Table 1. Serotype and sequence type of blood and posterior nares isolates

| Serotype | Blood | | Nasopharynx | | Remarks[1)] |
|---|---|---|---|---|---|
| | Sequence type | No. of isolates | Sequence type | No. of isolates | |
| 6B | ST90, ST2983 | 3, 2 | ST90, ST2983 | 6, 2 | ST2983, DLV of ST90 (*xpt*, *ddl*) |
| | | | ST902 | 1 | |
| | | | ST5846[2)] | 1 | |
| 19F | ST236, ST115 | 2, 1 | ST236, ST257 | 10, 1 | ST115, DLV of ST236 (*spi*, *ddl*)<br>ST257, DLV of ST236 (*aroE*, *ddl*) |
| 23F | ST1437 | 3 | ST1437, ST5845[2)] | 2, 1 | ST5845, SLV of ST1437 (*gdh*, *gki*) |
| | | | ST242, ST5841[2)], ST5844[2)] | 5, 1, 1 | ST5841, DLV of ST242 (*gdh*, *recP*)<br>ST5844, SLV of ST242 (*xpt*) |
| 14 | ST13 | 2 | ST13, ST2922 | 1, 1 | ST2922, SLV of ST13 (*xpt*) |
| | | | ST5240[3)] | 1 | |
| 19A | ST3111 | 2 | ST3111 | 6 | |
| | | | ST320 | 1 | |
| | | | ST5842[2)] | 1 | |
| 10A | ST6412[2)] | 1 | ST1263 | 1 | |
| | | | ST5236 | 1 | |
| 6A | | | ST3787 | 2 | |
| 6C | | | ST5241 | 1 | |
| 9V | | | ST280 | 2 | |
| 15A | | | ST63 | 3 | |
| 15B | | | ST199 | 1 | |
| 15C | | | ST5843[2)] | 1 | ST5843, SLV of ST199 (*spi*) |
| 33F | | | ST5840[2)] | 2 | |
| 11A | | | ST99 | 1 | |
| 18B | | | ST3594 | 1 | |
| 23A | | | ST5246 | 1 | |
| 34 | | | ST3116 | 1 | |
| 35B | | | ST558 | 1 | |
| 38 | | | ST393 | 1 | |
| Untypeable | | | ST1106 | 1 | |

1): SLV/DLV, single-/double-locus variant.
2): newly identified sequence type in this study.
3): MICs could not be determined (see text).

PCV7. Shibata Hospital participated in this surveillance study from the beginning, and the period of this study corresponds to the period just prior to the introduction of PCV7. The children in Shibata City and the surrounding area did not receive PCV7 in this period. In spite of this, *S. pneumoniae* serotype 19A—well recognized as a major replacement serotype following PCV7 introduction in USA and other countries (2,3,5,7,20)—was isolated in 12.5% and 12.7% of the blood and nasopharyngeal cultures, respectively. These rates are much higher than those observed before PCV7 introduction in USA (2,3,20) and Canada (5) and are comparable to those that had been observed in France (7). In a country-wide survey in Japan, Chiba et al. reported 12 pediatric invasive cases by serotype 19A *S. pneumoniae* (6.2%) out of a total of 193 cases from 2006 to 2007 (15). MLST analysis showed that the ST of a nasopharyngeal isolate of serotype 19A was ST320. *S. pneumoniae* with this serotype and ST has been isolated from multiple regions in Japan (unpublished data) and from many other countries, including USA, Venezuela, Spain, Italy, China, and South Korea (http://spneumoniae.mlst.net/sql/burstspadvanced.asp). The penicillin G MIC of all the ST320 isolates obtained from patients in Japan was 2–4 μg/mL. However, the 2 blood isolates of serotype 19A obtained in this study showed a penicillin G MIC of 0.03 μg/mL and belonged to ST3111. At all of the 7 loci [*aroE, ghd, gki, recP, spi, xpt,* and *ddl*] in the pneumococcal MLST analysis, the alleles differed between ST320 and ST3111 (ST320 [4, 16, 19, 15, 6, 20, 1] and ST3111 [61, 60, 67, 16, 10, 104, 14]). In addition, ST5842 [10, 16, 150, 1, 13, 1, 29], isolated from a swab sample, had allele numbers that were different from those of both ST320 and ST3111. These findings suggest that multiple serotype 19A variants have already spread in children in the Shibata City region; these variants may cause respiratory infections and would cause invasive infections. The invasive infection surveillance in 10 prefectures showed that various STs, including ST320 and ST3111, have been observed in serotype 19A isolates (unpublished data). In Japan, routine immunization with PCV7 has been recently initiated in 2011, and the reduction in the number of invasive and respiratory infection cases caused by the vaccine-serotype *S. pneumoniae* is anticipated, as has been observed in USA and other countries (2–11). PCV13, however, is not yet available in Japan. The domestic phase III study is still on-going in 2011. This situation raises concern about the rapid replacement of the PCV7 serotypte by non-PCV7 serotypes, as has been observed in USA (2,3,20). Replacement by serotype 19A (ST320), in particular, would be serious because of its high

