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Abstract

The prevalence of penicillin non-susceptible Streptococcus pneumoniae (PNSSP) is increasing among isolates from acute otitis media (AOM). Repeated episodes of antibiotic exposure are a well-known risk factor for the isolation of PNSSP although otitis-prone or recurrent AOM cases frequently require repeated courses of antibiotic treatment. In order to evaluate the chronological alteration of S. pneumoniae during recurrences of AOM, strains of S. pneumoniae were isolated from 11 patients, each of whom had experienced 2-4 episodes of AOM, were examined. Every bacterial specimen obtained from a single episode of recurrent AOM was examined by PCR-based penicillin-binding protein (PBP) assay, serotyping, and amplified fragment length polymorphism (AFLP), then compared to other samples from the same case. Two cases (18.2%) showed strain diversity during repeated antibiotic treatments by serotyping or PBP-assay. By AFLP analysis, 6 cases (54.5%) demonstrated heterogeneous strains during recurrent AOM. Clonal survivors of previous episodes of AOM were not always the cause of subsequent episodes of AOM, even in otitis-prone cases.

KEYWORDS: acute otitis media, amplified fragment length polymorphism(AFLP), penicillin non-susceptible Streptococcus pncumoniac.

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Original Article

Genetic Alteration of Penicillin Non-Susceptible *Streptococcus pneumoniae* Observed throughout Recurrence of Acute Otitis Media Detected by Amplified Fragment Length Polymorphism Analysis

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The prevalence of penicillin non-susceptible *Streptococcus pneumoniae* (PNSSP) is increasing among isolates from acute otitis media (AOM). Repeated episodes of antibiotic exposure are a well-known risk factor for the isolation of PNSSP although otitis-prone or recurrent AOM cases frequently require repeated courses of antibiotic treatment. In order to evaluate the chronological alteration of *S. pneumoniae* during recurrences of AOM, strains of *S. pneumoniae* were isolated from 11 patients, each of whom had experienced 2-4 episodes of AOM, were examined. Every bacterial specimen obtained from a single episode of recurrent AOM was examined by PCR-based penicillin-binding protein (PBP) assay, serotyping, and amplified fragment length polymorphism (AFLP), then compared to other samples from the same case. Two cases (18.2%) showed strain diversity during repeated antibiotic treatments by serotyping or PBP-assay. By AFLP analysis, 6 cases (54.5%) demonstrated heterogeneous strains during recurrent AOM. Clonal survivors of previous episodes of AOM were not always the cause of subsequent episodes of AOM, even in otitis-prone cases.

Key words: acute otitis media, amplified fragment length polymorphism (AFLP), penicillin non-susceptible *Streptococcus pneumoniae*.

Streptococcus pneumoniae is the most common causative pathogen of acute otitis media (AOM), accounting for 35% to 45% of all AOM cases [1]. *S. pneumoniae* was once highly susceptible to penicillin; however, recent epidemiological studies have demonstrated that penicillin non-susceptible *S. pneumoniae* (PNSSP) (Minimal Inhibitory Concentration: MIC $\geqq$ 0.1 μg/ml) now causes a large proportion of pneumococcal AOM cases [2, 3]. A recent survey in the United States indicated that the proportion of invasive disease caused by PNSSP ranged from 8% to 34% [4]. The prevalence of PNSSP is even greater in Japan: PNSSP was detected in 43.5% of the cases of acute infection caused by *S. pneumoniae* in 1996 [5]. Suetake also reported that penicillin-resistant *S. pneumoniae* (PRSP) was isolated from 62% of *S. pneumoniae* in the AOM of children below the age of 2 [6]. The PNSSP endemic is now an emerging health problem.

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Two major risk factors have already been reported for the development of PNSSP: day-care attendance and a recent history of antibiotic exposure [4, 7]. Repeated antibiotic exposure increases the risk of nasopharyngeal carriage of PNSSP 2-5 times [7]; Dagan *et al.* [8] and Leach *et al.* [9] also reported that MIC of the *S. pneumoniae* contained in the nasopharyngeal carriage was dramatically changed after antibiotic treatment. Most of these reports basically depend on biological markers including serotypes and antibiogram. The determinant of these markers, however, can be transmitted by horizontal gene transfer in the case of *S. pneumoniae* [10]. Thus the change of the biological markers themselves might not be direct evidence of the alterations of the clones [11].

