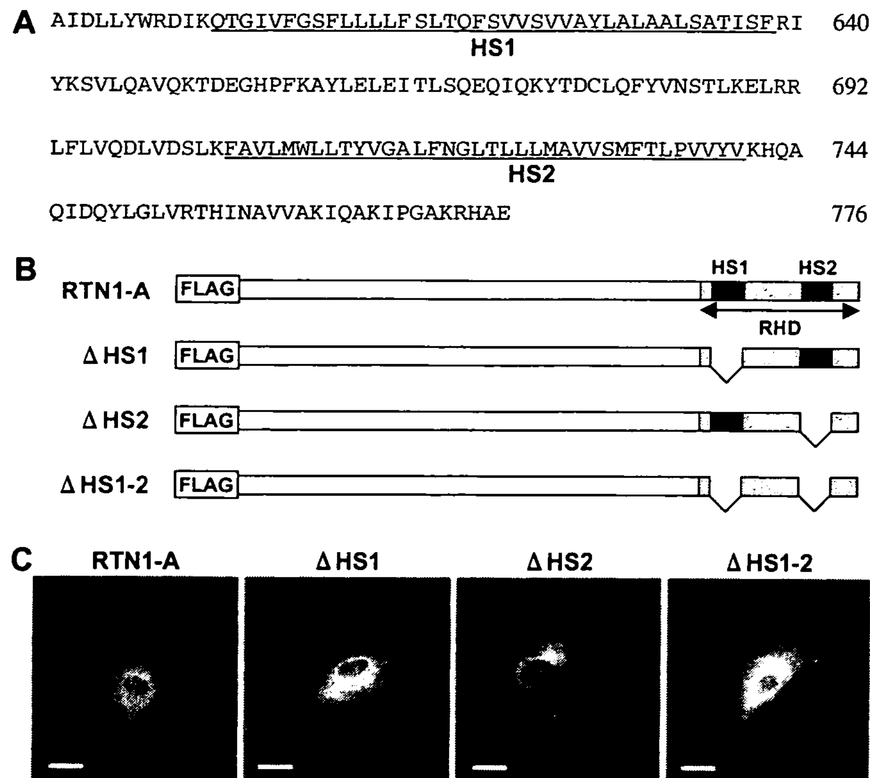

Fig. 1. Localization of wild-type RTN1-A and deletion mutants in CV-1 cells. (A) The amino acid sequence of the RHD of human RTN1-A. HS1 and HS2 are underlined. (B) Schematic structure of FLAG epitope-tagged RTN1-A and the hydrophobic segment-deleted mutants. (C) CV-1 cells expressing the indicated constructs were analyzed by immunocytochemistry using anti-FLAG M2 monoclonal IgG and Alexa488-labeled anti-mouse IgG. Fluorescent images were obtained using a fluorescence microscope. Bars, 10 μm.

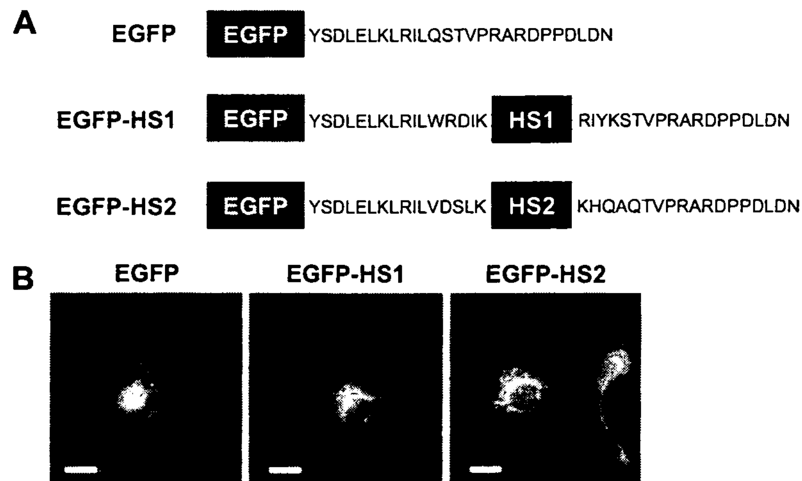

Fig. 2. Localization of EGFP tagged with the hydrophobic segment to the ER. (A) Schematic presentation of HS1- and HS2-tagged EGFP constructs. pEGFP-C3 (Clontech), a mammalian expression vector, harbors a cDNA for EGFP with a C-terminal extension consisting of 26 amino acid residues. HS1 and HS2 were inserted into the extension, respectively. (B) CV-1 cells expressing the indicated constructs were fixed and fluorescent images were obtained using a fluorescence microscope. Bars, 10 μm.

