of either TEM-1 or ROB-1 type β-lactamase.[8,9] The other is designated as β-lactamase nonproducing ampicillin resistant (BLNAR), and involves a decreasing affinity of PBPs to β-lactams caused by conformational changes with genetic mutations.[10-12] Recent studies revealed that increasing resistances to β-lactams in BLNAR strains were closely related to mutations in the *ftsI* gene encoding PBP3, which mediates septum peptidoglycan formation.[13,14] The substitutions in the *ftsI* gene have been classified into the following three groups: group I, His is substituted for Arg-517 (Arg517His) near the KTG motif; group II, Lys is substituted for Asn-526 (Asn526Lys) near the KTG motif; group III, three residues (Met-377, Ser-385, and Leu-389) near the SSN motif are replaced by Ile, Thr, and/or Phe (Met377Ile, Ser385Thr,and/or Leu389Phe, respectively), in addition to the replacement of Asn526Lys. Isolates with susceptible or intermediate ampicillin resistance are commonly found in groups I and II, and isolates in group III are associated with a higher level of ampicillin resistance.[15]

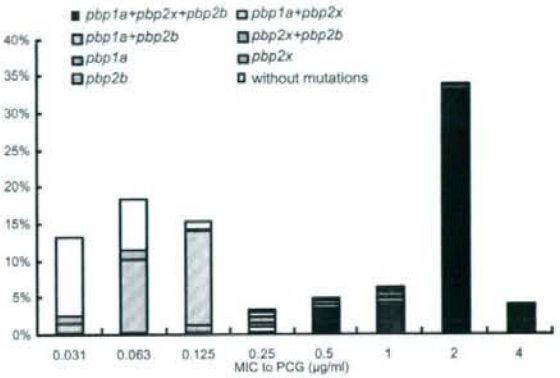

Fig. 1. Correlation between susceptibility to penicillin G (*PCG*) and mutations in penicillin binding protein (*pbp*) genes. *MIC*, minimal inhibitory concentration

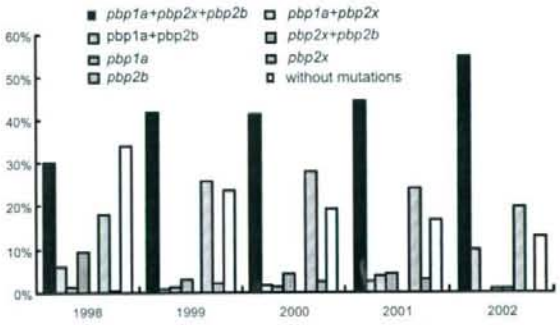

Fig. 2. Annual distributions of *pbp* gene mutations in *S. pneumoniae*

Antimicrobial resistance in *Streptococcus pneumoniae*

S. pneumoniae is the most important pathogen for AOM, and 20%–35% of AOM is caused by *S. pneumoniae*. The high incidence of PRSP strains has recently been a global issue. Resistance strains are appearing all over the world nowadays. Recent reports indicate that the rates of PRSP were 54.8% in Korea, 43.2% in Hong Kong, 38.6% in Taiwan, 71.4% in Vietnam, 29.3% in Japan, 12% in the United States of America, and 2% in Germany.[2,16] Almost 76% of strains had a mutation in their PBP 1a, 2b, and 2x in Japan. They were classified into seven genotypic classes after polymerase chain reaction (PCR) identification of abnormal *pbp1a*, *pbp2x*, and *pbp2b* genes: (i) penicillin-susceptible *S. pneumoniae* (PSSP) isolates with no abnormal *pbp* genes (24.2%); (ii) genotypic penicillin-intermediate *S. pneumoniae* (gPISP) isolates with only an abnormal *pbp2x* gene [gPISP (*2x*)] (26%); (iii) with only an abnormal *pbp1a* gene [gPISP (*1a*)] (0.1%); (iv) with only an abnormal *pbp2b* gene [gPISP (2b)] (2.2%); (v) gPISP isolates with abnormal *pbp1a* and *pbp2x* genes (2.8%); (vi) gPISP isolates with abnormal *pbp2x* and *pbp2b* genes (2.2%); (vii) genotypic penicillin-resistant *S. pneumoniae* (gPRSP) isolates with three abnormal *pbp* genes (38.5%). Almost 95% of strains had abnormal ppb 2x gene mutations (Fig. 1). The minimal inhibitory concentration (MIC) MIC_{50} and MIC_{90} of the strains with mutations in the three *pbp* genes to PCG were ≥2 μg/ml, whereas strains without mutations in either *pbp* genes were ≤0.03 μg/ml and 0.06 μg/ml, respectively. The MIC_{50} and MIC_{90} of the strains with mutations in *pbp2x* were 0.06 μg/ml and 0.125 μg/ml, respectively. On the other hand, the MIC_{50} and MIC_{90} of strains with mutations in two types of *pbp* genes (*pbp1a* and *pbp2x*, *pbp1a* and *pbp2b* or *pbp2x* and *pbp2b*) varied in the range between 0.125–0.5 μg/ml and 0.5–4 μg/ml, respectively. Annual changes of mutations in the three PBP genes were assessed for 5 years. Strains with mutations in three PBP genes gradually increased from 1998 to 1999. Then the increase of PBP-mutated strains became slower from 1999 to 2001. In 2002, the strains increased again. On the other hand, strains without mutations in PBP genes decreased in number. The strains with mutations in *pbp2x* gradually increased from 1998 to 2000, and then gradually decreased in numbers from 2001 to 2002 (Fig. 2). In the age distribution of mutations in PBP genes, strains with mutations in all three PBP genes were frequently identified among children younger than 2 years old (48.9% vs. 35.2%, $P < 0.01$) (Fig. 3). The other strains showed similar prevalences between the two age groups.

S. pneumoniae resistance to macrolides has also been a big concern all over the world, and the rates of resistance were 70%–80% in Japan, 92.1% in Vietnam, 86% in Taiwan, 80.2% in Korea, 76.8% in Hong Kong, and 30% in the USA.[2,17,18] The majority of the strains had *mefA* (32.5%) or *ermB* (34%) and *mefA* and *ermB* (3.4%) gene-mediating macrolide resistance. Susceptibilities to clarithromycin of strains with *mefA* gene, *ermB* gene, and both were 1–4 μg/ml, >64 μg/ml, and >64 μg/ml, respectively. Macrolide-resistant genes were frequently identified among penicillin nonsusceptible strains (PISP + PRSP)[2,17] (Fig. 4).

PRSP causes a three times higher incidence of intractable AOM than PSSP. Serotypes or serogroup 19F, 23F, and

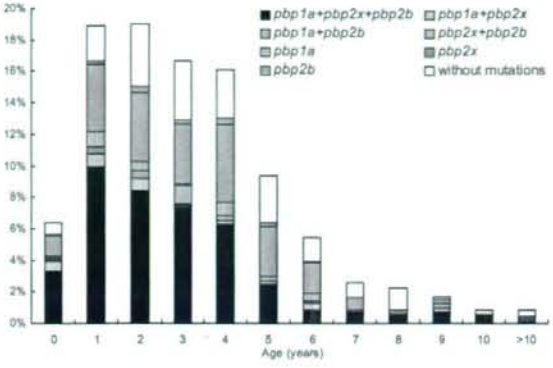

Fig. 3. Age distribution of mutations in *pbp* genes

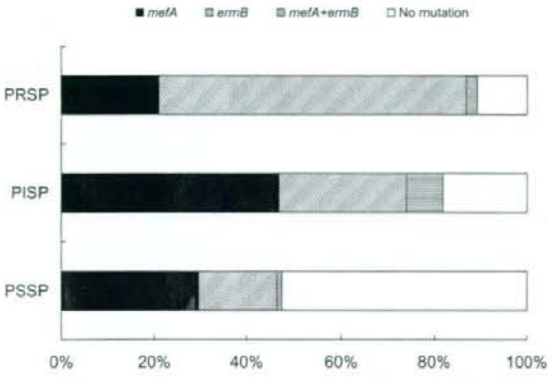

Fig. 4. Macrolide-resistant genes and susceptibility to PCG. *PRSP*, penicillin-resistant *Streptococcus pneumoniae*; *PISP*, penicillin-intermediate *Streptococcus pneumoniae*; *PSSP*, penicillin-susceptible *Streptococcus pneumoniae*

6 are the most prevalent serotypes, followed by serotype 3, serotype 9V, and serotype 7F all over the world. The increasing incidence of PRSP is nowadays a great concern all over the world, and most strains have also been getting multidrug resistant. Conjugated pneumococcal vaccine does reduce the colonization of *S. pneumoniae* in AOM patients, but the efficacy of vaccine in AOM patients still remains controversial.