The aim of this study is to examine the alterations of *S. pneumoniae* throughout recurring AOM by using molecular typing methods. For this evaluation, we applied newly developed a PCR-based genotyping procedure, amplified fragment length polymorphism (AFLP) [12], which has recently been used for DNA fingerprinting [13]. This technique offers a higher level of discrimination and more rapid results than previous typing techniques [14], and the results obtained from this procedure can be comparable with pulse field gel electrophoresis analysis (PFGE) in the study of *S. pneumoniae* [15]. In this report, we are the first to apply AFLP for the clinico-epidemiological study of *S. pneumoniae*.

Materials and Methods

Bacterial samples. All clinical samples were obtained from patients with AOM treated at the Department of Otolaryngology, Okayama University Medical School, and other cooperating otolaryngology clinics between June 1997 and July 1997. The diagnosis of AOM was based on signs of acute infection of the tympanic membrane or drainage from the spontaneous perforation. Tympanocentesis was performed if the tympanic membrane was bulging or full; the middle ear effusion was then collected. In patients with draining AOM, the external ear canal was mechanically cleaned and the discharge at the spontaneous perforation of tympanic membrane was swabbed. The nasal cavity was also mechanically cleaned and samples were taken from the nasopharynx; it has been reported that the pathogen that causes AOM resides here [16]. All specimens were immediately plated onto sheep blood agar and chocolate agar and incubated overnight at 35 °C. Colonies identified as *S. pneumoniae*, based on the results of Gram stain, α-hemolysis, and optochin susceptibility [17], were used in the following studies.

Susceptibility test. The MIC for benzylpenicillin (PC-G, Meiji Seika Kaisha, Ltd., Tokyo, Japan) was measured by broth microdilution methods according to the guidelines of the Japanese Society of Chemotherapy. The susceptibility of these strains to antimicrobial agents was determined using cation-adjusted Mueller-Hinton broth (Difico, Liverpool, NSW, Australia) supplemented with 5% defibrinized horse blood.

According to the National Committee for Clinical Laboratory Standards, *S. pneumoniae* with a MIC value of 2 μg/ml or higher was defined as penicillin-resistant *S. pneumoniae* (PRSP), those with a MIC of 0.1-1 μg/ml were defined as penicillin-intermediate *S. pneumoniae* (PISP), and those with a MIC below 0.06 μg/ml were considered penicillin-susceptible (PSSP). In the present report, both PISP and PRSP samples were classified as PNSSP.

Serotyping. All pneumococcal samples were serotyped by the capsular swelling technique using an Antisera ™kit (Staten Seruminstitut, Copenhagen, Denmark).

DNA preparation. Genomic DNA was extracted from a colony on each plate using the phenol-chloroform method. The concentration of extracted DNA was measured by means of a spectrophotometer (Gene Quant II™, Amersham Pharmacia Biotech, Inc., Piscateway, NJ, USA) and adjusted to 100 ng/μl for later use.

PCR assay of penicillin-binding proteins. Alterations in the genes that encode each of penicillin-binding proteins (PBPs) were examined by PCR. As a positive control, a pair of primers that were designed to amplify the *lytA* gene of *S. pneumoniae* was used. Other primers for the amplification of *pbp* (the gene that encodes PBP) *1a*, *2x*, and *2b* were prepared according to the method described in a previous report [18]. Amplification reactions were carried out as previously described [18]. Briefly, the PCR reaction had a total volume of 100 μl containing 1 μl of bacterial DNA, 2.0 pmol of each primer, 200 μM of each dNTP (Toyobo Co., Ltd., Osaka, Japan), 0.25 U of *Taq* DNA polymerase, and 10x PCR buffer (Takara Shuzo., Ltd., Shiga, Japan). After the initial denaturation step at 94 °C for 2 min, the samples were amplified under the following thermal conditions: 94 °C for 20 sec, 57 °C for 20 sec, and 72 °C for 15 sec, for 30 cycles, with an additional

extension time at 72 °C for 10 min. The PCR products were visualized following electrophoresis on a 3% agarose gel and ethidium bromide staining. The results of PCR were classified as follows: if all of the *pbp* bands were detected at the predicted sizes, such samples were classified as "S" and assumed to be penicillin-susceptible strains without any alterations in *pbp 1a*, *2x*, or *2b*. If at least one of these *pbp* bands was not detected, then the samples were classified as "I" strains and assumed to have incomplete or partial resistance to penicillin from mutations in at least one of the *pbps*. If no bands were observed in spite of the successful PCR amplification verified by a positive control, the samples were classified as "R" and assumed to be penicillin-resistant strains.