of the protein individually. ΔHS1-2 mutant protein lacking both HS1 and HS2 showed cytosolic localization, indicating that ΔHS1-2 has no primary sequence region that can associate with the ER membrane. It has been reported that several families of transmembrane ER proteins contain dilysine-based ER retention motifs (KK*XX*-COOH) in their cytosolic tails [17]. The RHD shared by three RTN1 family proteins has a putative ER retention motif (KRHAE) at its C-terminus. EGFP constructs tagged with HS1 or HS2 could localize predominantly to the ER, suggesting that the C-terminal retention motif is dispensable for the ER retention. This result is coincident with that mouse

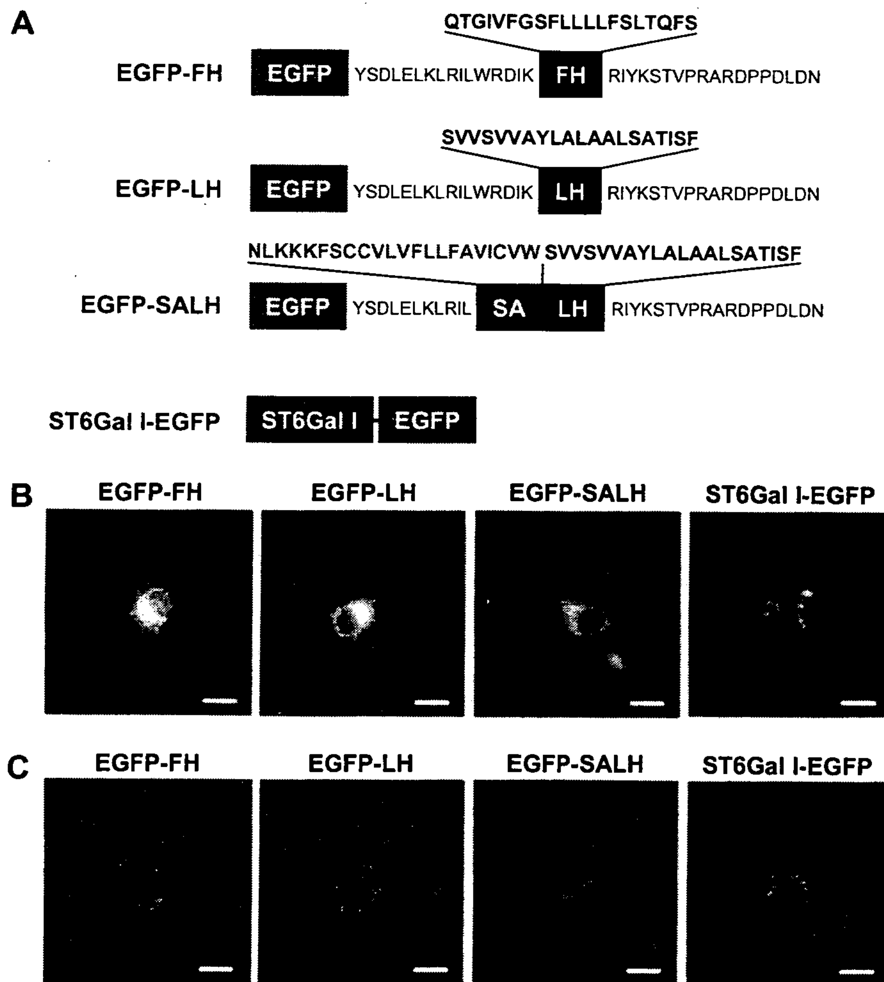

Fig. 3. The length of the hydrophobic segment is a determinant for ER localization. (A) Schematic presentation of EGFP constructs tagged with the truncated or fused hydrophobic segment. In EGFP-SALH, the former half of HS1 and preceding five amino acid residues (WRDIK) were replaced with a signal-anchor (SA; amino acids 5–26) of ST6Gal I. SA consists of 17 uncharged residues preceded by five residues (NLKKK). (B) Accumulation of EGFP-FH and EGFP-LH to the Golgi. CV-1 cells expressing the indicated constructs were fixed, and fluorescent images were obtained using a fluorescence microscope. ST6Gal I-EGFP was used as a control for Golgi localization. (C) Effect of nocodazole on the localization of EGFP-FH and EGFP-LH. Before fixation, CV-1 cells expressing the indicated constructs were treated with 20 μg/ml nocodazole (Sigma) for 2 h. Bars in (B) and (C), 10 μm.

RTN3 fused to N-terminus of EGFP localizes to the ER [18]. It has been reported that the transmembrane domains of mouse CD8β and yeast Gas1p precursor contain the ER localization and retention signals [19,20]. The length and hydrophobicity of the C-terminal transmembrane domain of Gas1p precursor modulates the ER retention. In the case of rat Tom20, a mitochondrial outer membrane protein, increase of the length or hydrophobicity of the N-terminal transmembrane domain induces localization shift of Tom20 from mitochondria to secretory compartments, the ER, the Golgi, and the plasma membrane [21]. Thus, these findings