Antimicrobial resistance in *Haemophilus influenzae*

H. influenzae is the second leading pathogen that causes AOM in children. Most AOM is caused by nontypeable *H. influenzae* (NTHi). Since the first reports of ampicillin-resistant strains of *H. influenzae* in 1974 from the USA, the major mechanism of the antimicrobial resistance of *H. influenzae* has been considered to be either TEM-1 or ROB-1 types of β-lactamase.[19-21] The prevalence of β-lactamase-producing strains increased markedly up to 15.2% in 1983–1984, 36.4% in 1994–1995, and 31.3% in 1997–1998 in the United States.[19,20] BLNAR strains had been isolated at low frequencies in the 1980s, but BLNAR strains have rapidly increased at the rate of 19.5% in the 1990s.[22] Recently, the rate of BLNAR was 58.1% in Korea, 37% in Japan, 0%–33% in Europe, and 4%–10.1% in the USA.[19-21,23,24]

In 2003, the Japan Society of Infectious Diseases in Otolaryngology conducted the 4th nationwide surveillance of causative pathogens responsible for upper respiratory tract infectious diseases in Japan in order to define the contemporary surveillance of antimicrobial-resistant pathogens. According to the criteria for the susceptibility of *H. influenzae* to AMP by the Clinical and Laboratory Standards Institute (CLSI), *H. influenzae* isolates were divided into 61.0% susceptible strains (MIC ≤ 1 μg/ml), 37 (14.0%) intermediately resistant strains (MIC = 2 μg/ml), and 66 (25.0%) resistant strains (MIC ≥ 4 μg/ml). Five strains produced TEM-type β-lactamase. These were divided into 3 (1.2%) strains with mutations in the *ftsI* gene (gBLPACR: genotypic β-lactamase-producing amoxicillin-clavulanate-resistant) and 2 (0.8%) strains without mutations in the *ftsI* gene gBLPAR (genotypic β-lactamase-producing ampicillin-resistant). According to PCR-based genotyping, 172 (65.1%) isolates had mutations in the *ftsI* gene without producing β-lactamase (gBLNAR: genotypic β-lactamase-nonproducing ampicillin-resistant). These were 98 (37.1%) strains with group I/II mutations in a variable mutated region (Group I/II gBLNAR) and 74 (28.0%) strains with group III mutations in a highly mutated region (Group III gBLNAR). The other 87 (33.0%) isolate were gBLNAS (genetically β-lactamase nonproducing ampicillin-susceptible) strains with mutations in neither the *ftsI* gene nor the *bla* gene (Table 1).[15] The Group III gBLNAR strains showed resistance to both penicillin and cephalosporin. Among the 61 gBLNAR strains with mutations in the *ftsI* gene, 6 clones were identified. As the MIC to AMP increased, the frequencies of clonal dissemination were getting higher. Six (25%) strains among 24 strains with MIC to AMP 4 μg/ml, 6 (23.0%) strains among 26 strains with MIC to AMP 8 μg/ml, and 7 (63.6%) strains among strains with MIC to AMP ≥ 16 μg/ml showed similar PFGE patterns (Fig. 5).[15] PBP gene-mutated *H. influenzae* is not only resistant to AMP, but also has reduced susceptibility to cephalosporin. Nowadays, the minimum inhibitory concentration to ampicillin is increasing rapidly, and the strains are becoming resistant to third-generation cephalosporin. Such a prevalence of BLNAR strains with mutations of the *ftsI* gene has been alarmingly high in Japan. The resistant *H. influenzae* pathogen will disseminate in different ways from penicillin-resistant *S. pneumoniae*. Consequently, we need to continue careful surveillance for BLNAR strains of *H. influenzae* in patient populations, and continue our efforts to understand why these antibiotic-resistant strains are becoming more prevalent. PCR-based genotyping and study of molecular characteristics bring us useful information to continue our surveillance of this resistant pathogen.

Table 1. Correlation between PCR-based genotyping and susceptibility to AMP

PCR-based genotype	No. of isolates	No. of isolates with MIC to AMP (μg/ml) of:										MIC (μg/ml)			Susceptibility (%)*		
		≤0.12	0.25	0.5	1	2	4	8	16	32	≥64	MIC_{50}	MIC_{90}	Range	S	I	R
gBLNAS	87	3	35	46	3							0.5	0.5	0.12–1	100	0	0
Group I/II gBLNAR	98	5	23	21	18	23	6	2				0.5	2	0.12–8	68.4	23.5	8.1
Group III gBLNAR	74				7	14	18	24	10	1		4	16	1–32	9.5	18.9	71.6
Group I/II gBLPACR	1										1	128	128	128	0	0	100
Group III gBLPACR	2										2	>128	>128	>128	0	0	100
gBLPAR	2										2	64	>128	64 ≥ 128	0	0	100
Total	264	8	58	67	28	37	24	26	10	1	5	0.5	8	0.12 ≥ 128			

*S, susceptible; I, intermediate; R, resistant
AMP: ampicillin

Clinical immunology in acute otitis media

Otitis media is the most common disease seen in childhood. *S. pneumoniae*, NTHi, and *Moraxella catarrhalis (M. catarrhalis)* are the most frequent pathogens in about 35%–40%, 30%–35%, and 10%–15%, respectively, of all episodes. Protection against the disease due to these pathogens may depend on pathogen-specific antibodies. In the case of *S. pneumoniae*, the protective antibody has been thought to be directed mainly toward capsular polysaccharide antigens.[25] Capsular polysaccharides of *S. pneumoniae* are type-specific and poorly immunogenic in children younger than 2 years old.[26,27] Some pathogen-specific antibodies may be directed against protein immunogens such as pneumococcal surface protein A (PspA) of *S. pneumoniae*, P6 of NTHi, and UspA of *M. catarrhalis*.

In our immunological study in children with otitis media,[28–31] otitis-prone children were not unusually vulnerable to infections except those resulting in otitis media. This fact seems to refute the presence of a broad immunological deficit in the children. However, children who had recurrent episodes of otitis media caused by *S. pneumoniae* or nontypeable *H. influenzae* did not mount a normal response to PspA, PCP-IgG_2, and P6 during the episodes, and failed to have a secondary immune response on repeated challenge. It is likely, therefore, that these children will not respond adequately to immunization with PspA or P6 vaccines. Otitis-prone children also fail to respond appropriately to pneumococcal antigens, and thus may not be immunized effectively with a vaccine for otitis media that contains pneumococcal polysaccharides. Selective immunological derangements in otitis-prone children may therefore be more widespread than previously believed. Effective active immunoprophylaxis against otitis media will be possible only when the mechanism of the immunological defect in otitis-prone children is understood.

Immune response to PspA of *S. pneumoniae* in children with acute otitis media

A number of recent publications have described the importance of PspA in both disease production and immunity. PspA is attached to the surface of the pneumococcus by the C-terminal end of the molecule, and much of the immune response elicited by immunization in animals is directed against the N-terminal α-helical portion of the molecule.[32] The PspA gene is expressed in all strains of pneumococci, regardless of their capsular serotype.[33] Antibody responses to PspA in animals protect against sepsis and nasopharyngeal colonization.[33] Although PspA is a heterologous protein, there is a high degree of serological cross-reactivity among different PspA molecules from the two major families of PspA.[34] A single recombinant PspA protein is capable of inducing protection against pneumococcal strains of diverse capsular serotypes and different PspA serotypes in animal models. Thus, it is hypothesized that a single PspA

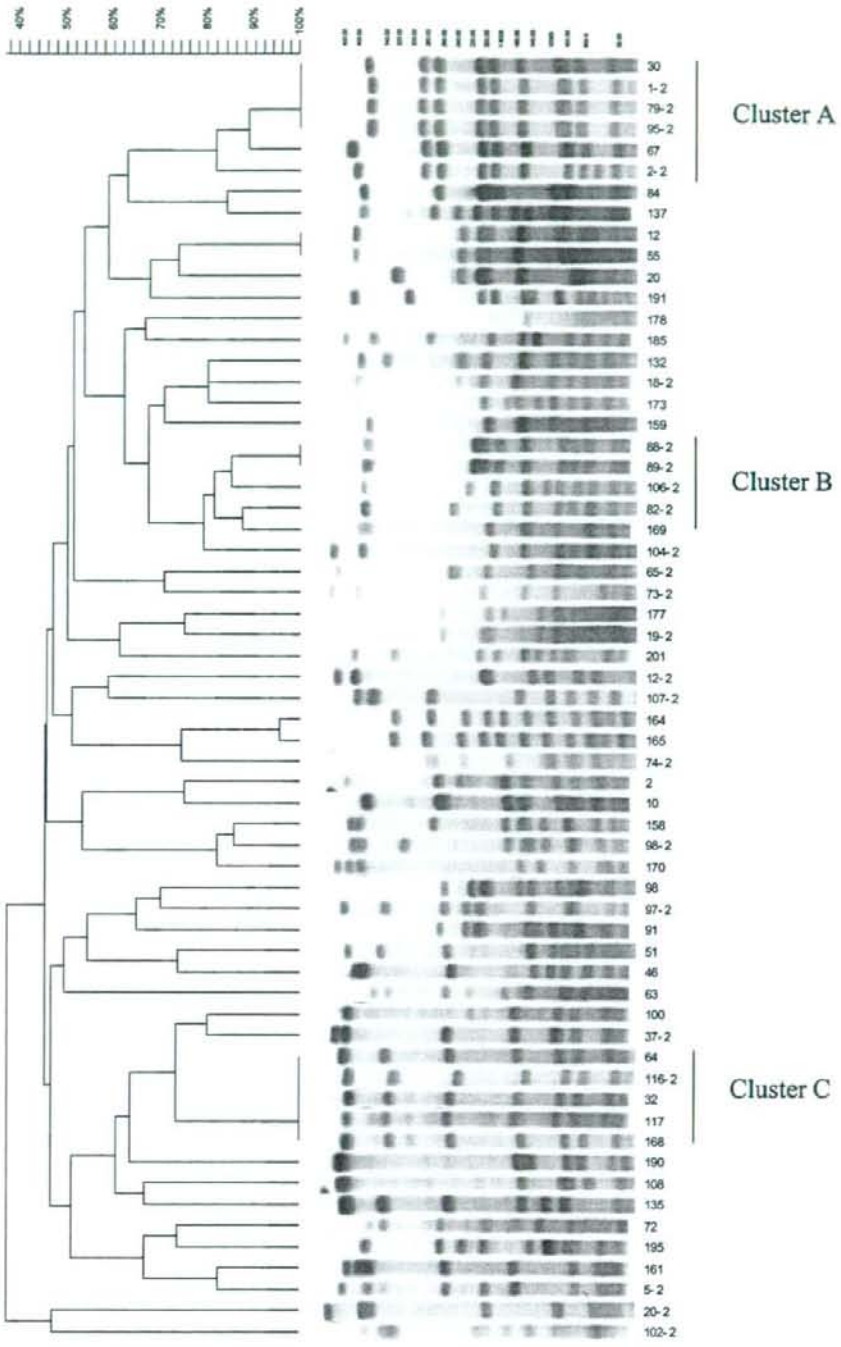

Fig. 5. Genetic identity and/or high relatedness among β-lactamase nonproducing ampicillin-resistant (BLNAR) isolates. Pulsed field gel electrophoresis (PFGE) dendrogram (unweighted pair group method with arithmetic means) of 61 BLNAR strains (MIC 4 μg/ml). Dice coefficients are shown above the dendrogram. Isolates with 80% relatedness on the dendrogram are considered to be strongly genetically related

protein may be able to provide protection against multiple diverse strains of *S. pneumoniae*.[35]