AFLP: amplified fragment length polymorphism. First, genomic DNA (10 ng) was digested in a total volume of 11 μl containing 1 U *Mse* I (New England BioLabs [NEB], Hertfordshire, England), 5 U *EcoR* I (NEB), 10 U T4 DNA ligase (NEB), 1.0 μl of 10X T4 DNA ligase buffer with ATP (NEB), 0.5 g/ml bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO, USA), 1.0 μl of 0.5 M NaCl, *Mse* I adapter pairs (Perkin-Elmer Co. [PE], Foster City, CA, USA), and *EcoR* I adapter pairs (PE). The sequence of the *Mse* I adapter and the *EcoR* I adapter are shown in Table 1. The reaction mixtures were incubated at 25 °C for 10 h, heated at 65 °C for 10 min to inactive the ligase, and stored at − 20 °C. Then 189 μl TE buffer was added to the restriction-ligation reaction products for preselective amplification.

Next, a reaction mixture was prepared which contained 4.0 μl diluted DNA arrived at by restriction-ligation, 0.5 μl AFLP *EcoR* I core primer sequence (*EcoR* I adapter- specific, termed *EcoR* I + O) (PE), 0.5 μl AFLP *Mse* I core primer sequence (*Mse* I adapter-specific, termed *Mse* I + O) (PE), and 15 μl AFLP Amplification Core Mix™ (PE), containing buffer, nucleotides, and ampli *Taq* DNA Polymerase (Roche Molecular Systems, Inc., Palo Alto City, CA, USA).

All PCR assays were performed in a TP2000 thermal cycler (Takara). The following conditions were used for preselective PCR amplification: preheating at 72 °C for 2 min, followed by a cycle of 94 °C for 20 sec, 56 °C for 30 sec and 72 °C for 2 min repeated for 20 cycles. The PCR products were then diluted with TE buffer to 0.05 times of the initial concentration and held at 4 °C.

The reaction mixture then contained 1.5 μl diluted amplification products, 0.5 μl *Mse* I primer sequence (*Mse* I + O), 0.5 μl *EcoR* I primer sequence (*EcoR* I + 0) which was labeled with the blue fluorescent dye 5-carboxyfluorescein (5-FAM), and 7.5 μl AFLP Amplification Core Mix. These primers, shown in the Table 1, were used both for the preselective PCR and the selective PCR amplification.

The following conditions were used for selective PCR amplification: preheating at 94 °C for 2 min, followed by a cycle of denaturation at 94 °C for 20 sec, annealing for 30 sec, and an extension at 72 °C for 2 min repeated for 30 cycles. The annealing temperature began at 66 °C for the first cycle; the temperature was then decreased 1 °C at every cycle for the next 9 cycles. Finally, the annealing temperature was kept at 56 °C for the remaining 20 cycles. A final extension at 60 °C for 30 min was added. The selective amplification products were stored at − 20 °C.

The products of the AFLP reactions were loaded and run on a ABI PRISM 310 Genetic Analyzer (PE) and the results were interpreted using GeneScan ver. 2.1 software (PE). Injection was performed at 15 kV for 12 sec. Electrophoresis was performed at 13.0 kV for 35 min at 60 °C. Bands between 50 bp and 450 bp were then detected. The software detected 50–2000 peaks.

Table I Selective primer and adapter sequences for the *Mse* I/*EcoR* I to AFLP

Identifier	Sequence
Mse I recognition site	5′-T ↓ TAA-3′
EcoR I recognition site	5′-G ↓ AATTC-3′
MseI adaptor	5′-TACTCAGGACTCATC GAGTCCTGAGTAGCAG-5′
EcoR I adaptor	5′-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5′
Mse I-O	5′-GATGAGTCCTGAGTAA-3′
EcoR I-O-FAM	5′-GACTGCGTACCAATTC-3′

Mse I/*EcoR* I: Restriction Enzyme
FAM, labeled blue flouorescent dye 5-carboxyfluorescein.

