grown overnight in brain heart infusion (BHI) broth (BBL, Becton Dickinson Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD, USA) supplemented with 5% digested horse blood at 37°C.

Immunoblot assay

The harvested bacteria were washed 2 times with sterile phosphate buffered saline (PBS). After washing, the bacteria were fixed in 75% ethanol, washed and resuspended in PBS, and stored at −80°C until used. Whole cell preparations from six strains of NTHi were heated at 95°C for 5 min then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% separating gels. Three identical patterns of OMP bands on SDS-PAGE were found among these six strains (data not shown). The pairs of identical strains were; H05-15 and H05-19, H04-03 and H04-06, and H98-224 and H99-115, respectively. We therefore employed H05-19, H04-06 and H99-115 in this study. Whole cell preparations of three strains were applied SDS-PAGE and then transferred to nitrocellulose membrane and incubated with a 1:5,000 dilution of anti-P2 rabbit sera (Yi et al. 1997). The isolated P2 protein from the NTHi strain and the anti-P2 rabbit sera were generous gifts from Dr. TF Murphy, (Buffalo, NY, USA). Horseradish peroxydase-conjugated donkey anti-rabbit IgG at 1:10,000 dilution (GE Healthcare, Bio-Sciences Corp., Piscataway, NJ, USA) was used for detection of rabbit antibodies.

Genotyping with pulsed-field gel electrophoresis (PFGE)

After digesting the genomic DNA of NTHi with *Sma*I (Takara Shuzo Co., Shiga), PFGE was performed on these three strains as described previously (Yano et al. 2000).

Polymerase chain reaction (PCR) and sequence of P2 gene

The nucleotide sequence of the PCR amplicon of *ompP2* gene was performed according to the previously published method (Hiltke et al. 2002). Briefly, bacterial genomic DNA was isolated from 400 μl of an overnight NTHi broth culture employing a Wizard genomic DNA purification kit (Promega Biotech). Using the Gene Amp PCR kit (Applied Biosystems, Tokyo) the *ompP2* gene was amplified using approximately 75 ng of bacterial DNA and primers a and b (Table 1). Reactions were carried out using a Gene Amp PCR system 9700 thermalcycler (Applied Biosystems). The reactions consisted of an initial hold for 3 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 90 sec. The PCR products were purified with the ExoSAP-IT enzyme (GE Healthcare, Bio-Science Corp.) according to the instructions of the manufucture. DNA sequencing procedure was performed by primer walking method using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and primers shown in Table 1. The product was then loaded on ABI PRISM 3100 DNA Analyzer (Applied Biosystems) to determine the DNA sequence.

Intratracheal immunization with ethanol-killed bacteria

In order to develop protective immunity in the lungs against NTHi strain, 50 μl of ethanol-killed bacteria (H05-19 strain, H04-06 strain or H99-115 strain) at a concentration of 4×10^9 colony-forming units (CFU)/ml suspended in PBS was intratracheally administered to each mouse on day 0, 7 and 14 (Kurita et al. 2006). Day 0 was defined as the day of the first intratracheal (IT) immunization. Two different types of serial three IT immunizations were performed in this study. In the first type, one of these strains (H05-19 strain, H04-06 strain or H99-115 strain) was immunized on day 0, 7 and 14.

TABLE 1. Sequence of primers used in polymerase chain reaction and sequencing of ompP2 of NTHi.

Primer	Sequences
a	5′ACGCGGATCCTGGTGTTGTTTATAACAACG3′ (forward)
b	5′GGTGAAGTAAAACTTGGTC3′ (forward)
c	5′AGGCTTATTAGTCTCTCTAG3′ (forward)
d	5′ATCAGGATCCTTAGAAGTAAACGCGTAAACCTAC3′ (reverse)
e	5′CCATAGACATTAAGTATCTTCC3′ (reverse)
f	5′GCGCCTAATACTAAACCATC3′ (reverse)

In the second type, H05-19 strain was immunized on day 0, followed by the H04-06 strain on day 7 and then the H99-115 strain on day 14. Four or five animals were employed for each type of IT immunization. The procedure was performed under anesthesia induced by an intraperitoneal injection of 0.2 ml of a solution containing 3 mg of ketamine and 0.1 mg of xylazine. Heparinized blood was obtained, and the plasma separated, and bronchoalveolar lavage (BAL) was performed at the indicated times after the initial immunization as previously described (Kurita et al. 2006). Plasma and BAL fluid were stored at –80°C until used.

Determination of P6 or strain-specific antibodies

The P6-specific antibody titer in plasma or BAL fluid was determined by ELISA according to a previously published method (Kurita et al. 2006). The strain-specific antibody titer to the whole cell preparation of NTHi in plasma or BAL fluid was also determined by a previously described method, with minor modifications (Sethi et al. 2004). Because of the limitations in measuring the avidity of the strain-specific antibody to the whole cell preparation of NTHi, IgA specific and its avidity to OMP in BAL fluid were determined by ELISA employing 0.5 M sodium thiocyanate (Anttila et al. 1998). The OMPs from each NTHi strain were prepared as previously described (Kurita et al. 2006). A P6 preparation (1 μg/ml), the whole bacterial preparation (10^8 CFU/ml) or the OMP preparation (1 μg/ml) was used as the coating antigen. The isolated P6 protein was a generous gift from Dr. N. Yamanaka, Wakayama, Japan. In whole cell ELISA, 200 μl of 5% bovine serum albumin in PBS was used as the blocking solution and 50 μl of a plasma sample or BAL fluid was then added, followed by incubation at 37°C for 30 min. The plate was washed and treated with 50 μl of alkaline phosphatase-conjugated goat anti-mouse IgM, IgG, or IgA (Zymed, San Francisco, CA, USA). The optical density (OD) was measured at 405 nm. The end-point titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an OD_{450} of $\geqq$ 0.1 OD unit above the OD_{450} of negative control samples obtained from non-immunized mice.

Cell proliferation assay

In vitro antigen specific cell proliferation assays were performed using the Premix WST-1cell proliferation assay system (TAKARA, Tokyo) according to manufacture's instructions. Mononuclear cells were prepared from pulmonary lymph node (LN) of mice that had received three serial IT challenges of ethanol-killed NTHi as described above (Kurita et al. 2006). LN cells, at a concentration of 5×10^6 in 200 μl of Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS), were added to the well of a 96 well cell culture plate in triplicate and were stimulated at 37°C for 72 hrs with or without antigens at concentrations of 1 or 5 μg/ml. Ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO, USA) was used as a control antigen. After incubation, the assay was developed by the addition of a premix WST-1 solution (10 μg/well) to each well. OD was measured at 450 and 600 nm after 4 hrs incubation at 37°C. These data were calculated by subtracting the OD_{600} value from the OD_{450} value.

Bacterial clearance of NTHi strain in mice

Each live strain of NTHi (H05-19 strain, H04-06 strain or H99-115 strain), at a dose of a 4×10^8 CFU/mouse, was intratracheally challenged to mice which had previously received one of four different types of the serial three IT immunization of ethanol-killed NTHi strains at one week intervals, or to untreated mice. Five animals were employed in each type of IT immunization followed by bacterial challenge. Quantitative bacterial cultures of lung tissue from mice that had been euthanized with pentobarbital were performed on agar 4 hrs or 12 hrs after the bacterial challenge as previously described (Kurita et al. 2006). The lungs were removed asceptically and homogenized in 9 ml of sterile saline per gram of lung tissue prior to culturing. The detection limit of bacterial cultures of the lung tissue was 10^3 CFU/g.

Statistical analysis

Statistical analyses were performed by one-way ANOVA and multiple comparison method by Bonferroni-Dunn's test or the unpaired Student's *t*-test. Data were considered to be statistically significant, if the *p* values were less than 0.05. All data are expressed as the mean ± S.D.