Immune responses to PspA in the sera of various age groups in the general population and in the nasopharynx of 30 children monitored from birth until 1 year of age were evaluated[29] (Fig. 6). IgG was the dominant serum antibody to PspA. In the first 2 years of life, comparable amounts of IgM and IgG antibodies were observed. In older people, IgG antibodies to PspA predominated over IgM antibodies. The level of IgA antibodies to PspA in serum remained low

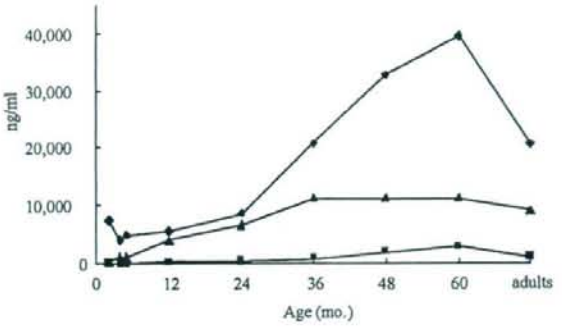

Fig. 6. Antibody to pneumococcal surface protein A (PspA) of *S. pneumoniae* in serum according to age

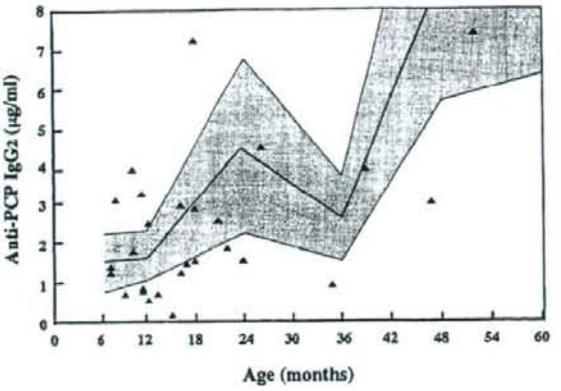

Fig. 7. Anti-PCP IgG2 in otitis-prone children. Normal values are plotted as a shaded area encompassing 2 SD around the mean. Otitis-prone children are represented as individual points (*small black triangles*). *PCP*, polysaccharide capsular protein

during the first 2 years of life. Although IgA was the dominant antibody to PspA in airway secretions, it was detected in a minority of children. Even the majority of children previously colonized with *S. pneumoniae* lacked antibody to it in their secretions. A decline in PspA IgG antibody concentrations was noted in sera from adults, and this was reflected in a similar decline in the proportion of total IgG represented by PspA-specific IgG. Epidemiological studies with *S. pneumoniae* indicate that acquisition of the strain and length of colonization decreases with increasing age, suggesting that maturation of the immune system in some way plays a role in controlling colonization patterns.[36]

The antibody response was evaluated in children with acute otitis media due to *S. pneumoniae*.[30] The age of the children had a range of 4–32 months. The mean IgG, IgM, and IgA antibody responses to PspA in sera from children at the acute and convalescent stages were 4864 vs 5831 ng/ml, $P < 0.05$, 1075 vs 3752 ng/ml, $P < 0.05$, and 67 vs 93 ng/ml, nonsignificant, respectively.

Studies of natural immunity to pneumococcal infections have focused almost exclusively on antibodies directed against the capsular polysaccharides. Although the introduction of conjugate vaccine has given satisfactory protective responses to polysaccharides in young children and raised expectations regarding a capsular-based vaccine, there are still more than 90 individual types of capsule in the pneumococcus. A single protein immunogen capable of eliciting protective antibodies would be attractive when compared with the need to include multiple polysaccharides in a vaccine for young children. This study showed that the majority of children responded to an infection by *S. pneumoniae* by making antibody to PspA. Nevertheless, the specific antibody to PspA may not always be protective to middle-ear infections of *S. pneumoniae* owing to several factors. Mucosal antibodies, which are expected to be those most crucial for protection at the respiratory surface, might not be parallel to serum antibodies. Moreover, it is quite possible that anti-PspA antibodies elicited during otitis media may be protective against invasive disease even if protection against otitis media is not always achieved.

Antibody response to pneumococcal capsular polysaccharides (PCP) in normal and otitis-prone children[31]

Antipneumococcal capsular polysaccharide (PCP) IgG_2 antibodies were measured by the quantitative enzyme-linked immunosorbent assay (ELISA). A polyvalent pneumococcal vaccine (Pneumovax; Merck Sharp & Dohme, West Point, PA, USA) containing 23 serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) was used as a coating antigen. In healthy children, the total IgG2 level was lowest at 6 months of age. The level increased until 2 years of age, and then gradually decreased until 4 years of age. Thereafter, at 4–5 years, it increased again. Anti-PCP IgG2 was lowest at 6 months of age. The level increased until 2 years of age and decreased at 3 years of age. Thereafter, at 4–5 years, it increased again. In otitis-prone children, subnormal levels of total IgG, and anti-PCP IgG were defined if the concentration was lower than 2 SD below the mean for that age in normal children. Five of 36 otitis-prone children (13.9%) showed subnormal levels of total IgG. Thirteen of 27 otitis-prone children (48.1%) showed subnormal levels of anti-PCP IgG2 antibody (Fig. 7). The number of children with subnormal levels of total IgG2 was not higher in the otitis-prone group than in the normal group ($P = 0.1484$). However, the number of children with subnormal levels of anti-PCP IgG antibody was significantly higher in the otitis-prone group than in the normal group (anti-PCP IgG2, $P < 0.01$).

Immune responses to P6 of NTHi in children with acute otitis media

NTHi is frequently associated with recurrent and chronic episodes of middle ear disease.[37] One of the major outer membrane proteins of NTHi, P6, is highly conserved among

strains, is antigenically stable, serves as a target for bactericidal antibody, and has been proposed as a possible candidate for vaccine formulation.[38–40]

The serum antibody response to P6 was studied in otitis-prone and normal children by ELISA.[28] The study group consisted of 43 children, who ranged in age from 1 to 92 months and were included in a prospective study of otitis media. Thirty of the subjects were classified as otitis-prone because they had had four or more episodes of otitis media in the first year of life, or six or more episodes of otitis media by the second year, or needed placement of tympanostomy tubes. The other 13 children were considered to be healthy and had had two or fewer episodes in the first year of life, or three or fewer by the end of the second year.

In the general population, anti-P6 IgG antibody at birth was found at almost the same level as in adults, whereas no IgM or IgA antibodies specific for P6 were detected. Anti-P6 antibody levels in the three isotypes studied were lowest at 6 months of age and rose significantly after 2 years; IgG levels peaked at 10 years, whereas IgM and IgA peaked at 6 years. In every age group, IgG antibody specific for P6 was in the highest concentration among the three isotypes. Anti-P6 IgG antibody was detected in all individuals in each age group; however, IgM antibody specific for P6 was detected in all individuals older than 6 years of age, and IgA antibody specific for P6 was detected in all individuals only after 10 years of age. During the episode of otitis media, antibody levels in convalescent-phase sera exceeded those in acute-phase sera in 60% of cases. Sera obtained during acute and convalescent periods were screened for bactericidal antibody. Ten acute-phase sera possessed bactericidal antibody and 10 did not; all convalescent-phase sera had bactericidal antibody. When the paired sera were divided into two groups depending on the presence or absence of bactericidal antibody in the acute period and then analyzed for antibody to P6, a significant rise in anti-P6 antibody was detected in the group initially lacking bactericidal antibody.

In order to evaluate the immunological derangement in otitis-prone children, anti-P6 antibody levels were measured longitudinally in 30 otitis-prone and 13 healthy children on 93 and 32 occasions, respectively. The age at time of sampling varied between 1 and 92 months. Antibody levels increased seven-fold in the normal group for 36 months in comparison with less than three-fold in the otitis-prone group for 48 months. The levels of antibody in the normal group were significantly higher than those in the otitis-prone group after the age of 18 months. In general, individual antibody levels in otitis-prone individuals did not have an age-dependent rise. Furthermore, children who experienced two or more episodes of otitis media caused by nontypeable *H. influenzae* had no anamnestic antibody response to P6. Immunoglobulin IgM and IgA antibody responses to P6 in otitis-prone children reached a plateau after 18 months of age, and the anti-P6 IgM antibody level remained below the adult serum level even after 4 years of age. Differences between otitis-prone and normal children were not statistically significant. Antibody levels to P6 in otitis-prone children were measured, and anti-P6 IgG was defined if the concentration was lower than 2 SD below the mean for that age in normal children. As shown in Fig. 8, 11 of 20 otitis-prone children (55%) showed subnormal levels of anti-P6 IgG.[31]

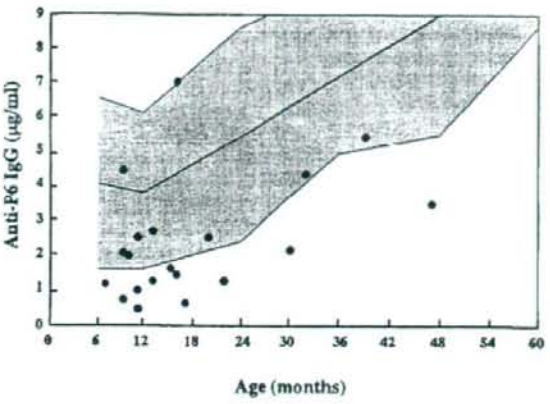

Fig. 8. Anti-P6 IgG in otitis-prone children. Normal values are plotted as a shaded area encompassing 2 SD around the mean. Otitis-prone children are represented as individual points (*small black circles*)

The failure to develop a good antibody response to common antigens may enable the pathogen to cause persistent or recurrent disease. As demonstrated by Harabuchi et al.[40] the level of anti-P6 antibody correlated to the severity of otitis media with effusion. The basis for these observed immunological abnormalities remains obscure. However, Kodama and Faden[41] suggested that in the case of NTHi, a lack of memory T lymphocytes might contribute to the poor antibody response despite repeated exposure to the pathogen. These results provide further information on the immunological aspects of otitis proneness.