Results

Presence of PNSSP in recurring acute otitis media. Of the 123 samples collected, 30 were classified as recurrent AOM cases with PNSSP. These 30 samples were obtained from 11 patients with recurrent AOM treated at 3 different otolaryngology clinics. No genetic or personal relationship existed among these patients. The patients ranged in age from 11

months old to 10 years old. All experienced a recurrence of AOM within 1 month of the initial diagnosis. Six cases had 2 episodes of recurrence, 2 cases had 3 episodes of recurrence, and 3 cases had 4 episodes of recurrence. Most of the cases were treated either with cefditoren pivoxil or amoxicillin throughout their repeated AOM episodes. One case was empirically treated with rokitamycin and another case was treated with cefpodoxime-proxetil. Both cases were later treated either with cefditoren pivoxil or amoxicillin.

Serotyping. Of the 30 samples, 6 could not be classified by serotype (n/t). Of the remaining 24 samples, 4 were serotype 6 (17%), 2 were serotype 9 (8%), 2 were serotype 14 (8%), 7 were serotype 19 (29%), and 9 were serotype 23 (38%). The serotypes from different AOM episodes are summarized in Table 2. One case apparently demonstrated a change in serotype throughout the repeated AOM episodes: Case #5 went from serotype 6 to serotype 19.

Mutations in PBP detected by PCR. The results of the PCR-based PBP assay are summarized in Table 2.

Table 2 Summary of serotyping and genotyping results regarding recurrent AOM

Case No.	Sample No.	Date	Drug	MIC (μg/ml)	*pbp*	Serotype	bp 74	75	76	81	87	91	113	120	142	144	157	159	162	192	219	222	239	315
1	T056	Jun. 16	CDTR (×2)	0.031	S	9	●	–	●	–	●	●	●	●	–	●	–	●	–	●	–	●	–	–
	T091	Jun. 23	CDTR + AMPC		I	9	●	–	●	–	●	–	●	●	–	●	–	●	–	●	–	–	–	–
2	T035	Jun. 14	CDTR (×2)	I	R	23	●	–	●	●	●	–	–	●	●	–	●	●	●	●	●	–	–	–
	T105	Jun. 25			R	23	●	–	●	●	●	–	–	●	●	●	●	●	●	●	●	●	–	–
	T261	Jul. 9			R	n/t	●	–	●	–	●	–	–	●	●	●	–	●	●	●	●	–	–	–
	T297	Jul. 14			R	n/t	●	–	–	–	●	●	–	●	–	●	–	●	●	●	●	●	–	–
3	T058	Jun. 17	CPDX	I	R	23	–	●	–	–	●	●	–	●	–	●	–	●	●	●	●	–	●	–
	T098	Jun. 24	CDTR (×2)		R	23	–	●	–	–	–	●	–	●	–	●	–	●	●	●	●	–	●	–
	T148	Jun. 30	CDTR + AMPC		R	23	–	●	–	–	–	●	–	●	–	●	–	●	●	●	●	–	●	–
	T221	Jul. 7			R	n/t	●	–	●	–	●	●	–	●	●	●	–	●	●	●	●	–	●	–
4	T057-1	Jun. 16	unknown	2	R	19	–	●	–	–	●	●	●	●	–	●	–	●	●	●	●	●	●	–
	T237	Jul. 7			R	n/t	–	●	–	–	●	●	●	●	–	●	–	●	●	●	●	●	●	–
5	T094-2	Jun. 23	CDTR + AMPC	I	I	6	●	–	–	●	●	●	●	●	–	●	–	–	●	●	–	●	●	–
	T151	Jun. 30			R	19	–	●	–	–	●	●	●	●	●	●	–	●	–	●	–	–	●	–
6	T074	Jun. 21	CDTR	I	R	23	–	–	●	–	–	●	–	●	–	●	–	●	●	●	●	–	●	–
	T122	Jun. 26	CDTR + AMPC		R	23	–	–	●	–	–	●	–	●	–	●	–	●	●	●	●	–	●	–
7	T059	Jun. 17	AMPC	0.031	I	14	–	–	●	–	●	●	●	–	●	●	–	●	–	●	–	●	–	–
	T100	Jun. 24	CDTR		I	14	●	–	–	–	●	●	–	–	●	●	–	●	●	●	●	–	–	–
8	T011	Jun. 8	AMPC + RKM	0.25	I	19	–	●	–	–	●	–	●	●	●	●	–	●	–	●	–	–	●	–
	T029	Jun. 10			I	19	–	●	–	–	–	–	●	●	●	●	–	●	–	–	–	–	●	–
9	T006	Jun. 8	CDTR + AMPC	I	R	6	●	–	–	●	●	●	●	●	●	●	–	●	–	–	–	●	●	●
	T028	Jun. 10			R	6	●	–	●	–	●	●	●	●	●	●	–	●	●	–	●	●	●	●
	T049	Jun. 15			R	6	●	–	●	●	●	●	–	●	●	●	–	●	●	–	●	●	●	●
	T209	Jul. 6			R	n/t	●	–	●	●	●	●	●	●	●	●	–	●	●	–	–	●	●	●
10	T003	Jun. 1	CDTR + AMPC	I	R	19	–	●	–	●	●	●	●	●	●	●	–	●	–	–	●	–	●	–
	T030	Jun. 11			R	19	–	●	–	–	●	●	●	–	●	●	–	●	–	–	–	–	●	–
	T103	Jun. 25			R	19	–	●	–	–	●	●	●	●	–	●	–	●	●	–	–	–	●	–
11	T048	Jun. 15	AMPC	I	R	23	–	–	●	–	–	●	–	●	–	●	–	●	●	●	●	–	●	–
	T083	Jun. 22	CDTR + AMPC		R	23	–	–	●	–	–	●	–	●	–	●	–	●	●	●	●	–	●	–
	T211	Jul. 6			R	n/t	–	–	●	●	–	●	–	●	–	●	–	●	●	●	●	–	●	–