demonstrate the participation of the transmembrane domain and the hydrophobic segment in selection of a targeting pathway and the intracellular localization. HS1 and HS2 of the RTN1 family are nearly twice as long as the usual transmembrane domain. EGFP-FH and EGFP-LH could not remain in the ER and accumulated in the Golgi. The length of the hydrophobic segments in EGFP-FH and EGFP-LH are 20 amino acid residues, a normal size as the transmembrane domain. It has been suggested that long hydrophobic segments of RTN4-C, DP1, and caveolin-1 form a hairpin structure [12,22]. RTN4-C and DP1 localize to the ER. Caveolin-1 accumulates in the ER and then targets to lipid droplets. We propose that the hydrophobic segments of the RTN1 family, which might also form a hairpin structure, determine the ER localization and retention.

The RTN proteins are produced without an N-terminal signal peptide. The signal peptide in the nascent polypeptide destined for the ER membrane is recognized by a signal recognition particle [23], and the translocation of the polypeptide across the ER membrane occurs cotranslationally [24]. The targeting and integration of the RTN proteins to the ER membrane might be posttranslational. To learn how the RTN proteins are targeted to the ER in detail, *in vitro* experiments will be required. There are interaction

proteins of the RTN1 family [9–11,25,26]. RTN-interacting proteins would possibly interfere with binding of the targeting factors to the RTN protein in the *in vitro* ER targeting assay. Judging from the results obtained in this work, factors involved in the ER targeting of the RTN protein would recognize only the hydrophobic segment, and other primary sequence regions of the RTN protein are dispensable. Thus, soluble proteins tagged with the hydrophobic segment of the RTN protein (e.g. EGFP-HS1 and EGFP-HS2) would be ideal substrates for the *in vitro* experiments.

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SHORT REPORT

Antimicrobial susceptibility and genetic characteristics of *Haemophilus influenzae* isolated from patients with respiratory tract infections between 1987 and 2000, including β-lactamase-negative ampicillin-resistant strains