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Serotype Distribution and Penicillin Resistance of *Streptococcus pneumoniae* Isolates from Middle Ear Fluids of Pediatric Patients with Acute Otitis Media in Japan▽

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Out of 175 pneumococcal isolates from middle ear fluids, 26.3% were penicillin-resistant *S. pneumoniae* (PRSP). Serotypes 19F and 23F occurred most frequently among PRSP strains. The 7-valent pneumococcal conjugate vaccine (PCV) showed better coverage of PRSP strains (87.0%). The 7-valent PCV may reduce the prevalence of PRSP in Japan.

Acute otitis media (AOM) is one of the leading infectious diseases caused by *Streptococcus pneumoniae* (3, 9). In recent decades, penicillin-resistant *S. pneumoniae* (PRSP) has evolved into a global problem, especially with AOM (7, 12, 18, 21). The 7-valent pneumococcal conjugate vaccine (PCV) introduced in the United States and Europe has reduced the incidences of invasive pneumococcal disease and AOM (8, 13, 17, 25, 26). Nasopharyngeal carriage and transmission in children were also reduced (24). However, in Japan, use of the 7-valent PCV has not yet been licensed, and little is known about the distribution of pneumococcal serotypes. Prior to the introduction of 7-valent PCV in Japan, it is important to determine the distribution of pneumococcal serotypes in Japan as well as the prevalence of antimicrobial-resistant pneumococci associated with AOM. In this study, we evaluated the distribution of pneumococcal serotypes among children with AOM and determined the serotype coverage of pneumococcal vaccine formulas in Japan.

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One hundred seventy-five pneumococcal isolates were collected randomly from middle ear fluids of 175 children with AOM at outpatient clinics in different regions of Japan during nationwide surveillance from February 2006 to June 2007. The range and quartiles of the ages of the subjects were 1 to 127, 12.8 (25th percentile), 17.0 (median), and 42.3 (75th percentile) months. Susceptibilities to penicillin G (PCG) were tested, and breakpoints were defined according to the Clinical and Laboratory Standards Institute (CLSI) (6). All isolates were serotyped or serogrouped by the capsular quelling method with pneumococcal capsule-specific antisera (Statens Serum Institut, Copenhagen, Denmark) as recommended by the manufacturer. The chi-square test and Fisher's exact test were used for categorical variables to test for significant differences between groups with Prism 4 (GraphPad Software, Inc.). A P value of <0.05 was considered statistically significant.

Out of 175 pneumococcal isolates, 63 (36.0%), 66 (37.7%), and 46 (26.3%) were penicillin-susceptible *S. pneumoniae* (PSSP), penicillin-intermediate-resistant *S. pneumoniae* (PISP), and PRSP, respectively (Table 1). The predominant serotype was 19F (19.4%), followed by 23F (14.9%), 14 (11.4%), 6B (11.4%), 6A (9.1%), and 3 (9.1%). The distribution of *S. pneumoniae* serotypes among PCG-susceptible strains was significantly different from that among PCG-resistant strains ($P < 0.01$). Serotype 3 was the most prevalent PSSP serotype (odds ratio [OR], 78.2; 95% confidence interval [CI], 4.6 to 1,330; $P < 0.01$), while serotype 19F (OR, 55.8; 95% CI, 3.4 to 929; $P < 0.01$) and serotype 23F (OR, 17.8; 95% CI, 2.4 to 135.1; $P < 0.01$) were frequently identified as drug-resistant *S. pneumoniae* (both PRSP and PISP). The proportions of serotypes 6A, 6B, and 14 were not different for PCG-susceptible and PCG-resistant isolates. The five predominant serotypes (19F, 23F, 6B, 6A, and 14) represented about 95.7% of the strains identified as PRSP. On the basis of age, PRSP strains were identified in 31.8% of children ≤2 years old, while PSSP strains were identified in 50.0% of children ≥3 years old. Serotypes 6A, 6B, 19F, and 23F were prevalent in children ≤2 years old, while serotype 3 was frequently identified in children ≥3 years old.

The total percentages (95% CIs) of serotypes covered by the 7-valent, 10-valent, 13-valent, and 23-valent pneumococcal vaccines were 60.6 (53.3 to 68.7), 61.7 (54.5 to 68.9), 82.9 (77.3 to 88.4), and 73.7 (67.2 to 80.2), respectively (Table 2). The percentages (95% CIs) of serotypes identified as PRSP and covered by the 7-valent, 10-valent, 13-valent, and 23-valent pneumococcal vaccines were 87.0 (77.2 to 96.7), 87.0 (77.2 to 96.7),

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TABLE 1. Serotype distribution and penicillin resistance of *S. pneumoniae* isolates from middle ear fluids of Japanese pediatric patients with AOM, based on age

Serotype	No. (%) of isolates from indicated age group											
	≤2 yr				≥3 yr				All			
	PSSP	PISP	PRSP	Total	PSSP	PISP	PRSP	Total	PSSP	PISP	PRSP	Total
1						1 (1.5)		1 (1.5)		1 (0.6)		1 (0.6)
3	3 (2.8)			3 (2.8)	13 (19.1)			13 (19.1)	16 (9.1)			16 (9.1)
4					1 (1.5)			1 (1.5)	1 (0.6)			1 (0.6)
6A	5 (4.7)	6 (5.6)	3 (2.8)	14 (13.1)	1 (1.5)		1 (1.5)	2 (2.9)	6 (3.4)	6 (3.4)	4 (2.3)	16 (9.1)
6B	7 (6.5)	9 (8.4)	3 (2.8)	19 (17.8)	1 (1.5)			1 (1.5)	8 (4.6)	9 (5.1)	3 (1.7)	20 (11.4)
7F	1 (0.9)			1 (0.9)					1 (0.6)			1 (0.6)
9V					2 (2.9)			2 (2.9)	2 (1.1)			2 (1.1)
9N	1 (0.9)			1 (0.9)					1 (0.6)			1 (0.6)
10A	3 (2.8)			3 (2.8)	3 (4.4)			3 (4.4)	6 (3.4)			6 (3.4)
11A	1 (0.9)			1 (0.9)	1 (1.5)			1 (1.5)	2 (1.1)			2 (1.1)
12F	1 (0.9)			1 (0.9)	1 (1.5)			1 (1.5)	2 (1.1)			2 (1.1)
14	1 (0.9)	7 (6.5)	2 (1.9)	10 (9.3)	3 (4.4)	6 (8.8)	1 (1.5)	10 (14.7)	4 (2.3)	13 (7.4)	3 (1.7)	20 (11.4)
15B		2 (1.9)		2 (1.9)	1 (1.5)	1 (1.5)		2 (2.9)	1 (0.6)	3 (1.7)		4 (2.3)
18C	2 (1.9)			2 (1.9)	1 (1.5)			1 (1.5)	3 (1.7)			3 (1.7)
19A	1 (0.9)			1 (0.9)	4 (5.9)			4 (5.9)	5 (2.9)			5 (2.9)
19F		11 (10.3)	13 (12.1)	24 (22.4)		4 (5.9)	6 (8.8)	10 (14.7)		15 (8.6)	19 (10.9)	34 (19.4)
22F					1 (1.5)			1 (1.5)	1 (0.6)			1 (0.6)
23F	1 (0.9)	5 (4.7)	12 (11.2)	18 (16.8)		5 (7.4)	3 (4.4)	8 (11.8)	1 (0.6)	10 (5.7)	15 (8.6)	26 (14.9)
Group 23		2 (1.9)	1 (0.9)	3 (2.8)		3 (4.4)		3 (4.4)		5 (2.9)	1 (0.6)	6 (3.4)
non23	2 (1.9)	2 (1.9)		4 (3.7)	1 (1.5)	2 (2.9)	1 (1.5)	4 (5.9)	3 (1.7)	4 (2.3)	1 (0.6)	8 (4.6)
Total	29 (27.1)	44 (41.1)	34 (31.8)	107 (100)	34 (50.0)	22 (32.4)	12 (17.6)	68 (100)	63 (31.2)	66 (32.7)	46 (22.8)	175 (100)

95.7 (89.8 to 100), and 87.0 (77.2 to 96.7), respectively. Among children ≤2 years old, the total percentages (95% CIs) of serotypes covered by the 7-valent, 10-valent, 13-valent, and 23-valent pneumococcal vaccines were 68.2 (59.4 to 77.0), 69.2 (60.4 to 77.9), 86.0 (79.4 to 92.6), and 72.9 (64.5 to 81.3), respectively. Among these same children, the percentages (95% CIs) of serotypes identified as PRSP and covered by the 7-valent, 10-valent, 13-valent, and 23-valent pneumococcal vaccines were 88.2 (77.4 to 99.1), 88.2 (77.4 to 99.1), 97.1 (91.4 to 100), and 88.2 (77.4 to 99.1), respectively (Table 2). Among children ≥3 years old, the total percentages (95% CIs) of serotypes covered by the 7-valent, 10-valent, 13-valent, and 23-valent pneumococcal vaccines were 48.5 (36.7 to 60.4), 50.0 (38.1 to 61.9), 77.9 (68.1 to 87.8), and 75.0 (64.7 to 85.3), respectively. Among these same children, the percentages (95% CIs) of serotypes identified as PRSP and covered by the 7-valent, 10-valent, 13-valent, and 23-valent pneumococcal vaccines were 83.3 (62.2 to 100), 83.3 (62.2 to 100), 91.7 (76.0 to 100), and 83.3 (62.2 to 100), respectively (Table 2).