pbp, genetic alteration detected by PCR described by Zenni *et al* [16]. S, all 3 *pbps* (*pbp* 1a, 2x and 2b) were detected by PCR; I, at least one of the 3 *pbps* could not be detected; R, none of 3 *pbps* was amplified. MIC (μg/ml) for penicillin G.

(●): The presence or (–): absence of a polymorphic peak as determined by AFLP analysis was indicated in the table. Each case number represent different patient while a sample number indicates each sample that was isolated during each AOM episode.

AMPC, amoxicillin; CDTR, cefditoren pivoxil; CPDX, cefpodoxime proxetil; RKM, rokitamycin; (×2), shadow; similar band pattern twice as standard dosages; (n/t), not tested.

Only one strain, T056, revealed no apparent alterations in *pbp 1a*, *2x*, or *2b*. Six strains were classified as "I" and 23 strains were classified as "R", indicating that at least one *pbp* alteration was observed in most of the strains examined here. The greatest changes in *pbp* throughout the repeated episodes of AOM were observed in Cases #1 and #5.

AFLP. In this study, 101 peaks were detected in the samples. Eighty-three of them were common peaks, so the other 18 peaks were utilized as markers representing polymorphisms. The raw AFLP waves are shown on Fig. 1. Table 2 summarizes the polymorphic results of the present data, showing the presence of a peak by a closed circle (●) and the absence of the peak by a minus