resistance to penicillin and non-susceptible phenotype to meropenem. The prospective survey of pneumococcal infection in both children and adults, together with intensive laboratory analysis, will be necessary for detecting the very early stage of the replacement. We do anticipate the early introduction of PCV13 in Japan.

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**Conflict of interest** None to declare.

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# My continuing efforts toward the eradication of the vaccine-preventable diseases from Japan

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ABSTRACT

When compared to the Western countries, unfortunately, the progress toward the eradication of vaccine-preventable diseases in Japan has been relatively slow and hampered by some problems inherent to Japan. Concerning this, I have been working on the vaccine-preventable diseases, that is, measles, influenza, *Haemophilus influenzae*, and *Streptcoccus pneumoniae* infections. My first work was to produce monoclonal antibodies against measles virus proteins, and by using these antibodies, I established the immunofluorescent measles virus detection system in tissue samples. I constructed the 'Hokkaido measles-zero strategy' to eliminate measles from Hokkaido prefecture, the northernmost island of Japan, within 5 years since 2001 with very kind efforts of pediatricians in Hokkaido and those who are engaged in political activities. Coworkers and I established the disease entity of influenza-associated encephalopathy. And finally, I worked as a member of some groups that accumulated the basic data on the occurrence of bacterial meningitis in Japan and urged the government to introduce the *H. influenzae* type b conjugate vaccine and the pediatric 7-valent pneumococcal conjugate vaccine in Japan. I would like to express many thanks to all of my colleagues for their contributions to these achievements.



## 1. Introduction

When compared to the Western countries, unfortunately, the progress toward the eradication of vaccine-preventable diseases in Japan has been relatively slow and hampered by some problems inherent to Japan. Concerning this, I have been working on the vaccine-preventable diseases, that is, measles, influenza, *Haemophilus influenzae*, and *Streptococcus pneumoniae* infections. In this opportunity I summarize my works on these diseases and hope that this article provides readers with some useful information.

## 2. Measles

### 2.1. Production of monoclonal antibodies against virus proteins by use of the mouse hybridoma technique

My first work was to produce monoclonal antibodies against measles virus proteins under the instruction of professor Erling Norrby of Karolinska Institute [1]. Briefly, mouse hybridoma cell lines were produced by fusion of P3x63 Ag8 myeloma cells with spleen cells collected from BALB/c mice immunized with purified measles virions. Selected measles antibody producing hybridoma cell lines were passaged intraperitoneally in mice, and ascites fluids were collected. Seventeen lines produced high titers of hemagglutination inhibiting and hemolysis-inhibition antibodies. Homogenous immunoglobulin products carrying measles antibody activity were demonstrated by imprint immunoelectrophoresis of ascites materials. A representative result using hemagglutinin-highly specific clones 02 and 03 is shown in Fig. 1.

### 2.2. Immunofluorescent staining of lung tissues from patients with measles pneumonia using monoclonal antibodies

By use of the monoclonal antibodies which are mentioned above, I established the immune fluorescent measles virus detection system using tissue samples [2]. A representative result is presented in Fig. 2, which shows a successful detection of measles virus antigens in a lung tissue from a patient with a primary immunodeficiency syndrome who died of giant cell pneumonia. In this context, I found that the measles antigens cannot be detected in a lung tissue of an immunocompetent patient with interstitial, but not giant cell, pneumonia (Fig. 3, data unpublished). This result clearly demonstrated that the measles pneumonia in an immunocompetent host, unlike giant cell pneumonia in an immunocompromised host, is not a result of direct invasion by the virus itself.