Results

Immunoblot assays of whole-cell lysates and PFGE

To determine whether the P2 molecules of the three strains of NTHi (H05-19 strain, H04-06 strain or H99-115 strain) are different in molecular size, an immunoblot assay of whole cell lysates of these strains was performed using anti-P2 rabbit sera (Fig. 1A). The anti-P2 antisera

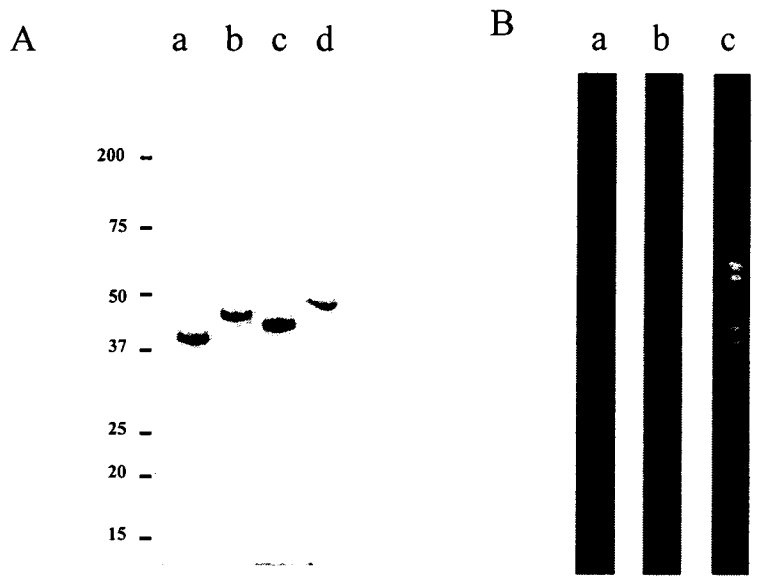

Fig. 1. Immunoblot assay of bacterial lysates of three NTHi strains and the purified P2 protein with anti-P2 sera (A) and pulse-field gel electrophoresis of *Sma*I-digested genomic DNA of three NTHi strains (B). Whole cell preparations of H05-19 strain (a), H04-06 strain (b) and H99-115 strain (c) and the purified P2 (d) were transferred to a nitrocellulose membrane and the P2 bands were visualized by mean of an anti-P2 rabbit serum. The PFGE patterns of *Sma*I-digested DNA from H05-19 strain (a), H04-06 strain (b) and H99-115 strain (c).

detected P2 molecules of these strains and the purified P2 protein, with different molecular sizes between molecular markers of 37 and 50 kDa. In addition, PFGE also demonstrated different patterns among the three strains (Fig. 1B).

OmpP2 sequence analysis

Analyses of the deduced amino acid sequences of *ompP2* of the three strains of NTHi (H05-19 strain, H04-06 strain or H99-115 strain) demonstrated sequence heterogeneity in all eight loop regions (Fig. 2). Therefore, all three strains of NTHi used in this study possess different P2 epitopes.

P6-specific cell proliferation of LN cells

In all of the three serial IT immunizations, except for the three serial IT immunizations by H05-19 strain, a concentration-dependent increase in cell proliferation was found in cultured cells isolated from pulmonary LN of mice in the presence of P6 at 1 and 5 μg/ml (Fig. 3A-D). In contrast, no cell proliferation was found in cultured cells isolated from the pulmonary LN of mice that had received any of the three types of serial IT immunizations in the presence of OVA at 1 and 5 μg/ml.

P6-specific immunoglobulins (Igs) in plasma and BAL fluid

The induction of P6-specific IgG and IgM, but not IgA, were found in plasma of mice that received any of the three types of serial IT immunizations (Table 2). In contrast, P6-specific IgG, IgM or IgA were undetectable in BAL fluids of mice that received any of the three types of serial IT immunizations.

Strain-specific Igs in plasma and BAL fluid by ELISA using the whole cell preparations

In plasma of mice that received any of the three types of serial IT immunizations, strain-specific Igs were found, except for the case of mice that received three serial IT immunizations by strain H04-06 (Table 3). Only strain H04-06-specific IgA, but not strain H05-19 or strain H99-

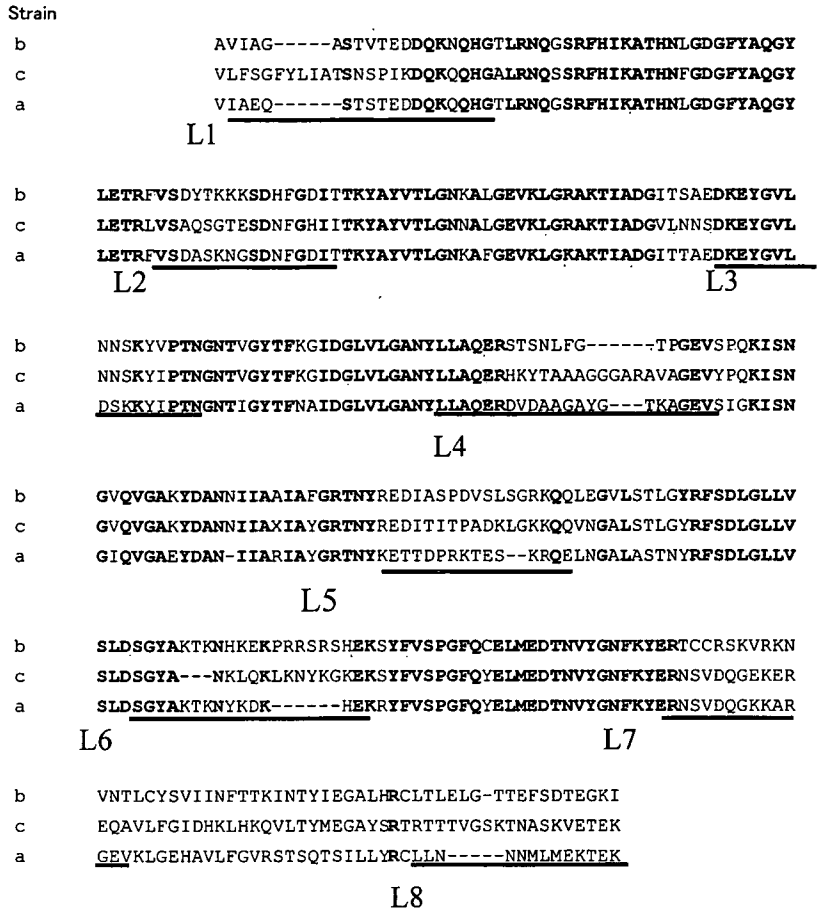

Fig. 2. Amino acid sequence of the *ompP2* regions of the three NTHi strains isolated from the same patient with COPD. Identical residues are shown in the bold. Strain a denotes H05-19 strain, strain b denotes H04-06 strain, and strain c denotes H99-115 strain, respectively. The loops 1 (L1) through loops 8 (L8) are indicated as the underlined parts.

115-specific IgA, was found in the plasma. In most types of the serial IT challenges, strain-specific IgA was detected in BAL fluids (Table 3). In case of the three serial challenges of the H04-06 strain, only strain H04-06-specific IgA was found in BAL fluids. No detectable levels of strain H05-19 or strain H99-115-specific Igs were found in BAL fluids of these mice.

Strain-specific IgA and its avidity in BAL fluid by ELISA using OMP preparations

In order to evaluate the functional strain-specific IgA, we next examined strain-specific IgA and its avidity in BAL fluids of mice (Table 4). In BAL fluids of mice that received three serial immunizations of strain H05-19 or H99-115 or three different strains, an increased IgA or its avidity specific to the OMP of strain H05-19 and H99-115 were found, compared to the OMP of strain H04-06. A similar increase in IgA and its avidity specific to OMP of strain H04-06 was shown only in BAL fluids of mice that had received three serial immunizations of strain H04-06, compared to those specific to OMP of strain H05-19 or H99-115.