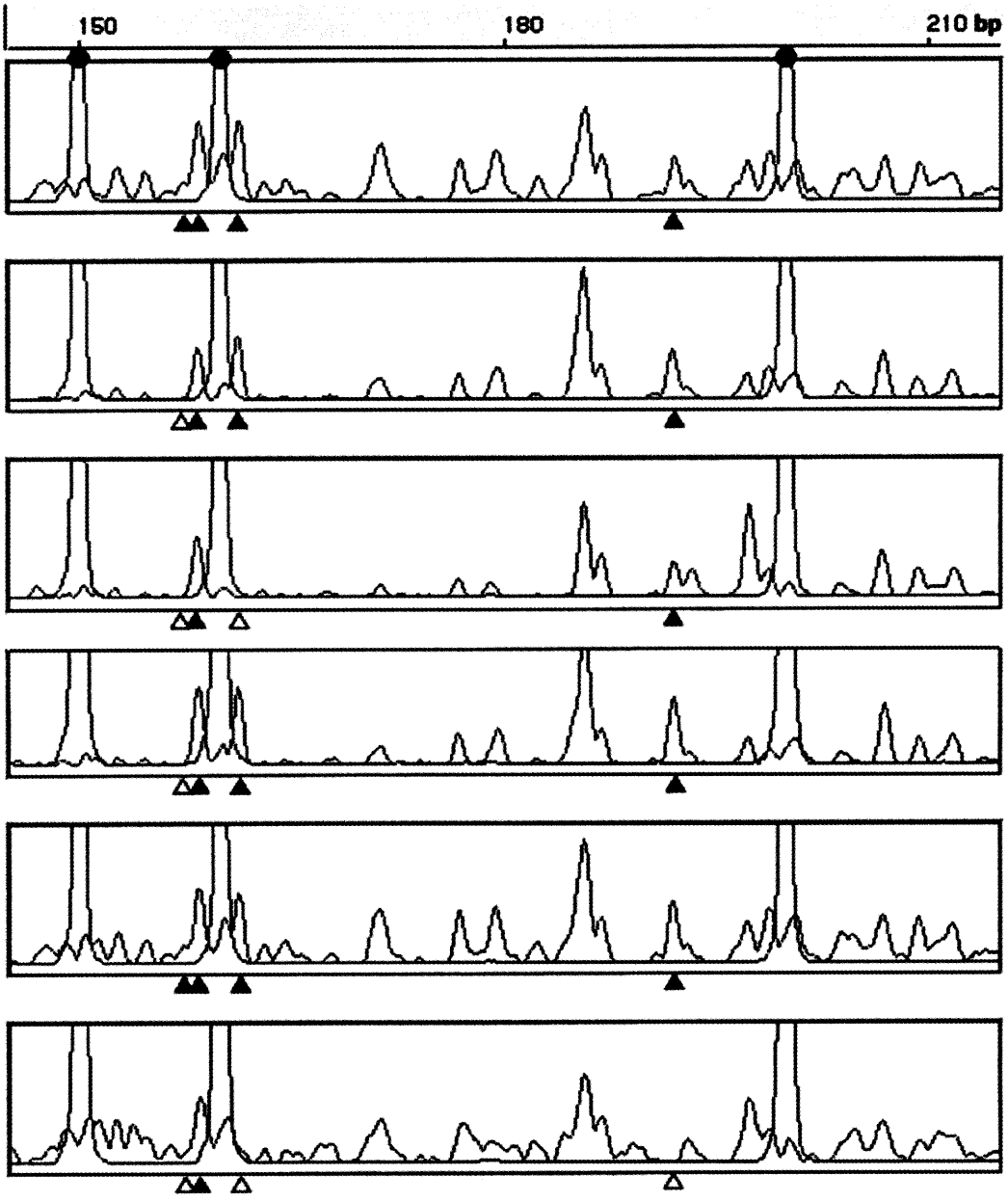

Fig 1 Typical result of AFLP with chromatogram using an automated sequencer.
Triangles indicate the presence (▲) or absence (△) of specific peaks after AFLP. Circle (●) indicate size markers.

symbol (-). Five cases (#1, #4, #6, #8, and #11) demonstrated highly similar (more than 16 out of 18, 88.9 %) genotyping patterns among samples. Conversely, 6 cases (#2, #3, #5, #7, #9, and #10) demonstrated relatively heterogeneous results, showing more than 4 different peaks among samples. A controversial result was observed in Case #3 when samples T058, T098, and T148 demonstrated highly similar polymorphic patterns and only sample T221 demonstrated a different fingerprinting pattern.

Discussion

The present study demonstrated that 6 of the 11 recurrent pneumococcal AOM cases (54.5%) included genetically diverse strains of *S. pneumoniae*, with highly and similarly altered *PBP* genes. This implies that the strain that causes the initial AOM is not always responsible for the recurrence; subsequent episodes of AOM may be caused by genetically unrelated strains. Strains that are genetically different from those in the initial AOM may originate from: 1) strains with de novo gene alterations great enough to be recognized as different genotypes, although this presumes that the genetic change involves a considerably large part of the *S. pneumoniae* gene, 2) strains that are introduced from outside of the nasopharyngeal ecosystem, or 3) 2 or more genetically different strains of *S. pneumoniae* colonizing in the nasopharynx reservoir. The fact that the change could happen within several days [19] makes the possibility of de novo gene alteration unreasonable. On the contrary we could not demonstrate that any major genetic alterations had occurred during treatment in the remaining 5 recurrent AOM cases. There remains the possibility that the strain that caused the initial AOM survived to cause the subsequent episodes of AOM. However, 4 (#1, #4, #6, #8) of the 5 cases included only 2 episodes of AOM, and it is possible that studying more recurrences, over a longer period of time, would reveal subsequent genetic alterations.