Currently available pneumococcal vaccines are designed to elicit antibodies to the capsular polysaccharides of the most common pneumococcal serotypes. Serotyping of pneumococcal isolates from patients is an important tool for monitoring the effectiveness of pneumococcal vaccines (4, 8, 14). However, there are a limited number of clinical trials, and the precise distribution of serotypes among children with AOM in Japan still remains unclear (1, 5). Extreme diversity of distribution in pneumococcal serotypes is a common phenomenon and is of great concern for researchers in this field. As documented in previous reports, penicillin-resistant strains were frequently identified among children ≤3 years old (11). The serotype distributions among Japanese children with AOM were similar to those reported for children from the United States and Europe (2, 10, 15, 18). While the 7-valent PCV has demon-

TABLE 2. Serotype coverage of pneumococcal vaccines among Japanese pediatric patients with AOM, based on age and penicillin susceptibility

Vaccine type (established formula)	No. (%) of isolates from indicated age group					
	≤2 yr		≥3 yr		All	
	PRSP (n = 34)	Total (n = 107)	PRSP (n = 12)	Total (n = 68)	PRSP (n = 46)	Total (n = 175)
7-Valent (4, 6B, 9V, 14, 18C, 19F, 23F)	30 (88.2)	73 (68.2)	10 (83.3)	33 (48.5)	40 (87.0)	106 (60.6)
10-Valent (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F)	30 (88.2)	74 (69.2)	10 (83.3)	34 (50.0)	40 (87.0)	108 (61.7)
13-Valent (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F)	33 (97.1)	92 (86.0)	11 (91.7)	53 (77.9)	44 (95.7)	145 (82.9)
23-Valent (1, 3, 4, 5, 6B, 7F, 8, 9V, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 22F, 23F, 33F)	30 (88.2)	78 (72.9)	10 (83.3)	51 (75.0)	40 (87.0)	129 (73.7)

strated its efficacy in the United States and Europe, the formulation of a vaccine based on the existing data from the United States and from European countries may not be optimal for some Asian countries, because the distributions of *S. pneumoniae* serotypes differ among nations (8, 17, 25, 26). The 7-valent PCV covers most of the strains of PRSP found in Japanese children, especially those <3 years old. These findings have implications for the potential use of the 7-valent PCV in Japan. Serotype 6C was recently discovered among serotype A pneumococcal strains (19, 20). Consequently, the cross-protection provided against serotype 6C by the currently available pneumococcal vaccine containing antibodies against 6B may be inadequate, and the vaccine may reduce the prevalence of serotype 6A but not serotype 6C (19, 20). We must therefore pay attention to the prevalence of serogroup 6 before and after the introduction of 7-valent PCV. The shift in serotype replacement over time also may eventually make a given vaccine formulation obsolete. Several studies have reported a change in serotype after vaccination, with the increases in serotypes 3, 6A, 19A, and 23A being of particular concern (16, 22–23). Serotype 19A emerged as the most frequent cause of invasive pneumococcal disease in the United States after the introduction of the 7-valent PCV (22). In Japan, where the 7-valent PCV has not yet been licensed, the prevalence of the serotype 19A strain is currently low. Continuous surveillance is necessary to monitor the distribution of antimicrobial-resistant pneumococci and their serotypes before and after the introduction of the 7-valent PCV.

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Prevalence of Streptococcus Invasive Locus (*sil*) and Its Relationship with Macrolide Resistance among Group A *Streptococcus* Strains▽

A recent study by Bidet et al. (1) reported the molecular epidemiology of the streptococcal invasive locus (*sil*) in the group A streptococcus (GAS), an organism which caused invasive infections in French children. The authors demonstrated the prevalence of *emm* type toxin genotypes among 74 invasive GAS isolates from French children. The authors PCR amplified and characterized the locus DNA of *sil* from invasive isolates, but there were no data concerning noninvasive isolates. It seems that the invasive locus was present not only in invasive isolates but possibly also in noninvasive isolates. Therefore, we conducted a study in which our aims were (i) to examine the prevalence of *Streptococcus pyogenes* exotoxins in relationship to the *sil* gene in invasive and noninvasive isolates of GAS, (ii) to define whether *sil* was predominantly present only in invasive isolates or also in noninvasive isolates of GAS, and (iii) to characterize the relationship between GAS and macrolide resistance.

To set up our hypothesis, we examined 242 noninvasive isolates (tonsillitis, 170 isolates; rhinosinusitis, 51 isolates; and acute otitis media, 21 isolates) and 13 invasive isolates (septicemia, 5 isolates; purulent arthritis, 4 isolates; meningitis, 2 isolates; necrotizing fasciitis, 1 isolate; and peritoneal abscess, 1 isolate) of GAS, which were isolated from individual patients. *emm* typing of GAS strains was performed by DNA sequencing according to the recommendations of the Division of Bacterial and Mycotic Diseases, the Centers for Disease Control and Prevention, and the *emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). Multiplex PCR was used for toxin gene (*speA*, *speB*, *speC*, *speF*, *speG*, *speH*, *speJ*, *ssa*, and *smeZ*) profiling, as described by Schmitz et al. (5). PCR detection of the *sil* locus was performed according to the method described by Bidet et al. (1). Macrolide resistance genes of GAS were determined by the PCR methods described by Weber et al. (6). To study the degree of macrolide resistance, MICs of azithromycin to all strains were determined by broth microdilution, using the standard method (2). All the experiments were conducted in duplicate.

Among the 242 noninvasive isolates, 11.98% (29/242) harbored the *sil* gene in their genomic DNA. The *emm* types and the toxin gene profiles of *sil*-positive isolates are shown in Table 1. In noninvasive strains, the *sil* locus was detected in 9 out of 33 *emm* types found in the collection (27.27%), and 41.4% (12/29) of the *sil*-positive isolates belonged to *emm* type 4. *emm* type 4 (12 isolates), *emm* type 48 (3 isolates), and *emm* type 94 (6 isolates) represented 72.41% (21/29) of the *sil*-positive isolates. All of the *sil*-positive noninvasive isolates carried *speB* alleles, but 68.96% of strains carried *speC*. There were no significant differences between the toxin gene profile of the *sil*-positive isolates and that of the *sil*-negative isolates, except for *smeZ*, which was 10.3% of the *sil*-negative isolates but 31% of the *sil*-positive noninvasive isolates. Seventy-five percent of *emm* type 4, 75% of *emm* type 48, 100% of *emm* type 94, 100% of *emm* type 53, 100% of *emm* type 54, and 100% of *emm* type 102 isolates harbored the *sil* gene in their DNA.

Although we used limited numbers of invasive isolates, 15.4% of the invasive GAS isolates harbored the *sil* gene, which is consistent with data from a previous study of invasive strains, which showed that 16% carried the *sil* gene (1). One hundred percent of *emm* type 87 and 100% of *emm* sequence type 1732 were positive for the invasive locus. Thirty percent of the *sil*-negative invasive isolates carried *speA* alleles, but all *sil*-positive isolates were negative for the *speA* gene. All strains were positive for the *speB* gene. Fifty percent of the *sil*-positive isolates were positive for *speC*, but 30% of the *sil*-negative isolates were positive for *speC*. There is no statistical significance in the prevalence of the *sil* gene among invasive and noninvasive isolates (Fisher's exact test, $P = 0.499$).

Among 255 invasive and noninvasive isolates, 16.86% (3 were invasive, and 40 were noninvasive; total, 43/255) of the isolates were azithromycin resistant and were positive for macrolide-resistant genes (Table 2). Among these strains, 65.12% (28/43), 13.95% (6/43), and 20.93% (9/43) of the strains possessed the *mef*(A), *erm*(B), and *erm*(TR) genes, respectively. All *sil*-positive isolates were sensitive to azithromycin and were negative for macrolide resistance genes (Fisher's exact test, $P < 0.006$).

From these result, we concluded that *sil* is present not only among invasive isolates but also among noninvasive isolates, with similar prevalences (15.4% versus 11.98%, respectively). To our knowledge, this is the first report to show the prevalence rates of *sil* in both invasive and noninvasive isolates of GAS in Japan. The predominant *emm* types that harbored *sil* were *emm* type 4, *emm* type 94, and *emm* type 48. Hidalgo-Grass et al. identified *sil* in the invasive serotype M14 clone, the organism that caused necrotizing fasciitis in Israel (3). In our study, *sil* was absent from *emm* type 3 isolates, a finding comparable to that in a previous study and associated with GAS invasive diseases worldwide (3). The *sil* locus was confirmed by direct sequencing of several representative PCR-

TABLE 1. Characteristics of streptococcal toxin gene profile of invasive and noninvasive *sil*-positive isolates[a]

Isolate type	*emm* type	Sequence type	No. of isolates	Pyogenic exotoxin				
				speA	*speB*	*speC*	*speH*	*smeZ*
Noninvasive	1		1	+	+	−	−	+
	1		1	−	+	−	−	+
	4		4	+	+	+	−	−
	4		2	−	+	+	−	−
	4		5	−	+	+	−	+
	4		1	−	+	+	+	+
	11		1	−	+	+	−	−
	48		3	−	+	+	−	−
	53		1	−	+	+	+	+
	54		1	−	+	+	−	−
	75		2	−	+	−	−	−
	94		4	−	+	−	−	−
	94		1	−	+	+	−	−
	94.1		1	−	+	−	−	−
	102.2		1	−	+	+	−	−
Invasive	87		1	−	+	+	−	−
		1732	1	−	+	−	−	−

[a] Characteristics of streptococcal toxin gene profile indicating the presence (+) and absence (−) of invasive and noninvasive *sil*-positive isolates.

TABLE 2. Relationship between *sil*-positive and macrolide-resistant genes and invasive and noninvasive GAS[a]

sil gene	No. of isolates				
	Macrolide resistance gene				Total
	Positive			Negative	
	mef(A)	*erm*(B)	*erm*(TR)		
Positive	0	0	0	31	31
Negative	28	6	9	181	224
Total	28	6	9	212	255

[a] Significant differences are based on a Fisher's exact test P value of <0.006.

amplified products and comparing those with the previous sequence. The overall prevalence of the *sil* locus in invasive isolates was the same as that from a previous study (16% versus 15.4%, respectively) (1). Up to now, there was no study which showed the status of noninvasive strains with the *sil* gene. When we examined noninvasive strains, the *sil* gene was found in 12% of isolates, which is not a remarkably different rate from that found in invasive isolates. All *sil*-positive isolates were negative for macrolide resistance genes, which were irreversibly important for clinical practice. Future studies should focus on a better understanding of the role of *sil* in the pathogenesis of GAS infection and its relationship with macrolide resistance. A recent candidate vaccine based on the M protein failed to elicit antibodies to serotype M4, and *sil*-encoded proteins might represent alternative vaccine targets for this serotype (4). The results of this study should contribute to a better understanding of the pathogenesis of GAS, as well as the epidemiology of GAS-associated disease, and to the establishment of methods for the prevention of diseases caused by GAS in Japan.

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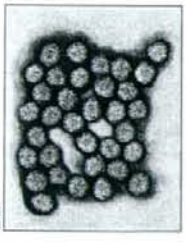
pain above the midpoint of the scale (pain >3), which is interpretable even if the data are ordinal. All of our major conclusions for the average pain rating also hold with these measures (see tables 1 and 2).