Bacterial clearance in the lungs

We next examined the bacterial clearance of each NTHi strain in the lungs of mice that received three serial IT challenges. The bacterial clearance of strain H05-19 was significantly faster in cases of three serial IT challenges of strain

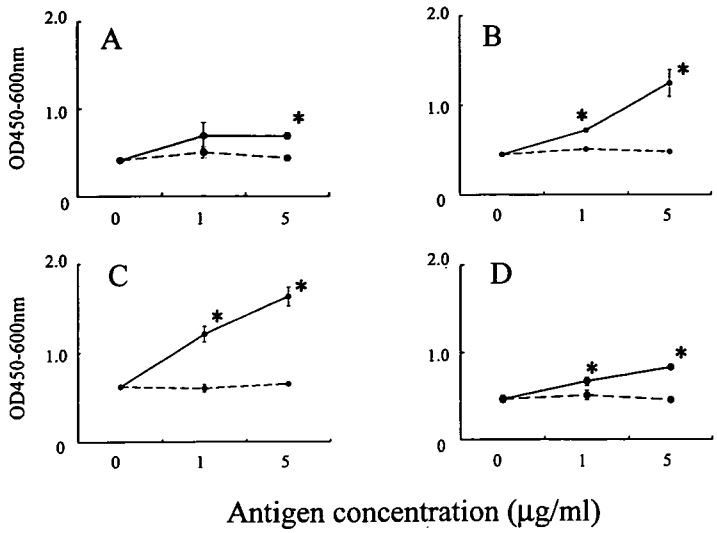

Fig. 3. In vitro cell proliferation using pulmonary lymph node cells from mice that received three serial IT challenge of killed-NTHi strain with a one-week interval in the presence of P6 (solid line) or ovalbumin (broken line). The IT immunizations include three serial immunizations of strain H05-19 (A), three serial immunizations of strain H04-06 (B), three serial immunizations of strain H99-115 (C) and three serial immunizations in the following order: strain H05-19, strain H04-06 and strain H99-115 (D). Values represent the mean ± S.D. of four animals.
*$p < 0.01$ (vs ovalbumin).

TABLE 2. Induction of P6-specific IgM and IgG in plasma in mice after three serial IT challenges of killed-NTHi strain with a one-week interval by ELISA.

Intrtracheal immunization	Ig class	Reciprocal $\log_2$ P6-specific titer in plasma
Serial three immunizations	IgM	7.0 ± 1.154
with H05-19 strain	IgG	6.5 ± 0.76
Serial three immunizations	IgM	5.5 ± 1.05
with H04-06	IgG	4.83 ± 0.75
Serial three immunizations	IgM	8.0 ± 0.89
with H99-115	IgG	6.17 ± 0.41
Serial three immunizations	IgM	7.83 ± 0.37
with three different strains	IgG	7.5 ± 0.76

Ig, immunoglobulin. Values represent the mean ± S.D. of four animals.

H05-19 or strain H99-115 than in untreated mice 12 hrs after the IT challenge (Fig. 4, upper column). No significant enhancement in the bacterial clearance of strain H05-19 was found in mice that received the three serial IT challenges of strain H04-06 or three different strains at 12 hrs post-challenge. Similarly, the bacterial clearance of strain H99-115 was significantly faster in the lungs of mice that received the three serial IT challenges of strain H05-19 or strain H99-115 than in untreated mice at 12 hrs post-challenge (Fig. 4, lower column). No significant enhancement of H99-115 strain was noted in mice that received the three serial IT challenges of strain H04-06 or the three different strains, although the bacterial densities of H99-115 strain in the lungs of mice receiving IT challenges of different strains tended to be lower than untreated mice. A

TABLE 3. Induction of strain-specific IgM, IgG and IgA in plasma and bronchoalveolar lavage (BAL) fluid of mice after three serial IT challenges of killed-NTHi strain with a one-week interval by ELISA using the whole cell preparations.

Intrtracheal immunization	Ig class	Reciprocal $\log_2$ titer in plasma			Reciprocal $\log_2$ titer in BAL fluid		
		anti H05-19	anti H04-06	anti H99-115	anti H05-19	anti H04-06	anti H99-115
Serial three immunizations	IgM	5.15 ± 0.41	6.0 ± 0	10.7 ± 0.58	N.D.	N.D.	N.D.
with H05-19 strain	IgG	10.5 ± 0.84	12.2 ± 1.47	12.0 ± 1.41	5.67 ± 0.68	5.83 ± 1.47	7.5 ± 1.29
	IgA	N.D.	7.83 ± 1.33	N.D.	7.0 ± 0.82	5.5 ± 1.22	6.83 ± 1.34
Serial three immunizations	IgM	N.D.	6.5 ± 0.58	N.D.	N.D.	N.D.	N.D.
with H04-06	IgG	N.D.	10.0 ± 0.89	7.6 ± 0.89	N.D.	N.D.	N.D.
	IgA	N.D.	N.D.	N.D.	N.D.	5.17 ± 0.75	N.D.
Serial three immunizations	IgM	N.D.	N.D.	8.0 ± 0.82	N.D.	N.D.	N.D.
with H99-115	IgG	10.5 ± 0.55	10.2 ± 1.17	10.8 ± 0.96	5.33 ± 0.58	4.25 ± 0.5	4.5 ± 0.58
	IgA	N.D.	6.0 ± 0	N.D.	4.5 ± 1.29	4.4 ± 0.55	6.8 ± 0.84
Serial three immunizations	IgM	N.D.	N.D.	9.17 ± 0.69	N.D.	N.D.	N.D.
with three different strains	IgG	12.17 ± 0.41	12.8 ± 0.41	13.0 ± 0	4.83 ± 0.41	4.67 ± 0.52	5.83 ± 0.37
	IgA	N.D.	6.33 ± 0.52	N.D.	6.0 ± 0.63	5.0 ± 0.63	4.67 ± 0.47

Ig, immunoglobulin. N.D., not detectable. Values represent the mean ± S.D. of four animals.

TABLE 4. The strain-specific IgA and its avidity in bronchoalveolar lavage (BAL) fluid of mice after three serial IT immunizations of killed-NTHi strain with a one-week interval by ELISA using the outer membrance preparations.

Intrtracheal immunization	IgA or IgA avidity	Reciprocal $\log_2$ titer in BAL fluid		
		anti H05-19	anti H04-06	anti H99-115
Serial three immunizations	IgA	10.81 ± 3.19	3.55 ± 0.95	9.36 ± 2.16
with H05-19 strain	IgA avidity	4.68 ± 0.69	2.34 ± 1.44	6.06 ± 1.59
Serial three immunizations	IgA	3.51 ± 0.20	6.10 ± 0.40	3.64 ± 0.22
with H04-06	IgA avidity	3.23 ± 0.19	5.13 ± 1.29	3.27 ± 0.20
Serial three immunizations	IgA	5.35 ± 1.76	2.85 ± 0.55	8.09 ± 1.36
with H99-115	IgA avidity	3.81 ± 0.66	1.85 ± 0.55	4.33 ± 0.25
Serial three immunizations	IgA	6.92 ± 0.52	4.64 ± 1.73	7.35 ± 0.89
with three different strains	IgA avidity	5.41 ± 0.78	3.25 ± 0.61	4.97 ± 0.43

Values represent the mean ± S.D. of four animals.

significant enhancement in bacterial clearance of strain H04-06 was found only in mice that received the three serial IT immunizations of the homologous strain, compared to untreated mice, at 4 hrs post-challenge (Fig. 4, middle column). No significant enhancement of H04-06 strain was found in mice that received the three serial IT immunizations of strain H05-19 or H99-115 or the three different strains.