Although examination of biological markers of *S. pneumoniae* could contain the potential risk for misinterpretation because of the tendency of horizontal gene transfer [11], most of the previous reports that concerned the alteration of *S. pneumoniae* after antibiotic treatment were limited in view of the serotyping or MIC profiling to several antibiotics. Dagan *et al.* demonstrated that 19 of 120 (16%) of the patients treated by antibiotics carried the new *S. pneumoniae* in the nasopharynx after 3 to 4 days of treatment [19]. Cohen *et al.* reported that a total of 51 of 465 (11%) acquired new *S. pneumoniae* after treatment [20]. Only a few reports were available regarding the alterations of *S. pneumoniae* examined by genotyping analysis. Yano *et al.* examined the genotypes of nasopharyngeal flora of 6 day-care attendants who had repeated episodes of upper aerodigestive tract infection including AOM [21]. Only 2 of them demonstrated 2 episodes of AOM caused by *S. pneumoniae*. PFGE showed that one case possessed the common genotyping pattern. Cohen *et al.* applied RAPD (random amplified polymorphic DNA) analysis for the classification of *S. pneumoniae* isolated from before and after antibiotic treatment and found that 16 of 94 children (17%) carried genetically new *S. pneumoniae* after antibiotic treatment [22]. Although some previous studies claimed that RAPD analysis is less reliable and highly dependent on optimal conditions for PCR [23], almost no information regarding the RAPD analysis used in the report was provided [22].

The presence of PNSSP potentially implied the persistence of PNSSP even after treatment. PNSSP was frequently (10 out of 11) isolated from the first bout of AOM episodes under this study project. Thus, a less frequent alteration of the genetic pattern could be suspected. It is very difficult to compare these previous reports because the number of the samples is not great enough for statistical analysis, but the present data demonstrated a relatively higher incidence of the alteration. This could partly account for why the higher discriminating power of genotyping could successfully demonstrate the difference between strains.

AFLP analysis added the feasibility of restriction fragment length polymorphism (RFLP) analysis to the flexibility of PCR-based technology. Because of this feature, AFLP analysis provides more feasible results than does RAPD. Sloos *et al.* directly compared 5 different typing methods for the study of *S. epidermidis*, including RAPD, PFGE, AFLP as well as plasmid typing and quantitative antibiogram [24]. Among these 5 methods, PFGE and AFLP were concluded to be the most accurate typing methods. In addition, because AFLP results can be analyzed on an automated sequencer, this procedure is potentially labor-saving for a large-scale epidemiological study, especially compared to PFGE. Because of these benefits, this procedure has been used for determining the genotypes of several types

of bacteria [12] and then applied for epidemiological study for these strains [15]. For the study of *S. pneumoniae*, however, van Eldere *et al.* were first used AFLP simply to compare its results with of those of PFGE that is widely used for the gold standard of epidemiological study of *S. pneumoniae* [15]. They examined the same samples using both procedures obtaining almost equal results. In this report, we were the first to apply AFLP to the epidemiological study of recurrent AOM.

Recurrent AOM is now an increasing disease in children. Bruce *et al.* reported that the incidence of recurrent otitis among preschool children had increased from 18.7% in 1981 to 26% in 1988 [25]. Recurrent otitis media naturally requires repeated courses of antibiotic treatment, and as demonstrated in this report, probably accelerated the turnover of *S. pneumoniae*. Because the use of antibiotics could work as a selective pressure for the evolution of *S. pneumoniae*, less susceptible microorganisms could be selected and persistently carried throughout human society by means of this repetition process. Thus, for the prevention of the spread of PNSSP as well as for the treatment of recurring AOM, more attention should be paid to the dynamics of *S. pneumoniae* in nasopharyngeal carriage.

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