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SUMMARY

The minimum inhibitory concentration (MIC) of five antibiotics and the presence of resistance genes was determined in 163 *Haemophilus influenzae* isolates collected over 13 years (1987–2000) in four two-yearly sampling periods from patients with respiratory tract infections. The prevalence of β-lactamase-negative ampicillin-susceptible strains was approximately 80% over the sampling period although fewer strains (65·9%) were recovered in the period 1995–1997. TEM-1 type β-lactamase-producing strains were less frequent starting at 15·6% and declining to 2·2% in the final sampling period. Low-β-lactamase-negative ampicillin-resistant (BLNAR) strains were uncommon in 1987–1989 (2·2%), peaked to 19·5% in 1995–1997, but fell back to 11·1% by 2000. Fully BLNAR strains were not detected until the last sampling period (6·7%). The MICs of ampicillin, levofloxacin, cefditoren and ceftriaxone remained stable but there was an eight-fold increase in the MIC of cefdinir over the sampling period. Pulsed-field gel electrophoresis of DNA digests showed that three representative BLNAR strains were genetically distinct and 11 DNA profiles were identified among 17 low-BLNAR strains. These data suggest that the number of genetically altered BLNAR and low-BLNAR strains are increasing in Japan.

Haemophilus influenzae can cause a variety of infections, including otitis media, bronchitis, pneumonia and meningitis [1, 2]. In the past, the activity of two β-lactamases, TEM-1 and ROB-1, accounted for almost all isolates with decreased susceptibility to ampicillin [3]. At present, the global prevalence of β-lactamase-negative ampicillin-resistant (BLNAR) *H. influenzae* remains low [4, 5], but the proportion of clinical BLNAR isolates is rapidly increasing, and has now reached more than 20% in Japan [6]. The characteristics of antimicrobial resistance of these strains are a serious concern for clinical prescribing. BLNAR strains have a resistance mechanism that decreases the affinity of ampicillin for penicillin-binding proteins (PBPs) [7]. The resistance phenotypes are classified according to substitutions at three positions of the *ftsI* gene which mediates septal peptidoglycan sysnthesis allowing the classification of strains as BLNAR or low-BLNAR by PCR [8].

In total, 163 strains of *H. influenzae* were isolated from sputum from patients with respiratory tract infections in Nagasaki University and its affiliated hospitals. These strains were selected at random and were divided into four groups, 45 strains between 1987 and 1989, 32 between 1991 and 1993, 41 between 1995 and 1997, and 45 between 1998 and 2000. Strains were capsule typed by slide agglutination with antisera (Difco Laboratories, Detroit, MI, USA) and

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Table. *Annual changes of the prevalence of each resistance class and antimicrobial susceptibility to five antibiotics*

Year	BLNAS[a]	BLPAR[b]	Low-BLNAR[c]	BLNAR[d]		MIC80 of five antibiotics (μg/ml)				
						AMP[e]	CFX[f]	CFR[g]	CFN[h]	LFX[i]
A 87–89	82·2	15·6	2·2	0	Total	0·5	0·008	0·25	0·016	0·032
					BLNAS	0·5	0·008	0·25	0·016	0·032
					BLPAR	16	<0·004	0·5	0·016	0·032
					Low BLPAR	1·0	0·008	0·5	0·016	0·032
B 91–93	78·1	6·3	15·6	0	Total	0·5	0·008	0·5	0·016	0·032
					BLNAS	0·5	0·008	0·25	0·016	0·032
					BLPAR	32·0	<0·004	0·25	0·008	0·032
					Low BLPAR	1·0	0·016	1·0	0·016	0·032
C 95–97	65·9	12·2	19·5	0	Total	0·5	0·008	1·0	0·016	0·032
					BLNAS	0·5	0·008	0·5	0·016	0·032
					BLPAR	32·0	<0·004	0·5	0·016	0·032
					Low BLPAR	1·0	0·016	1·0	0·032	0·032
D 98–00	80·0	2·2	11·1	6·7	Total	1·0	0·016	2·0	0·032	0·032
					BLNAS	0·5	0·016	1·0	0·032	0·032
					BLPAR	4·0	<0·004	0·13	0·008	0·063
					Low BLPAR	1·0	0·016	1·0	0·063	0·032
					BLNAR	1·0	0·125	8·0	0·125	0·032

[a] β-lactamase-negative ampicillin susceptible strains. [b] β-lactamase-producing ampicillin-resistant (TEM-1 type) strains. [c] Low β-lactamase-negative ampicillin-resistant strains. [d] β-lactamase-negative ampicillin-resistant strains. [e] Ampicillin. [f] Ceftriaxone. [g] Cefdinir. [h] Cefditoren. [i] Levofloxacin.