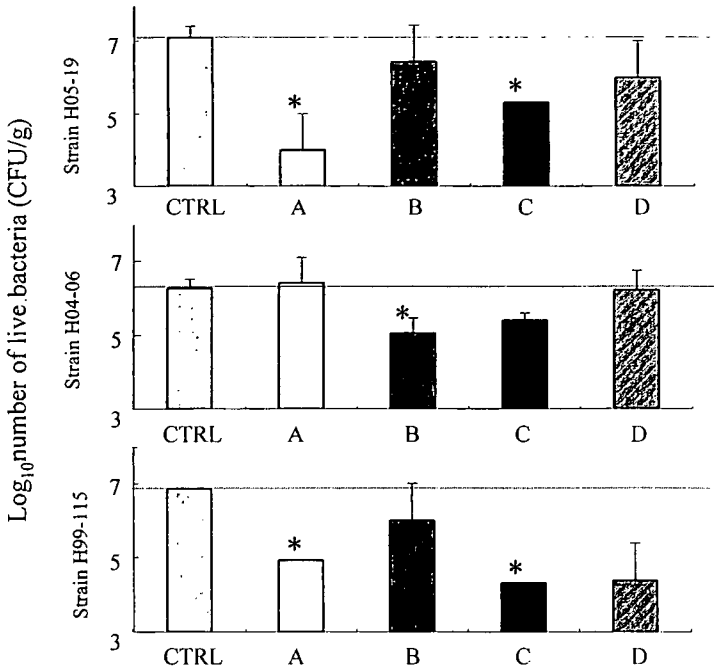

Fig. 4. The effect of three serial IT immunizations of killed-NTHi strain with a one-week intervals on the bacterial clearance of live NTHi strain H05-19, strain H04-06, and strain H99-115 are shown in the upper, middle and lower panels, respectively. The IT immunizations included three serial immunizations of strain H05-19 (A), three serial immunizations of strain H04-06 (B), three serial immunizations of strain H99-115 (C) and three serial immunizations in the following order: strain H05-19, strain H04-06 and strain H99-115 (D). The IT challenge of live strain of NTHi was done 3 weeks after the initial IT immunization with killed-NTHi. Untreated mice were used as controls (CTRL). The mice were euthanized and a quantitative bacterial culture of lung tissue was performed 12 hrs after the IT challenge for live strain H05-19 and live strain H99-115, and 4 hrs after the IT challenge for live strain H04-06. Values represent the mean ± S.D. of five animals.
*$p < 0.001$ (vs control).

Discussion

A recent study reported that the proliferative response of peripheral lymphocytes to P6 is associated with a relative protection from exacerbation by NTHi (Abe et al. 2002). We recently demonstrated a linkage between P6-specific T cell proliferation in LN cells and the induction of P6-specific IgA and IgG in BAL fluids by ELISA in mice that received repeated IT challenges of OMP of NTHi in mice (Kurita et al. 2006). Although T cell proliferation specific for P6 in LN cells was found in all mice that received the three serial IT immunizations of a single strain or three different strains of ethanol-killed NTHi in this study (Fig. 3), no P6-specific Igs including IgA, were detected in BAL fluids, but P6-specific IgG and IgM was detected in plasma. These data support the in vitro antigen presentation of P6 by dendritic cells contained in LN cells after degradation of the intratracheally challenged killed-NTHi strain. The antigen presentation in LN cells could lead to the production of Ig in plasma after the initial challenge of the OMPs of NTHi. The repeated IT immunization of OMP of NTHi, however, was required for the induction of IgG or IgA in the BAL fluids (Kurita et al. 2006). Repeated IT challenges of ethanol-killed NTHi strain failed to induce P6-specific IgG or IgA in BAL fluids in this study, because the P6 molecule, a cross-reactive epitope, is not exposed on the surface of the NTHi strain and is involved in anchoring the outer membrane (Nelson et al. 1991; Sethi and Murphy 2001). The detection of strain-specific

IgA in BAL fluids in most types of the three serial IT immunizations in the present study indicates the generation of cross-reactive IgA in the airways.

The three serial IT immunizations of a single strain significantly enhanced the bacterial clearance of the homologous strain from the lungs of mice (Fig. 4). No cross-protection was found in mice that had received three serial IT challenge of strain H04-06. These data suggest that the enhancement in bacterial clearance is primarily strain-specific. We also found that the three serial IT immunizations with strain H05-19 or H99-115 induced a cross-protective effect in the lungs of mice (Table 4). This cross-protective effect between strain H05-19 and H99-115 was associated with strain-specific IgA as well as IgG in BAL fluids (Table 3). Since there is a distinction in the amino acid sequence of loops 5 and 6 in the P2 molecule between these two strains, a cross-protective effect may be conferred by surface antigens other than P2. Possible surface antigens responsible for this cross-protective effect may include P5 adhesin and lipooligosaccharide (Sethi and Murphy 2001; Hirano et al. 2003; Novotny and Balaletz 2003). Although bacterial clearance in the lung also tended to be enhanced after an IT challenge of live H05-19 or H99-115 strain in mice that previously received the three serial IT immunizations of three different strains, the levels of enhancement were not significant in the lung of these mice (Fig. 4). Collectively, these data demonstrate that three serial IT immunizations of a single strain could lead to the production of strain-specific IgA as well as IgG, subsequently leading to an enhanced bacterial clearance of the homologous strain in the lung. The association of the enhanced bacterial clearance of strain H04-06 in the lungs of mice after three serial repeated IT challenge of the homologous strain with the presence of strain-specific IgA, but not IgG, may underscore the importance of strain-specific IgA in BAL fluids for inhibiting bacterial adherence in the airway (Taylor et al. 1990; Kurono et al. 1991).

The presence of strain-specific IgA and IgG in BAL fluid was not always associated with an enhanced bacterial clearance in lungs, because the serial IT challenges of three different strains could lead to the production of strain-specific IgA and IgG against all three strains, but none of them developed protective immunity against NTHi. These findings provide support for the view that three serial IT challenges of a single strain is sufficient to induce the production of strain-specific IgA which is capable of inhibiting the adherence of the homologous strain to the airway epithelium, while repeated IT challenges by three different strains generate strain-specific IgA which lacks such activity (Taylor et al. 1990; Kurono et al. 1991). We, therefore, examined the issue of whether strain-specific IgA and its avidity were associated with an enhanced bacterial clearance in the lungs of mice (Kauppi-Korkeila et al. 1996; Breukels et al. 2002). As we expected, the increased IgA and its avidity specific to OMP of NTHi were associated with an enhanced bacterial clearance in the lungs of mice that had received serial three IT challenges of a single strain (Table 4). No significant bacterial clearance, however, was found in the lungs of mice that had received serial three IT immunizations with different strains despite the increased IgA and its avidity in BAL fluid. Further examinations are required to elucidate this discrepancy in mice that received serial three IT immunizations by different strains.

In summary, three serial airway immunizations with a single or three different strains of NTHi stimulated the production of cross-reactive IgG and IgA in BAL fluids, but only three serial IT challenges of a single strain could induce the enhancement in bacterial clearance of the homologous strain in the lung. This enhancing effect on bacterial clearance in the lungs is, therefore, primarily induced in a strain-specific manner. In addition, enhanced bacterial clearance of a hetrologous strain was also found after three serial IT immunizations of a single strain among two of a the three strains employed for bacterial challenge. Increased strain-specific IgA and its avidity in BAL fluids was associated with an enhanced bacterial clearance in mice that had received serial IT immunizations with a homologous strain, but not in mice that had received serial IT immunizations

with heterologous strains. The data herein suggest that P2 molecules and surface antigens other than P2 are involved in the development of pulmonary defense against NTHi. Our data also suggest that a host previously infected by a NTHi continues to be susceptible to infections by other strains of NTHi, and may explain the mechanism of recurrent bacterial exacerbations of COPD. Since only three strains of NTHi with different P2 epitopes were employed in this study, the conclusions drawn are limited. Further studies will be required for a complete understanding of the strain-specific pulmonary defense against NTHi.