Although we agree with Avendano and Van Soest that vignettes are potentially a fruitful way to anchor subjective data, the study that they cite[1] has little relevance to our results. We collected data on reported pain corresponding to particular moments in time. The vignette study presented hypothetical stories involving people with back conditions, and asked a panel to assess the extent to which the condition posed a work limitation. Respondents were not asked to rate the intensity of pain, and were not presented with vignettes concerning pain at specific moments. The vignette study thus does not shed any light on whether older individuals or women have higher thresholds for reporting pain.



Corbis

More relevant evidence is from studies that expose participants to a common pain stimulus and then ask them to rate the intensity. Such studies do not find consistent evidence of higher thresholds for reporting pain for older individuals.[2]

Rather than differential thresholds, we suspect that our findings result from the use of a more accurate diary method. The US Centers for Disease Control and Prevention's (CDC's) survey of number of days of pain recalled in the past month finds a monotonically increasing relation between pain and age, and higher reported pain for women than men.[3] Why would differential pain thresholds by age and sex affect our survey but not the CDC survey?

Finally, the diary method allows us to estimate pain intensity for the same individual as he or she engages in different activities. This controls for the main effects of personal characteristics on pain thresholds and other reporting biases. Thus, as far as the pattern of pain during various activities is concerned, our technique is robust to interpersonal differences in pain thresholds.

ABK and AAS are consulting senior scientists to the Gallup Organisation. AAS is associate chair of the scientific advisory board for Invivodata.

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Rotavirus vaccine for developing countries

The Article by Alexandre Linhares and colleagues (April 5, p 1181)[1] provides valuable information on the efficacy and safety of an oral rotavirus vaccine against rotavirus gastroenteritis in Latin American infants.

The vaccine had an efficacy of 82% against G-P homotypic G1P[8] infections and of 81% against P homotypic G3P[8], G4P[8], or G9P[8] infections. However, it afforded only 39% protection against G-P heterotypic G2P[4] strains.

Linhares and colleagues' study, and the accompanying Comment,[2] show that G1P[8] strains are the prevalent types all over the world, and that G2P[4] were less frequently isolated in the study. However, a study by Rahman and colleagues[3] in Bangladesh showed that, during 2001–05, G1P[8] was dominant (36·4%), followed by G9P[8] (27·7%), G2P[4] (15·4%), and G12P[6] (3·1%), but that, in 2005–06, G2P[4] was the most prevalent strain (43·2%) and G12P[6] became more prevalent (11·1%). The recently licensed RIX4414 (Rotarix) vaccine studied by Linhares and colleagues includes only P[8] specificity, with less efficacy against heterotypic non-P[8].

High temperatures, low humidity, high rainfall, and high river levels increase the incidence of rotavirus infections in Asia and Africa.[4] The currently available Rotarix vaccine might provide good coverage against rotavirus strains in wealthier countries, but for developing countries we might need a vaccine containing different rotavirus strains.

We declare that we have no conflict of interest.

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Alexandre Linhares and colleagues'[1] study on the effectiveness of rotavirus vaccine in Latin America, and the accompanying Comment[2] on the importance of circulating rotavirus strains, highlight the international interest in the potential heterotypic cross-protection offered by these vaccines. This question will be answered as the vaccines are used more widely and a greater diversity of strains is encountered.

However, the perception of "strain replacement" as the vaccines are introduced needs to be considered. A healthy dose of caution should be exercised in interpreting the potential emergence of new rotavirus strains—

China and India's challenges: the other end of the spectrum

In your Aug 16 Editorial,[1] you comment on a UNICEF report on maternal, newborn, and child survival in the Asia-Pacific region, focusing on China and India. The other end of the demographic spectrum is not blessed with the resources to produce such useful reports, but ageing and adult health will have a huge effect on the Asia-Pacific region and the world.

India and China represent more than 33% of the world's older population, with 275 million adults aged 60 years and older.[2] By the year 2019 in China and 2042 in India, the proportion of people older than 60 years will exceed that of people aged 0–14 years. The proportion of older adults living in the Asia-Pacific region will increase from 55% to 61% of the world's older population by 2030.

The Global Burden of Disease 2004 update[3] indicated that 44% of the total Chinese burden of disease and a quarter of the Indian burden is concentrated in those aged 45 years and older. Longevity continues to increase, but whether the extra years lived will be in good health is not known; nor is the expected burden for health systems.[4]

With their economies booming and health-care systems brewing, China and India are indeed at a crossroads.[5] Data are urgently needed to inform decision making, especially for adult health needs.

I declare that I have no conflict of interest.

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Wealth and child survival: India and Bangladesh

Your Aug 16 Editorial[1] emphasises that India is far from its target of reaching Millennium Development Goal 4 on child survival, despite its impressive rate of economic growth compared with the other south Asian nations. You state that India is spending only 3% of its gross domestic product (GDP) on health, which is less than the other countries in the Asia-Pacific region; however, India has actually been spending only 0·9% of its GDP on heath for the past two decades.[2] 2–3% of GDP is the predicted level of spending by the Indian Government by 2010.[2]

Although the link between poverty and child mortality is very strong, some countries are better at translating their economic growth into preventing child deaths. For example, India's gross national income (GNI) per head has increased by a staggering 82% from US$450 in 2000 to $820 in 2006, yet its child mortality rate only declined by 19% from 94 per 1000 births to 76 per 1000. Over the same period, Bangladesh saw a much smaller 23% increase in GNI per capita—from $390 in 2000 to $480 in 2006—but its child mortality dropped by 25% from 92 to 69 per 1000 births.[3,4] The maternal mortality rate also declined from 440 per 10 000 births in 1997 to 315 in 2001 in Bangladesh.[5]

All countries, even the poorest, can reduce child mortality if they pursue the right policies and prioritise their poorest families. Good government choices save children's lives but bad ones are a death sentence.

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Fractures are not in genes

J B Richards and co-workers (May 3, p 1505)[1] report that two gene variants of key proteins in bone metabolism, LDL-receptor-related protein 5 (LRP5) and osteoprotegerin, increase the risk of osteoporosis and osteoporotic fracture. They suggest that these genetic markers could be used to screen individuals who are most at risk of fracture, but the evidence provided is neither consistent nor convincing.

First, Richards and colleagues found that the risk allele of the *LRP5* gene increased the risk of osteoporosis (odds ratio 1·3, p=0·008) and osteoporotic fracture (1·3, p=0·002), whereas the risk allele of the osteoprotegerin gene increased the risk of osteoporosis (1·2, p=0·038) but not fracture (1·1, not significant). The clinical relevance of these weak associations seems modest, especially by comparison with falling.[2–4] A sideways fall increases the risk of hip fracture three to five times, and when such a fall causes an impact to the greater trochanter of the proximal femur, hip fracture risk is raised 20–30 times.[4] Even a simple measurement of bone-mineral density (BMD) functions better than the above-noted risk alleles: a 1 SD reduction in BMD doubles the fracture risk.[4] Could

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Formation of biofilm by *Haemophilus influenzae* isolated from pediatric intractable otitis media

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Abstract

Objectives: The aims of this study are to evaluate biofilm formation by nontypeable *Haemophilus influenzae* (NTHi) isolated from children with acute otitis media (AOM) and its relation with clinical outcome of the disease.
Methods: Biofilm formations by NTHi clinical isolates from pediatric AOM patients were evaluated by a crystal violet microtiter plate and a 98 well pin-replicator assay with a confocal laser scanning microscopy (CLSM). Optical density values of clinical isolates were compared with a positive control and the ratio of clinical isolates to a positive control was defined as biofilm formation index (BFI).
Results: 84.3% clinical isolates of NTHi were biofilm forming strains (BFI $\geq$ 0.4). The BFI represented the levels of biofilm formation and adherence on the surface. The identical strains isolated from both middle ear fluids (MEFs) and nasopharynx showed biofilm formation at the same level. The prevalence of biofilm forming isolates was significantly higher among the susceptible strains than resistant strains. The level of biofilm formation of NTHi isolated from AOM cases who was not improved by amoxicillin (AMPC) was significantly higher than that of NTHi isolated from AOM cases who was improved by AMPC.
Conclusion: We clearly showed the biofilm formation of clinical NTHi isolates from AOM children. In addition, the biofilm formed by NTHi would play an important role in persistent or intractable clinical course of AOM as a result of lowered treatment efficacy of antibiotics.

Keywords: Biofilm; Nontypeable *H. influenzae*; Acute otitis media; β-Lactamase non-producing ampicillin resistant (BLNAR); Genotype

1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) colonizes in the human nasopharynx and becomes a major causative pathogen responsible for both acute otitis media (AOM) and otitis media with effusion (OME) [1]. In recent years, NTHi has been shown to form biofilm *in vitro* and *in vivo* [2–7]. The biofilm is defined as a group of bacteria growing as a community in a self-produced polymeric matrix [8]. Residence within a biofilm brings several benefits to pathogens for being protected from environmental threats such as host immune defenses, antimicrobial agents and surfactants [6,9,10]. Bacteria in biofilms are highly resistant to immune mediated clearance [11]. However, the composition of the biofilm is quite diverse depending on the organism and the environment in which the biofilm was produced.

The clinical outcomes of AOM are closely related with eradication of pathogens from middle ear [12,13]. Intractable clinical course is associated with persistent bacterial infections in spite of antimicrobial treatments. The biofilm is recently considered to be involved into the persistent bacterial infections caused by NTHi [14]. Several studies show that the ability to form biofilms contributes the recurrent and/or persistence of AOM caused by NTHi [3,14–16]. The genomic DNA of NTHi is frequently identified in middle ear fluids (MEFs) of OME while conventional bacterial culture failed to identify NTHi [17–19]. The

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biofilm formation can explain the failure to culture NTHi from MEFs and resistance to antibiotics even though the strains are susceptible *in vitro*.

In the current study, an attempt was taken to better understand the role of biofilm formation in the pathogenesis of AOM caused by NTHi and for that purpose we evaluated biofilm formations by NTHi isolated from children with AOM in comparison with clinical outcome of the disease.