β-lactamase production was detected using a nitrocefin-impregnated disk (Becton Dickinson, Sparks, MD, USA). The minimum inhibitory concentration (MIC) of five antibiotics was determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards [9]. The antibiotics were: ampicillin (Meiji Seika Kaisha, Tokyo, Japan), levofloxacin (Daiichi Pharmaceutical Co., Tokyo), cefditoren (Meiji Seika Kaisha), cefdinir (Astellas Pharma Inc., Tokyo) and ceftriaxone (Chugai Pharmaceutical Co., Tokyo).

PCR was performed to identify resistance genes using a multiplex assay as described previously [8]. Four sets of primers were obtained from Wakunaga Pharmaceutical Co. (Hiroshima, Japan): P6 primers to amplify the P6 gene which encodes the P6 membrane protein specific for *H. influenzae*; TEM-1 primers to amplify a part of the bla_{TEM-1} gene; PBP3-S primers to identify an Asn526→Lys amino-acid substitution in the *ftsI* gene; and PBP3-BLN primers to identify an Asn526→Lys and Ser385→Thr amino-acid substitution in the *ftsI* gene.

Pulsed-field gel electrophoresis (PFGE) was performed as described previously [10] using *SmaI* digestion (Takara Shuzo Co., Shiga, Japan) and electrophoresis in a CHEF Mapper PFGE system (Bio-Rad Life Science Group, Hercules, CA, USA) was carried out at 6 V/cm with switch times of 0·47 and 63 s, and a run-time of 20 h. After staining with ethidium bromide, the interpretation of PFGE patterns was based on the criteria described by Tenover *et al.* [11]. Briefly, PFGE patterns were classified into four groups: identical in profile = indistinguishable; 1–3 bands difference = closely related; 4–6 bands difference = possibly related; and >7 bands difference = different.

The Table shows that of the 45 strains isolated from 1987 to 1989, 37 (82·2%) were classified as β-lactamase-negative ampicillin-susceptible (BLNAS) strains, seven strains produced TEM-1-type β-lactamase and were ampicillin resistant (BLPAR) and one strain was classified as low-BLNAR by PCR. The proportion of BLNAS strains fell in the ensuing two sampling periods but recovered in the final time period to 80%. The frequency of BLPAR strains fluctuated from 15·6% in the initial period through 6·3% and 12·2% to 2·2% in the final sampling period. Similar variation was observed for low-BLNAR strains with just 2·2% of strains expressing this phenotype in the first period but rising to almost 20% in the third period before falling back to 11%. Three BLNAR strains (6·7%) were detected only in the fourth sampling period. The respective MIC_{80} values (μg/ml) for the four periods against the strain collection