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Increased Rates of Intense Nasopharyngeal Bacterial Colonization of Vietnamese Children with Radiological Pneumonia

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ANH, D.D., HUONG, P.L.T., WATANABE, K., NGUYET, N.T., ANH, N.T.H, THI, N.T., DUNG, N.T., PHUONG, D.M., TANIMURA, S., OHKUSA, Y., NAGATAKE, T., WATANABE, H. and OISHI, K. *Increased Rates of Intense Nasopharyngeal Bacterial Colonization of Vietnamese Children with Radiological Pneumonia.* Tohoku J. Exp. Med., 2007, **213** (2), 167-172 —— Acute lower respiratory infection (ALRI), primarily pneumonia, is the leading cause of death in children under the age of five. Bacterial ALRI is preceded by asymptomatic bacterial colonization. Bacterial colonization, therefore, may have an important role in the development of pneumonia in children. This case-control study was conducted in order to determine if intense bacterial colonization was increased in the nasopharynx of pediatric patients with ALRI. One hundred-sixty four pediatric patients with ALRI and 70 healthy children < 5 years of age were enrolled in Hanoi, Vietnam between 2001 and 2002. Bacterial pathogens were isolated from nasopharyngeal secretions and quantitatively cultured. Of 164 patients, 91 were diagnosed as having radiological pneumonia (PN group) and 73 as having acute bronchitis (AB group). Intense growth of any bacterial pathogen ($\geqq 10^6$ colony-forming units/ml) was highest in the PN group (49.4%), followed by the AB group (28.8%), with healthy children having the lowest (17.1%). Patients with intense bacterial growth were more likely to develop pneumonia, but not acute bronchitis, than were patients with light or no bacterial growth. The results of this case-control study suggest that the vertical spread of intense bacterial pathogens colonized in the nasopharynx to the lower airway leads to bacterial pneumonia in children under the age of five. ——— radiological pneumonia; children; bacterial colonization; Vietnam; *Streptococcus pneumoniae*

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Acute lower respiratory infection (ALRI), primarily pneumonia, is the leading cause of death in children under the age of five. A recent report indicated that worldwide 1.9 million children died from ALRI in 2000, world-wide and that 70% of these deaths occurred in Africa and Southeast Asia (Williams et al. 2000). The two most common bacterial pathogens associated with pneumonia are *Streptococcus pneumoniae* and *Haemophilus influenzae* (World Health Organization 1991; Factor et al. 2005; Watanabe et al. 2005).

In children, pneumococcal disease is preceded by asymptomatic bacterial colonization (Parry et al. 2002; Bogaert et al. 2004). Bacterial colonization, therefore, plays a central role in pneumococcal diseases, and may provide the basis for the vertical spread of pneumococci as well as other invasive diseases (Bogaert et al. 2004). A previous study reported a high positive rate of pneumococcal colonization during otitis media caused by pneumococci, and an increased tendency of pneumococcal colonization during viral respiratory infections (Syrjanen et al. 2001). Another study also reported a mildly increased rate of bacterial colonization in children with radiological pneumonia, compared with control children (Levine et al. 2000). To date, however, the association between intense bacterial colonization and the occurrence of pneumonia in children has not been examined. We hypothesized that an intense bacterial colonization rather than a light colonization in the nasopharynx might spread into the lower airway and develop bacterial pneumonia in children. Therefore, this case control-study was designed to examine if intense nasopharyngeal bacterial colonization was increased in pediatric patients with under 5 years of age with ALRI in Hanoi, Vietnam.

Material and Methods

The subjects for this study were chosen from among pediatric patients with ALRI enrolled at either the National Pediatric Hospital or the Bach Mai Hospital, which are the tertiary hospitals in Hanoi, Vietnam between January 2001 and December 2002. Inclusion criteria were: 1) age < 5 years of age and a diagnosis of ALRI made within 24 hrs of admission: 2) clinical symptoms of a productive cough, fast breathing and a fever (body temperature) > 37.5°C: and, 3) the finding of crackles in the lung fields by auscultation of the lung. The body weight on admission, preceding episodes of acute upper respiratory infections (AURI) and prior antibiotic use before the onset of ALRI were recorded. Exclusion criteria were: 1) age > 5 years, 2) illness of a non-infectious etiology: and, 3) failure to consent to study participation.

Laboratory examinations involved a chest radiograph (PA view). Currently, the best available method for diagnosing pneumonia is radiography. Chest radiographs showing recent infiltrations are indicative of pneumonia, while the absence of infiltrations warrants a diagnosis of acute bronchitis. The patients with ALRI were, therefore, classified into the pneumonia (PN) and the acute bronchitis (AB) groups. Radiological findings by the World Health Organization Radiology Working Group, such as "a dense or fluffy opacity that occupies a portion or the findings whole of a lobe or the entire lung that may or may not contain air-bronchogram" were used (Cherian et al. 2005). All of the radiological films were examined by three experts who were blind to the clinical presentation—a radiologist, a pediatrician and a pulmonary physician. Seventy healthy children, from a local nursery school in Hanoi, were enrolled as the control (CT) group. None of the study participants had previously received a pneumococcal conjugate vaccine or a *Haemophilus influenzae* type b (Hib) vaccine before being enrolled in this study. All study procedures were approved by the Institutional Review Boards of National Institute of Hygiene and Epidemiology. Parents or guardians of all patients and healthy children provided written informed consent.

A quantitative culture and Gram's stain were performed simultaneously on nasopharyngeal secretions transnasally obtained from ALRI patients and from healthy children using two flexible swabs (Transwab^{R}, Medical Wire & Equipment Co., Ltd., Wiltshire, England). This Rayon swab is non-toxic to microorganisms and can provide both good absorption and retrieval of specimen. After weighing the nasopharyngeal swab samples on a microbalance, the volume of the sample was determined to be approximately 0.01 ml. Samples were diluted in Brain Heart Infusion broth (BBL, Becton Dickinson, Cockeysville, MD, USA), and ten-fold dilution was then prepared in saline, as described previously (Utsunomiya et al. 1998; Yoshimine et al. 2001). The

quantitative bacterial culture was carried out on trypticase soy agar (BBL, Becton Dickinson) containing 7% defibrinated rabbit blood and incubated in a 5% CO_2 incubator at 37°C overnight. The limitation of this bacterial quantitation method is 10^3 colony-forming units (CFU)/ml. The results of the quantitative culture of nasopharyngeal swab samples were classified into three categories: a) intense growth ($\geqq 10^6$ CFU/ml) of any bacterial pathogen; b) light growth ($< 10^6$ CFU/ml and $\geqq 10^3$ CFU/ml) of any bacterial pathogen; and, c) no bacterial growth ($< 10^3$ CFU/ml).

Statistical analysis

Age, body weight and the frequency of preceding episodes of AURI were compared between the cases (the PN group and the AB group) and the CT group or between the PN group and the AB group using the Mann-Whitney's U-test. Differences in the isolation rates of bacterial pathogens were compared between the cases (PN vs AB group), and the CT group and cases (PN, AB) using the χ^2 statistic. Odds ratios (OR) were calculated by comparing the PN or AB groups with the CT group. ORs with 95% confidence intervals that do not include 1.00 were considered statistically significant. Data were considered to be statistically significant at p-value < 0.05. The initial target sample sizes were chosen to ensure that there would be at least an 80% chance of detecting a 20% difference (i.e., 40% vs 20%) in the rates of nasopharyngeal bacterial colonization between patients and controls, using a two-sided test at a p-value of 0.05, according to previously published studies (Bogaert et al. 2004). Thus, the target sample size was 82 patients (PN and AB groups) and 82 controls (CT group).