2. Materials and methods

2.1. H. influenzae strains

A total 109 NTHi clinical isolates from 62 pediatric patients with AOM were used in this study. They were 40 females and 62 males ranging in age from 7 to 68 months (mean 20 months). Bacterial culture was performed before use of antibiotics. *H. influenzae* were identified and confirmed by colony morphology, Gram's staining, growth on chocolate but not on blood agar, the catalase test, and the X and V factor requirement. Productions of β-lactamase were examined using a nitrocefinase disc (Nippon Becton Dickinson Company Ltd., Tokyo, Japan). Serotyping of *H. influenzae* was determined by a slide agglutination procedure using type b antiserum (Denka Seiken, Tokyo, Japan). Until the study, the isolates were stocked in Litmus milk broth (Difco Laboratories, Detroit, MI, USA) containing 10% glycerol at −80 °C. Bacteria were reconstituted from frozen stocks and cultured on chocolate agar plates (Nippon Becton Dickinson Company Ltd., Tokyo, Japan) overnight at 37 °C. The strains were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with 10 mg/ml of hemin (Sigma Chemical Co., St. Louis, MO) and 2 mg/ml of NAD (Sigma) overnight at 37 °C. Biofilm positive (HI-139) and negative (HI-102) strains as controls were kindly provided from Prof. Hiroshi Watanabe, Department of Infectious Disease, University of Kurume. All clinical isolates were used at 3rd generation to prevent loss of phenotypic characteristics.

2.2. Susceptibility to ABPC

The minimal inhibitory concentration (MIC) to ampicillin (ABPC) was determined using the microbroth dilution method according to the procedure set forth by the Clinical and Laboratory Standards Institute (CLSI) (M 100-S17; 2007). The breakpoints recommended by the CLSI were used to define susceptibility patterns to ABPC. Briefly, the NTHi strains with MIC to ABPC ≤ 1 and ≥ 2 μg/ml were defined as susceptible and resistant, respectively.

2.3. Microtiter biofilm formation assay

The formation of biofilm by NTHi isolates was evaluated by a modification of the microtiter plate assay with crystal violet stain [20]. The assay is based on the ability of bacteria adhered on solid polystyrene surfaces. Briefly, NTHi isolates grown overnight in BHI broth were washed with sterile phosphate buffered saline (PBS) and diluted 1:200 in fresh BHI broth. Two hundred microliters of NTHi suspensions were inoculated into wells of a non-tissue culture treated polystyrene flat-bottomed 96-well microtiter plates (Nunc, Kracker Scientific, Inc, Albany, NY). The plates were incubated at 37 °C for 12–24 h aerobically in 5% CO_2 environment. Before the quantification of biofilm formations, the growth of NTHi was assessed by measuring the absorbance of optical density at 600 nm (OD_{600}). Culture media including unattached bacteria were decanted from the wells, and remaining planktonic NTHi cells were removed by rinsing with distilled water. The wells were air-dried and adhered bacteria were stained with 0.5% (w/v) crystal violet solution (Sigma) for 15 min. After rinsing with distilled water, bound crystal violet was released from NTHi cells by 30% of glacial acetic acid. This allowed us indirect measurement of biofilm formations on both bottom and sides of wells. The level of biofilm formations was quantified by measuring absorbance of OD_{570}.

In this study, we defined the ratio of OD_{570} of clinical isolates to OD_{570} of a standard strain producing biofilm (kindly provided from Prof. Watanabe, Division of Infectious Disease, Department of Infectious Medicine, Kurume University School of Medicine) as biofilm formation index (BFI). The mean-2 S.D. levels of BFI of the standard strain forming biofilm was 0.4. The BFI of a standard strain without biofilm formation was also 0.4. Thus, we defined BFI of 0.4 as cut off value and defined the strain showing BFI >0.4 as a strain forming biofilm in this study.

2.4. 96-Well pin-replicator assay

The biofilm formations and adherence of NTHi were evaluated by a 96-well pin-replicator assay with a confocal laser scanning microscopy (CLSM). The NTHi isolates grown in BHI broth until mid-log phase ($OD_{600} = 0.5$) at 35 °C were washed with sterile PBS and then diluted 1:200 in fresh BHI broth. One hundred microliters of the NTHi suspensions were inoculated into wells of a 96-well pin-replicator (Nunc) and incubated for 24 h at 35 °C with gentle shaking. After incubation, the pins were washed with sterilized PBS and stained with SYTO9 (6) and LPA-TRITC (EY laboratories, San Mateo, CA, USA) for 15 min at room temperature. The specimens were viewed with a Nikon TE-300 microscope attached to a BIO-RAD LSM Radiance 2100 confocal imaging system.

2.5. Polymerase chain reaction (PCR)-based genotyping

Genotypes of NTHi isolates were evaluated by PCR protocol reported elsewhere [21]. Briefly primers for the *ftsI* gene were used to amplify both the variable mutated locus

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(Asn-526 or Arg-517) and a highly mutated locus (Ser-385) [21]. A single colony of NTHi on chocolate agar plates were lysed in 30 μl of lysis solution (1 M Tris pH 8.9, 4.5 (v/v) nonidet P-40, 4.5 (v/v) Tween 20, and 10 mg/ml Proteinase K) for 10 min at 60 °C and for 5 min at 94 °C in the programmable thermal cycler (Gene Amp PCR System 9700, ParkinElmer, Norwalk, CT, USA). A total 50 μl of reaction mixture consisted of 2 μl of bacterial lysate, 0.8 μl of 10 mM of a dNTP mixture, 0.1 μl of *Taq* DNA polymerase, 2.5 μl of 10× PCR buffer, 0.5 μl of 25 mM $MgCl_2$, 5.0 μl Q-solution (Qiagen, Valencia, CA, USA), and 0.125 μl (100 μM) each of primer and distilled water. The mixture was subjected to denaturation at 94 °C for 10 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s and then further extension at 72 °C for 10 min. Amplified DNA fragments were analyzed using 3% agarose gel electrophoresis.

On the basis of PCR amplifications, NTHi strains were classified into five genotypes such as β-lactamase non-producing ampicillin susceptible (gBLNAS) strains without amino acid substitution in the *ftsI* and β-lactamase gene, β-lactamase nonproducing ampicillin resistance (gBLNAR) group I/II strains with amino acid substitution in the variable mutated locus of *ftsI*, gBLNAR group III strains with amino acid substitution in the highly mutated locus of *ftsI*, β-lactamase producing ampicillin resistance (gBLPAR) strains having *blp* gene but without amino acid substitution in the *ftsI* gene, and β-lactamase producing ampicillin clavulanate resistant (gBLPACR) strains having *bla* gene and amino acid substitutions in the *ftsI* gene. In this study, we have designated PCR-based genotypes to distinguish them from phenotypes, which are written without the introductory "g."

2.6. Restriction DNA fragment polymorphism analyzed by pulsed-field gel electrophoresis (PFGE)

The restriction fragment polymorphisms of *SmaI*-digested chromosomal DNA from NTHi isolates were evaluated by PFGE [22]. Briefly, NTHi cell plug were prepared and incubated with lysis buffer (0.25 M EDTA, 1% SDS, 10 mM Tris–HCl, pH 9.5, and 0.5 mg/ml of proteinase K) overnight at 50 °C. The restriction of genomic DNA was carried out with 80 U of *SmaI* (Takara Shuzo Co. Ltd., Ohtsu, Japan) for 20 h at 30 °C. The reaction was stopped by an equal volume of 0.5 M EDTA (pH 8.0). Electrophoresis was performed with a GenePath PFGE apparatus (Bio-Rad Laboratories, Hercules, CA) in 0.5× TBE buffer (1× TBE buffer: 0.1 M Tris–HCl, 0.1 M boric acid, 2 mM EDTA, pH 8.0). The gels were stained with ethidium bromide for 20 min and were photographed under UV light at 302 nm.

2.7. Statistical analysis

Statistical comparisons were carried out by the Mann–Whitney *U*-test between two groups and Kruskal–Wallis test among 3 groups and above. A p-value of <0.05 was considered statistically significant. Correlation between MEFs isolates and nasopharyngeal swab isolates was assessed by spearman test. Calculations were performed using the statistical software package Prism4 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Biofilm formation of NTHi

Among 70 NTHi clinical isolates from nasopharynx, 59 (84.3%) isolates were defined as biofilm forming isolates (BFI ≥ 0.4) by crystal violet assay (Fig. 1A). We further evaluated adhesion of NTHi to the polystyrene surface as well as the biofilm formation by CLSM. The HI-202 strain (BFI = 0.7) formed biofilm highly and adhered well on the polystyrene surface. In contrast, the HI-285 strain (BFI = 0.3) formed small quantity of biofilm and could

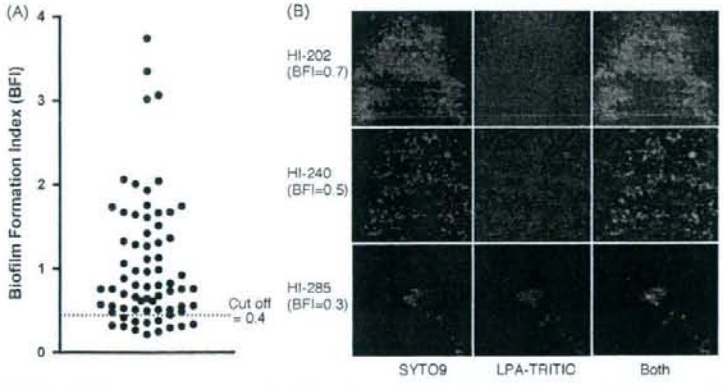

Fig. 1. Evaluation of biofilm formation of NTHi by crystal violet assay and confocal laser scanning microscope. (A) Crystal violet assay, (B) confocal laser scanning microscope (CLSM)

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not adhere on the surface (Fig. 1B). The HI-240 (BFI = 0.5) moderately formed biofilm and adhered on the surface. These results confirmed that the BFI by the crystal violet assay was highly correlated with the levels of biofilm formation and adherence on the surface.