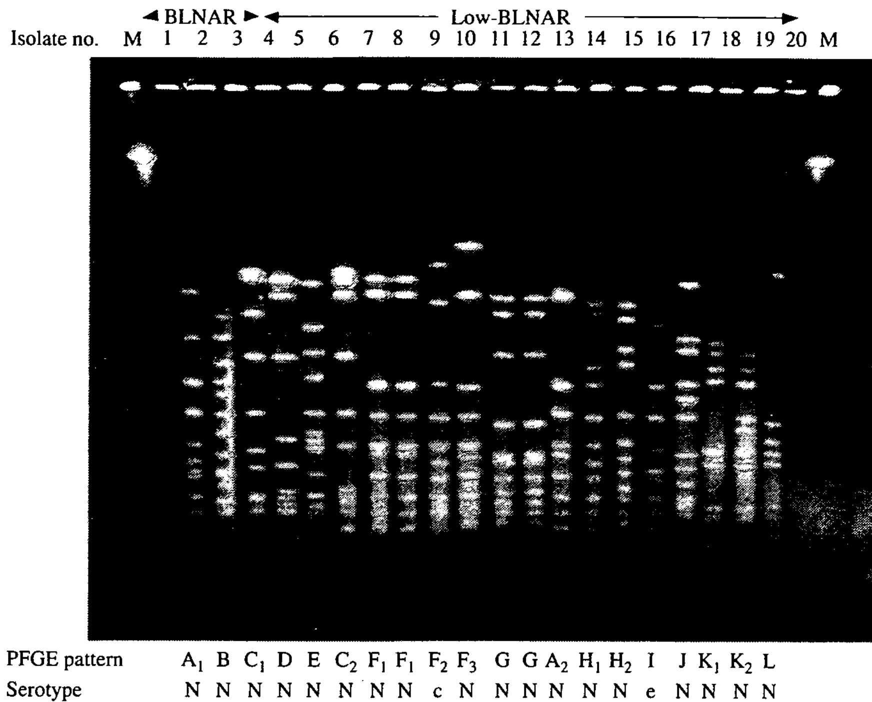

Fig. PFGE patterns of *Sma*I-digested DNA from three BLNAR and 17 low-BLNAR *H. influenzae* strains. Lanes 1–3, BLNAR strains; lanes 4–20, low-BLNAR strains. Coding of the PFGE patterns depicting group and subgroups and serotype status. N, Non-typable.

is also shown in the Table. The NCCLS susceptibility/resistant break-points for *H. influenzae* are 1 μg/ml for ampicillin, 2 μg/ml for levofloxacin, 1 μg/ml for cefdinir, and 2 μg/ml for ceftriaxone. The MIC of ampicillin, ceftriaxone, cefditoren and levofloxacin remained constant or within one doubling concentration over the years but resistance to cefdinir increased by eight-fold over the sampling period (Table).

The three BLNAR strains gave distinct DNA profiles in PFGE while 11 profiles were distinguished among the 17 low-BLNAR strains. Two of these profiles exhibited some similarities to profiles found in fully BLNAR strains. Three pairs of strains each exhibited similar patterns and four strains were grouped within the same pattern (Fig.). All but two of the 20 strains were non-typable with capsular antisera.

H. influenzae is one of the important pathogens associated with respiratory tract infections and thus acquisition of antimicrobial resistance raises concern. The prevalence of BLNAR strains was reported to be 2·4% in the United States between 2002 and 2003 [12], 1·3% in France in 1999 [13], and 9·3% in Spain between 1998 and 1999 [14]. Nevertheless, their global prevalence remains relatively low. However, BLNAR strains are spreading rapidly with increasing frequency in Japan with reported prevalence rates of 14·9% between 1996 and 1997 [15], and 23·1% between 1998 and 1999 [6], although BLNAR was identified by only MIC in these reports. We report here a prevalence of BLNAR strains of 6·7% by PCR between 1998 and 2000. An understanding of the characteristics of antimicrobial resistance of *H. influenzae*, especially BLNAR strains, is important not only for prescribing clinicians but also for formulation of practicable chemotherapy guidelines. Although *H. influenzae* strains are generally susceptible to the early cephalosporins [5, 12], the BLNAR and low-BLNAR strains recovered here showed a marginal increase in MIC to two of the three cephalosporins tested and an eight-fold increase in MIC of cefdinir which is consistent with a previous report from Japan [6].

PFGE of DNA macrorestriction fragments is a sensitive fingerprinting method for *H. influenzae* and this method was used by Karlowsky *et al.* [4] to demonstrate clonal dissemination of BLNAR strains in the United States between 2000 and 2001. However, the BLNAR and low-BLNAR strains found here displayed a variety of genetic backgrounds. It has previously been reported that *H. influenzae*, including resistant strains, can be transmitted at day-care centres or in the home [13, 16], and this may

be one reason for the spread of BLNAR strains in Japan. We did observe that some low-BLNAR strains isolated from different patients had similar PFGE patterns and therefore must consider that such strains could potentially spread in a community. Ongoing monitoring of *H. influenzae* resistance determinants is thought to be important and may help to predict how this organism responds to current antimicrobial regimens [17]. Despite the limitations of this pilot study, particularly the small sample size, it shows the value of surveillance of antimicrobial resistance levels and their genetic determinants in *H. influenzae* and further surveys specifically of BLNAR strains in the wider Japanese community should be undertaken to inform antimicrobial prescribing policy.

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DECLARATION OF INTEREST

None.

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旅行医学とトラベルクリニック

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