Results

Among the 220 patients with ALRI, 160 patients were subsequently enrolled and classified as the PN group (n = 91) and the AB group (n = 73). Fifty-six patients could not be diagnosed because chest radiographs either were unavailable or of poor quality. Males compromised 62.5% of the PN group, 72.6% of the AB group and 51.4% of the CT group (n = 70). Sixty-two patients (68.1%) of the PN group and 53 patients (72.6%) of the AB group had prior antibiotic use, respectively. Most of these were oral antibiotics. The mean ages (± S.D., months) were: 14.6 ± 11.8 for the PN group, 12.4 ± 9.4 for the AB group, and 15.0 ± 9.4 for the CT group. No significant age differences were found between cases (PN and AB) and controls (CT) (p = 0.11) or between the PN and AB groups (p = 0.40). No significant difference was found between the PN and AB groups with respect to demographic characteristics on admission, the mean ± S.D. for body weight (8.8 ± 2.4 kg for PN, 8.5 ± 2.1 kg for AB; p = 0.917) or the frequency of preceding episodes of AURI (91.2% for PN, 93.2% for AB; p = 0.801).

Intense growth of any bacterial pathogen was highest in the PN group (49.4%), followed by the AB group (28.8%), with the CT group having the lowest (Table 1). A significant difference was found in the frequency of intense growth of potential pathogens between the cases (PN and AB) and the controls (CT) (p = 0.001) and between the PN group and AB groups (p = 0.012).

Table 1. Frequencies of intense, light and no growth of potential pathogens in the nasopharynx from patients with acute lower respiratory infections and healthy children.

Bacterial colonization	Pneumonia (n = 91)	Acute bronchitis (n = 73)	Control (n = 70)
		No. case (%)	
Any potential pathogen with a heavy growth	45 (49.4)	21 (28.8)*	12 (17.1)**
Any potential pathogens with a light growth	8 (8.8)	11 (15.1)	4 (5.8)
No growth	38 (41.8)	41 (56.1)	54 (77.1)

**p = 0.001 (vs Pneumonia and Acute bronchitis).
*p = 0.012 (vs Pneumonia).

In contrast, no significant differences were found in the frequency of light growth or in the absence of bacterial growth among the three groups. Patients with intense bacterial growth were more likely to develop pneumonia, but not acute bronchitis, than were patients with light or no bacterial growth (Table 2). The pathogens most likely to exhibit intense growth in the PN group were *H. influenzae* and *S. pneumoniae*, followed by *Moraxella catarrhalis* (Table 3). No statistical significance was found in the frequency of a particular potential pathogen with intense growth among the three groups. In addition, isolates of *S. pneumoniae* and *H. influenzae* were highly resistant to β-lactam antibiotics. While most of pneumococci were genotypic penicillin-resistant *S. pneumoniae*, possessing altered *pbp 1a + 2x + 2b* genes (Watanabe et al., in press), most of *H. influenzae* strains had TEM-1 type β-lactamase gene (Watanabe et al. 2005).

TABLE 2. Association between intense growth of potential bacterial pathogens and the group of pneumonia or acute bronchitis.

Group	Total	OR	95% CI
Pneumonia	91	4.73	2.24, 9.96
Acute bronchitis	73	1.95	0.88, 4.35

OR, odds ratio; CI, confidence interval.

DISCUSSION

In this case-control study, a significant association between the increased frequency of intense bacterial growth and the PN group was found. Isolated bacterial pathogens, therefore, do not suggest the etiologies of ALRI. These data suggest that the vertical spread of bacteria pathogens from the nasopharynx to the lower respiratory tract subsequently might cause bacterial pneumonia. Furthermore, recent studies reported that strain-specific IgG found in sera plays a critical role in the recurrent exacerbation of chronic obstructive pulmonary diseases, and that the strain-specific protective immune response confers susceptibilities to infections by other strains of the same bacterial species (Sethi et al. 2002, 2004). Taken together, our present data suggest that a higher load of bacterial colonization, therefore, leads to bacterial pneumonia, especially in non-immuned hosts to the certain bacterial strain found in the upper airway.

More than 90% of patients with ALRI had the preceding episodes of AURI in this study. Since the frequency of nasopharyngeal pneumococcal colonization was increased in children with viral respiratory tract infections (Syrjanen et al. 2001), the patients in the present study may have developed radiological pneumonia associated with viral upper respiratory infections. This possibility is supported by a scenario in which respiratory virus-infected epithelial cells facilitate

TABLE 3. Isolation rates of potential bacterial pathogens exhibiting intense nasopharyngeal growth in patients with acute lower respiratory infections and healthy children.

Organism	Pneumonia (n = 91)	Acute bronchitis (n = 73)	Control (n = 70)
	No. positive case (%)		
Heamophilus influenzae	24 (26.3)	10 (13.7)	4 (5.7)
Streptococcus pneumoniae	18 (19.8)	13 (17.8)	7 (10.0)
Moraxella catarrhalis	7 (7.7)	1 (1.7)	1 (1.4)
Staphylococcus aureus	0 (0)	1 (1.7)	0 (0)
Escheriachiae coli	0 (0)	0 (0)	1 (1.4)
Enterobacter cloacae	1 (1.1)	0 (0)	0 (0)
Klebsiella ozaenae	0 (0)	0 (0)	1 (1.4)

pneumococcal adherence, resulting in enhanced bacterial growth in the nasopharynx and the development of bacterial pneumonia in these children (Ishizuka et al. 2003; Madhi et al. 2004; Poltola et al. 2005).

A significant association between intense bacterial growth of a particular bacterial strain, such as *S. pneumoniae* or *H. influenzae*, and the development of pneumonia was not observed in the present study. No difference was also found in the isolation rates of pneumococci exhibiting an intense growth in between the groups of PN and AB. This may be, in part, explained by the underestimation of the burden of pneumococcal pneumonia diagnosed by the standardized definition of "radiologically-confirmed pneumonia" (Madhi and Klugman 2007). Therefore, additional studies are required to determine whether intense bacterial colonization of a particular pathogen in the nasopharynx is associated with progression to pneumonia in children. The influence of prior antibiotic use on results for bacterial growth in the nasopharynx of pediatric patients with ALRI may occur. Syrjanen et al. (2001) demonstrated that oral antibiotics prior to sampling reduced pneumococcal carriage only temporarily. Since most of patients with ALRI had oral antibiotics before admission in this study, the isolation rates of bacterial pathogens from the PN and AB groups, but not the CT group, may have been reduced by prior antibiotic use.

In summary, the novel finding of the present study was the association between intense nasopharyngeal bacterial colonization and the presence of radiological pneumonia in children. This result suggests that intense bacterial colonization plays a role in the development of pneumonia in children under the age of five.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Possible Prevalence and Transmission of Acute Respiratory Tract Infections Caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* among the Internally Displaced Persons in Tsunami Disaster Evacuation Camps of Sri Lanka

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Abstract

Objective The objective of this prospective study was to investigate the status of acute respiratory tract infections caused by *Haemophilus influenzae* and *Streptococcus pneumoniae* in tsunami disaster evacuation camps.

Methods Nasopharyngeal swabs (NP) of 324 internally displaced persons (IDP) in 3 different tsunami disaster evacuation camps of Sri Lanka were collected between March 18th and 20th, 2005, and analyzed for MIC, β-lactamase production, serotypes, PCR and pulsed-field gel electrophoresis (PFGE).

Results Many IDP had respiratory symptoms and the prevalence of cough and/or sputum was 84%, 70.5% and 64.7% in the three camps. Twenty-one *H. influenzae* from 20 IDP and 25 *S. pneumoniae* from 22 IDP were isolated from the NP. All *H. influenzae* isolates were nontypeable, and 5 were β-lactamase producing. Seventeen pneumococci were susceptible, 5 showed intermediate resistance and 3 were fully resistant to penicillin G. Molecular analysis showed the 21 *H. influenzae* strains had 13 PFGE patterns and 25 pneumococci had 16 PFGE patterns. All 4 different PFGE patterns of *H. influenzae* strains were detected in a few IDP in camps 1 and 3, and 5 different PFGE patterns of serotype 3, 22A, 9A, 10A and 11A pneumococci were detected in a few IDP in camps 1 and 3.