3.2. Comparison of the biofilm formation between middle ear isolates and nasopharyngeal isolates

Thirty-nine pairs of NTHi strains isolated simultaneously from both middle ear fluids and nasopharyngeal swabs were evaluated for the biofilm formation. Thirty-six (92.3%) pairs showed same patterns on PFGE and designated as identical strains (data not shown). The BFIs of NTHi isolates from MEFs were well correlated with those of NTHi from NPS ($r = 0.646$, 95% CI = 0.384–0.812, $p < 0.01$) (Fig. 2).

3.3. Relevance of biofilm formation with genotypes and the susceptibility to ABPC

The median values of BFI in susceptible and resistant strains to ABPC were 1.13 and 0.97, respectively. The prevalence of biofilm forming strains (BFI ≥ 0.4) was significantly higher in the susceptible strains than those of the resistant strains ($p < 0.05$) while the mean levels of BFI between susceptible strains and resistant strains were similar with each other (Fig. 3). Among the 43 susceptible strains, 40 strains (93.0%) were of biofilm forming, whereas 29 (74.4%) of 39 resistant strains were of biofilm forming. The median value of BFI in gBLNAS, gBLNAR group I/II, and gBLNAR group III were 0.79, 1.33 and 0.97, respectively. The biofilm formation of NTHi also did not show any significant difference among genotypes (Fig. 4).

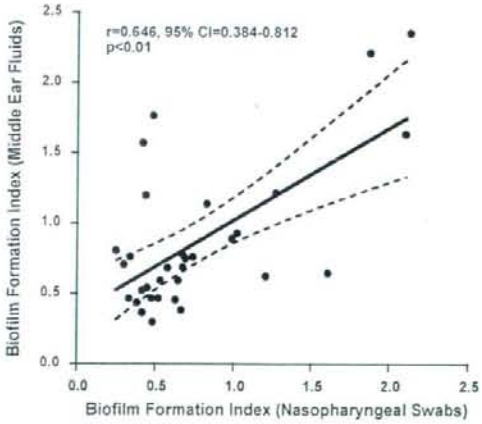

Fig. 2. Correlation of biofilm formation between middle ear and nasopharyngeal isolates. $r = 0.646$, 95% CI = 0.384–0.812, $p < 0.05$

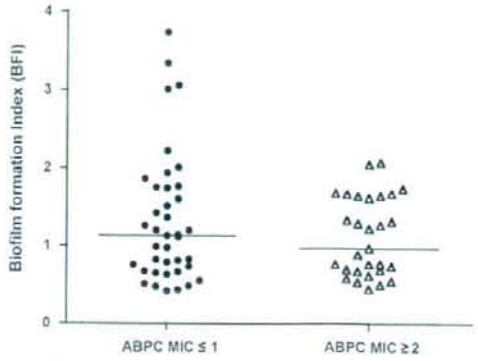

Fig. 3. Biofilm formation according to susceptibilities to AMPC. The median values of BFI in susceptible and resistant strains were 1.13 and 0.97, respectively.

3.4. Role of NTHi biofilm on clinical outcomes of AOM

To evaluate role of biofilm on clinical outcomes of AOM, AOM cases caused by NTHi with MIC to ABPC ≤ 1 μg/ml were classified as improved and non-improved AOM. Our preliminary study about the clinical outcomes of AOM based on the antimicrobial susceptibility of NTHi revealed that the cure ratio of AOM caused by BLNAR was about 30% while those by BLNAS was about 65% on day 14. We mentioned about these finding in results (data not shown). The levels of biofilm formation were compared between two groups. The improved case, 14 patients and 14 strains, was defined the cases that their tympanic membrane findings as redness, bulging and obliteration of landmark became normal within 5–7 days after treatments with amoxicillin (AMPC) (40 mg/kg). The non-improved case, 16 patients and 30 strains, was also defined the cases that their tympanic membrane findings did not become normal over the 14 days after the treatment with AMPC (40 mg/kg). Eleven (78.6%)

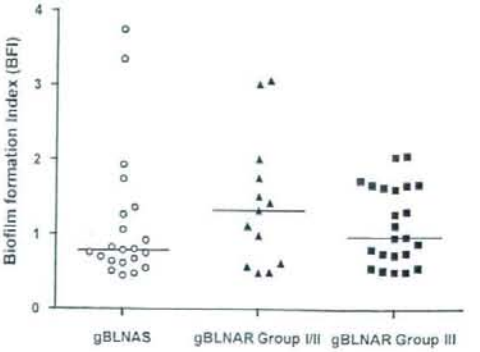

Fig. 4. Relevance of biofilm formation with genotypes of penicillin binding protein. The median value of BFI in gBLNAS, gBLNR group I/II, and gBLNAR group III were 0.79, 1.33 and 0.97, respectively.

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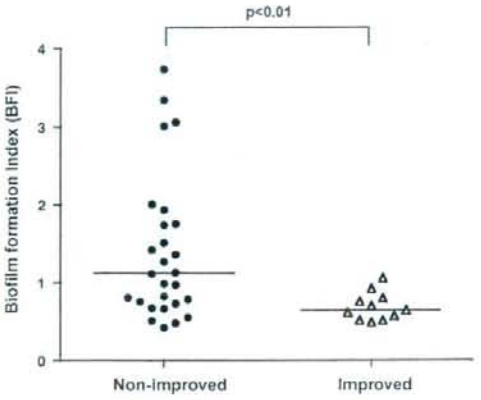

Fig. 5. Roles of NTHi biofilm on clinical outcomes of AOM. Improved case; 14 patients, 14 strains, non-improved case: 16 patients, 30 strains. The median values of BFI in non-improved cases and improved cases were 1.11 and 0.64, respectively.

of 14 isolates from the improved cases were biofilm forming strains. On the other hands, 27 (90.0%) of 30 isolates from non-improved cases were biofilm forming strains. There was no statistical significance in the prevalence of biofilm forming strains between two groups. The median values of BFI in non-improved cases and improved cases were 1.11 and 0.64, respectively. The BFIs of isolates from non-improved cases were significantly higher than those of improved cases ($p < 0.05$) (Fig. 5).

4. Discussion

Biofilm consists of aggregated bacteria surrounded by an extracellular matrix and has been implicated in several persistent bacterial infections [23]. In this report, we focused on the biofilm formation by clinical NTHi isolates and roles of biofilm by this pathogen on clinical outcomes of AOM.

The biofilm formation assays *in vitro* usually characterize clinical isolates and investigate the potential role of certain cell surface antigens in the biofilm formation [24]. The assay applied in this study was based on a use of crystal violet to stain bacteria grown in and attached to polystyrene microtiter plate wells reported by O'Toole and Kolter [20]. Crystal violet predominantly binds to bacterial cell surfaces and also potentially binds to other substances such as extruded DNA. However, there are some questions as to whether the crystal violet assay provides direct evidence for biofilm formation. Because the assay may measures the biomass of viable or possibly even lysed bacteria adherent to a polystyrene surface. In the current study, thus we evaluated the biofilm formation and adherence of live NTHi on the polystyrene surfaces by CLSM. With the increase of the level of biofilm formation determined by crystal violet assay, the levels of biofilm formation and adherence of NTHi on the polystyrene surface also increased. Thus we confirmed that values of biofilm formation evaluated by crystal violet assay could reliably represent the capability of biofilm and subsequent adherence of NTHi.

There have been no reports on the prevalence of biofilm forming strains of NTHi isolated from AOM. Since 83.4% of clinical NTHi isolates from AOM patients were biofilm forming strains in this study, a biofilm might contribute in the pathogenesis of AOM more than it was thought before. Murphy and Kirkham demonstrated that middle ear isolates of NTHi resided within a biofilm [24]. Recent studies reported various factors correlated with the biofilm formation [2,4,25–27]. Expression of lipooligosaccharide (LOS) epitope in a biofilm was altered to those of planktonically grown cells [28]. Swords et al. also showed that sialylation of NTHi LOS promoted biofilm formation and that the phosphorylcholine content of NTHi LOS increased when growing in a biofilm [29]. Further study of experimental otitis media using chinchilla model revealed that sialylated glycoforms of LOS incorporated into the biofilm formation by NTHi [23]. In contrast to these current precise observations, there was less information about biofilm formation related to phenotypes of NTHi [30].

In this study, the prevalence of biofilm formation isolates was higher in susceptible strains rather than resistant strains while the level of biofilm formation was not correlated with antimicrobial susceptibility or genotypes. In contrast, the level of biofilm formation by NTHi isolated from MEFs was similar to that of NTHi isolated from NPS. This is the first report on the biofilm formation related with antimicrobial susceptibilities. Chinchillas infected with a *lic1D* mutant of NTHi showed an increased early inflammatory response because of reduced biofilm formation, although the same mutant of NTHi did not show a defect in biofilm formation *in vitro* [31]. In a different study, *siaA* mutant and *wecA* mutant of NTHi showed reduced biofilm formation, but the alleged biofilms were formed by these mutants in a greater percentage of infected ears in a chinchilla model of otitis media [32]. These findings suggest that NTHi may show a specific phenotype to form biofilm as a natural feature of its life cycle.

The majority of papers on effects of biofilm formation on clinical prognosis of infectious diseases have especially focused on chronic obstructive pulmonary disease (COPD) [9,33,34]. On the other hand, few study have directly examined role of biofilm on the pathogenesis of human AOM. The impact of biofilm on clinical courses of AOM also has not been well documented yet. We found that the level of biofilm formation by NTHi was significantly higher in AOM cases not improved by treatments with AMPC than the cases improved by AMPC. These findings suggest that formation of biofilm by NTHi would be associated with persistent and/or intractable clinical outcome of AOM. NTHi could exist in both planktonic and biofilm states in middle ear cavity. The NTHi may survive in a biofilm and cause persistent or intractable clinical course of AOM. The

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NTHi persists in a biofilm despite intensive antibiotic therapies, and therefore, even antibiotics with clinically attainable concentration may not adequately clear infections. The current findings strongly suggest that a biofilms will be present in the intractable cases of AOM and may help to explain the lack of antibiotic efficacy for this disorder. Surgical drainage of MEFs by myringotomy to reduce the possible biofilm forming NTHi as well as development of effective anti-biofilm agents and antimicrobial agents or application of effective drug delivery systems to eradicate middle ear pathogens will be more important to cope with intractable AOM.

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