Conclusion Our data indicate acute respiratory tract infections caused by various types of *H. influenzae* and *S. pneumoniae* appear to have been prevalent, some of which were potentially transmitted from person to person in tsunami disaster evacuation camps.

Key words: *Haemophilus influenzae*, *Streptococcus pneumoniae*, tsunami, internally displaced persons, acute respiratory tract infection, evacuation camp

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Introduction

The tsunami that occurred on December 26th, 2004 struck southeast Asia and affected 12 countries. At least 310,000 people died, and many millions were left destitute (1). After the tsunami, many people continued to live in evacuation camps in the affected countries for various periods of times.

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It has previously been reported that acute respiratory infections were one of the leading causes of death among Bhutanese refugees in refugee camps in Nepal (2) and cases of pneumonia increased after the Hanshin-Awaji earthquake in Japan (3). *Haemophilus influenzae* and *Streptococcus pneumoniae* are bacteria that colonize the human nasopharynx and can cause a variety of infections, including otitis media, meningitis, bronchitis and pneumonia (4-6). It is well known that *H. influenzae* and *S. pneumoniae* can be transmitted from person to person at day care centers (7) or in the home (8, 9). Furthermore, these two organisms are becoming increasing resistant to antibiotics (4, 10). The objective of this prospective study was to investigate the status of acute respiratory tract infections caused by these two organisms in tsunami disaster evacuation camps in Sri Lanka.

Material and Methods

All studies described herein were approved by the Human Ethics Review Boards of Sri Lanka, and a signed consent form was obtained from each internally displaced person (IDP).

IDP, evacuation camps and bacterial strains.

Nasopharyngeal swabs (NP) were collected from 324 (100, 105, 119) IDP living in 3 different tsunami disaster evacuation camps in Batticaloa, the eastern district of Sri Lanka that was affected by the tsumani between March 18th and 20th , 2005. These 3 camps were selected at random among the camps which more than 500 IDP lived. Most IDP lived in the camps for a while after the tsunami. In the camps, we announced the content of our study to IDP who were there, and examined those who agreed with participation in our study. Numerous IDP had respiratory symptoms after entering the camps and the prevalence of IDP who had cough and/or sputum was 84%, 70.5% and 64.7% (totally 72.5%) in each camp. After collection of the NP, they were transferred from the camp to the Department of Microbiology of the University of Peradeniya, Sri Lanka within 24 hours to identify the organisms. As a result, 21 *H. influenzae* from 20 (10, 4 and 6 in each camp) IDP and 25 *S. pneumoniae* from 22 (6, 4 and 12 in each camp) IDP were isolated from the NP.

Serotyping and β-lactamase production.

H. influenzae isolates were serotyped by slide agglutination with antisera purchased from Difco Laboratories (Detroit, MI, USA) and β-lactamase production was detected by a nitrocefin impregnated disk (Becton Dickinson, Sparks, MD, USA). *S. pneumoniae* was serotyped on the basis of capsular swelling (quellung reaction) observed microscopically after suspension in pneumococcal diagnostic antisera (Statens Serum Institut, Copenhagen, Denmark).

Antimicrobial susceptibility test.

The Minimum inhibitory concentration (MIC) was determined by the agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (11). The susceptibility of 21 *H. influenzae* and 25 *S. pneumoniae* isolates to the following 18 antibiotics was tested: penicillin G (PCG, Meiji Seika Kaisha, Tokyo, Japan), ampicillin (AMP, Meiji Seika Kaisha), amoxicillin-clavulanic acid (GlaxoSmithKline, Tokyo), cefazolin (Astellas Pharma Inc., Tokyo), cefaclor (Shionogi Co., Osaka Japan), cefotiam (Takeda Chemical Industries, Osaka), ceftriaxone (Chugai Pharmaceutical Co., Tokyo), cefotaxime (Aventis Pharma, Tokyo), imipenem (Banyu Pharmaceutical Co., Tokyo), minocycline (Lederle (Japan), Tokyo), chloramphenicol (Sankyo Co., Tokyo), erythromycin (Dainippon Sumitomo Pharma Co., Osaka), clarithromycin (Taisho Pharmaceutical Co., Tokyo), clindamycin (Pfizer Japan Inc., Tokyo), gentamicin (Schering-Plough K.K., Osaka), levofloxacin (Daiichi Pharmaceutical Co.), vancomycin (Shionogi Co.), and sulfamethoxazole-trimethoprim (Shionogi & Co., Osaka).

Identification of resistant genes by PCR.

PCR was carried out for 21 of the *H. influenzae* isolates to identify resistant genes using mixed primers (Wakunaga Pharmaceutical Co., Hiroshima, Japan), as described previously (12). PCR was also performed for the 25 pneumococcal isolates to detect alterations in the penicillin-binding protein genes *pbp1a*, *pbp2x*, and *pbp2b* and macrolide resistance genes *mef (A)* and *erm (B)* using mixed primers (Wakunaga Pharmaceutical Co.), as described previously (13).

Pulsed-field gel electrophoresis.

Pulsed-field gel electrophoresis (PFGE) was performed on 21 *H. influenzae* and 25 *S. pneumoniae* isolates to determine genetic relatedness, as described previously (7). The DNA was digested with *Sma*I (Takara Shuzo Co., Shiga, Japan). CHEF Mapper Pulsed Field Electrophoresis Systems (Bio-Rad Life Science Group, Hercules, CA, USA) were used for the electrophoresis, with a potential of 6 V/cm, switch times of 0.47 and 63 seconds, and a run-time of 20 hours and 18 minutes. After staining with ethidium bromide, the interpretation of PFGE patterns was based on the criteria described by Tenover et al (14).

Results

Epidemiological characteristics of IDP in tsunami disaster evacuation camps.

Table 1 shows the sex, age, respiratory symptoms, antibiotic use within the past 48 hours, and the results of the swab cultures. Man to woman ratios of the sample was comparable for each camp. Regarding age distribution, the rate for children less than 5 years of age was similar. However, in camp 3 many children who were 6-15 years of age joined this study, whereas in camp 2 more adults tended to be involved. The rate of IDP who had cough and/or sputum

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Table 1. Epidemiological Characteristics of Internally Displaced Persons in Tsunami Disaster Evacuation Camps of Sri Lanka

		Camp 1 (n=100)	Camp 2 (n=105)	Camp 3 (n=119)
Sex	Male / Female	45 / 55	34 / 71	57 / 62
Age (years)	0- 5	14 (14.0%)	17 (16.2%)	17 (14.3%)
	6-15	15 (15.0%)	16 (15.2%)	48 (40.3%)
	16-25	16 (16.0%)	11 (10.5%)	19 (16.0%)
	26-35	26 (26.0%)	13 (12.4%)	18 (15.1%)
	36-45	10 (10.0%)	11 (10.5%)	6 (5.0%)
	46-55	7 (7.0%)	19 (18.1%)	6 (5.0%)
	56-65	9 (9.0%)	15 (14.3%)	2 (1.7%)
	66-	3 (3.0%)	3 (2.9%)	3 (2.5%)
Respiratory symptom	Cough	79 (79.0%)	68 (64.8%)	74 (62.2%)
	Sputum	61 (61.0%)	40 (38.1%)	57 (47.9%)
	None	16 (16.0%)	31 (29.5%)	42 (35.3%)
Antibiotic use within the past 48 hours	Used	16 (16.0%)	17 (16.2%)	2 (1.7%)
	Not used	80 (80.0%)	62 (59.0%)	93 (78.2%)
	Unknown	4 (4.0%)	26 (24.8%)	24 (20.2%)
Swabs culture	*S. pneumoniae*	6 (6.0%)	4 (3.8%)	12 (10.1%)
	H. influenzae	10 (10.0%)	4 (3.8%)	6 (5.0%)

Table 2. Distribution of MICs against Antibiotics for *H. Influenzae* and S. *Pneumoniae* Strains Isolated from The Nasopharyngeal Swabs from Internally Displaced Persons in Tsunami Disaster evecuation Camps of Sri Lanka

Antibiotic	*H. Influenzae* (n=21) MIC (μg/ml)			*S. pneumoniae* (n=25) MIC (μg/ml)		
	Range	50%	90%	Range	50%	90%
Penicillin G	0.125-128	0.25	16	≦0.004-4	0.016	2
Ampicillin	0.25-64	0.25	8	0.008-8	0.032	4
Amoxicillin-clavulanic acid	0.125-1	0.25	0.5	ND	ND	ND
Cefazolin	16-64	16	64	0.063-16	0.125	4
Cefaclor	4-16	8	16	0.25-128<	1	128<
Cefotiam	0.5-2	1	2	0.063-16	0.25	4
Ceftriaxone	≦0.004-0.016	0.008	0.008	≦0.004-4	0.032	1
Cefotaxime	0.008-0.032	0.016	0.032	0.008-8	0.032	2
Imipenem	0.25-8	4	8	≦0.004-0.5	0.008	0.25
Minocycline	1-2	2	2	0.125-64	0.5	16
Chloramphenicol	1-16	2	16	4-64	4	8
Erythromycin	2-4	4	4	0.063-128<	0.125	128
Clarithromycin	4-8	8	8	ND	ND	ND
Clindamycin	ND	ND	ND	0.032-128<	0.125	128
Gentamicin	0.5-4	1	2	4-16	8	16
Levofloxacin	0.016	0.016	0.016	1-4	2	4
Vancomycin	ND	ND	ND	0.125-1	0.5	0.5
Sulfamethoxazole-trimethoprim	8- 128<	128<	128<	2-128<	32	128<

ND: not determined

were 84%, 70.5% and 64.7% (totally 72.5%) in each camp. In particular, more than 60% in each camp had a cough. Although the extent of pre-antibiotic use was unknown, more than half the IDP had not used antibiotics within the past 48

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Table 3. Epidemiological Characteristics of *H. Influenzae* Strains Isolated from Internally Displaced Persons in Tsunami Disaster Evacuation Camps of Sri Lanka

Camp No.	Room No.	IDP No.	Isolate No.	Age (ys)	Symptom	Serotype	β-lactamase	MIC against AMP (μg/ml)	Resistance gene identified by PCR	PFGE pattern
1	1	1	1	2	Cough, Sputum	Nontypeable	-	0.25	None	A
1	1	1	2	2	Cough, Sputum	Nontypeable	-	0.25	None	B
1	1	2	3	6	Cough, Sputum	Nontypeable	-	0.25	None	B
1	2	3	4	0	Cough, Sputum	Nontypeable	-	0.25	None	B
1	3	4	5	34	Cough, Sputum	Nontypeable	-	0.25	None	C
1	3	5	6	12	Cough, Sputum	Nontypeable	-	0.25	None	C
1	4	6	7	4	Cough, Sputum	Nontypeable	-	0.25	TEM	D
1	5	7	8	1	Cough	Nontypeable	+	4	TEM	E
1	5	8	9	46	Cough, Sputum	Nontypeable	+	8	TEM	F
1	6	9	10	18	Cough, Sputum	Nontypeable	+	64	TEM	G
1	7	10	11	34	None	Nontypeable	-	2	TEM	H
2	8	11	12	61	Cough, Sputum	Nontypeable	-	0.25	TEM	B
2	9	12	13	9	Cough	Nontypeable	-	0.25	None	D
2	10	13	14	5	Cough, Sputum	Nontypeable	-	0.25	None	I
2	11	14	15	13	Cough, Sputum	Nontypeable	-	0.25	None	J
3	12	15	16	25	Cough, Sputum	Nontypeable	-	0.5	TEM	B
3	13	16	17	45	Cough, Sputum	Nontypeable	-	0.5	TEM	K
3	14	17	18	20	Cough, Sputum	Nontypeable	+	4	TEM	K
3	15	18	19	0	Cough, Sputum	Nontypeable	-	0.25	None	L
3	16	19	20	1	Cough, Sputum	Nontypeable	-	0.25	None	L
3	17	20	21	8	Cough, Sputum	Nontypeable	+	8	TEM	K

TEM: TEM-1-type β-lactamase gene

hours. *H. influenzae* and *S. pneumoniae* were detected in each of the camps. However, the detection rate in camp 2 tended to be lower than in the other camps. No IDP had both *H. influenzae* and *S. pneumoniae* simultaneously.

Antimicrobial susceptibility test and characteristics of organisms.

Table 2 shows the MIC range, the MIC_{50} and the MIC_{90} values for 18 antibiotics against 21 *H. influenzae* and 25 *S. pneumoniae* isolates from the NP. Twelve (60%) of the 20 IDP with *H. influenzae* isolates were children under 15 years of age. Twenty-one of the *H. influenzae* isolates were nontypeable, 16 (76.2%) were β-lactamase-negative, and 5 (23.8%) were β-lactamase-producing. Futhermore, 10 *H. influenzae* isolates had the TEM-1-type β-lactamase gene, including 5 β-lactamase-producing isolates. The MIC against AMP of one β-lactamase-negative *H. influenzae* isolate (Isolate No. 11) with TEM-1-type β-lactamase gene was 2 μg/ml and that did not have such resistant gene as β-lactamase-negative AMP-resistant (BLNAR) strains (Table 3). On the other hand, 18 (72%) of 22 IDP with *S. pneumoniae* were children less than 15 years of age. The 25 pneumococci were associated with 13 serotypes, including 3, 6A, 6B, 9A, 10A, 10F, 11A, 15B, 17A, 19A, 22A, 22F and 23F. Of the 25 *S. pneumoniae* isolates, 17 (68.0%) were susceptible, 5 (20.0%) showed intermediate resistance (MIC, 0.12 to 1.0 μg/ml) and 3 (12.0%) were fully resistant (MIC, > or = 2.0 μg/ml) to PCG. The macrolide resistance gene *erm(B)* was found in 2 of 3 fully resistant strains and the 3 of 5 intermediate resistant strains had alterations in *pbp1a*, *pbp2x*, and *pbp2b* with and without macrolide resistance gene *erm (B)* (Table 4).

Interpretation of PFGE.

Molecular typing by pulsed-field gel electrophoresis (PFGE) showed that the 21 *H. influenzae* strains consisted of 13 patterns (A-L) (Fig. 1) and the 25 *S. pneumoniae* strains consisted of 16 patterns (a-p) (Fig. 2). Regarding *H. influenzae*, PFGE pattern B isolates from 3 IDP and PFGE pattern C isolates from 2 IDP were detected in camp 1, and PFGE pattern K isolates with the TEM-1-type β-lactamase gene from 3 IDP and PFGE patten L isolates from 2 IDP were detected in camp 3. Regarding *S. pneumoniae*, PFGE pattern a isolates of serotype 3 were found for 3 IDP and PFGE pattern b isolates of serotype 22A were found for 2 IDP in camp 1, and PFGE pattern h isolates of serotype 10 A with the macrolide resistance gene *erm (B)* for 2 IDP, PFGE pattern f isolates of serotype 11A for 2 IDP and PFGE pattern j isolates of serotype 9A with alterations in the penicillin-binding protein genes *pbp1a*, *pbp2x*, and *pbp2b* for 2 IDP were detected in camp 3. Some of the IDP, in whom the same PFGE pattern of the organism was detected, lived in the same room at the camp, but not nescessarily all (Table 4). Two different *H. influenzae* isolates with PFGE pattern A and B were detected from one IDP (IDP No. 1, Table 3). Similarly, 2 different pneumococci (serotype 3 and 22A) with PFGE pattern a2 and b1, and 3 different pneumococci (serotype 17A, 22A and 22F) with PFGE pattern o, b2 and p were detected from each one IDP (IDP No. 3 and 22, Table 4).