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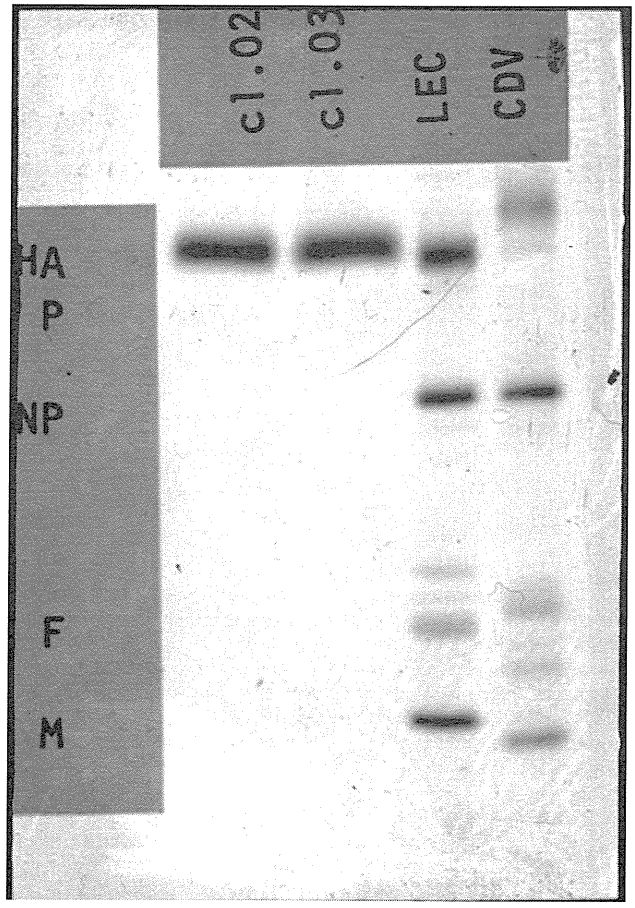


**Fig. 1.** Detection of HA antigen of measles virus by immunoprecipitation with using monoclonal antibody produced in hybridoma clones 02 and 03. HA; hemagglutinin, NP; nucleoprotein, F; fusion, M; matrix. LEC; measles virus reference strain. CDV; canine distemper virus.

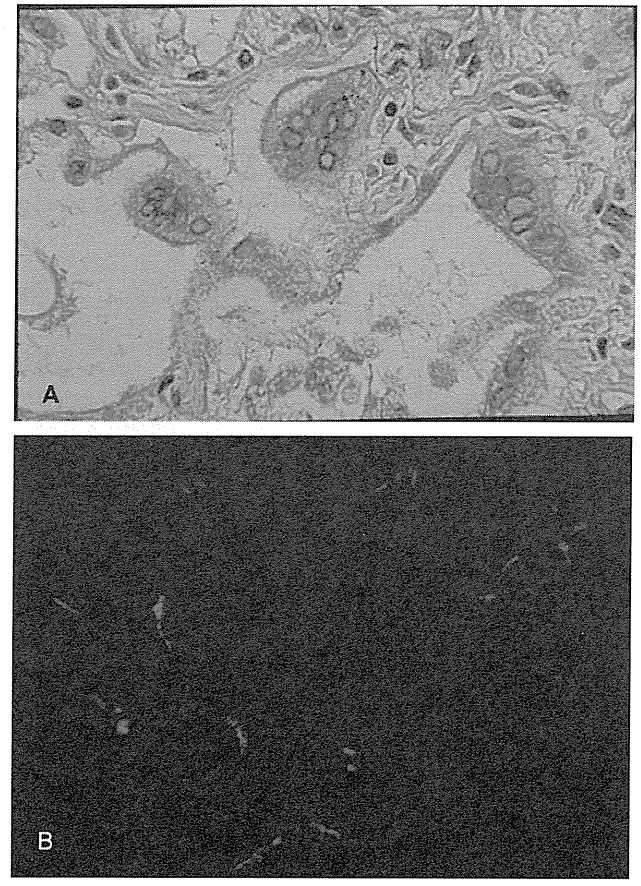


**Fig. 2.** Histological analysis of the lung tissue section from giant cell pneumonia. A; Hematoxylin–eosin staining. B; Immunofluorescent staining using monoclonal antibody against hemagglutinin of measles virus (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### *2.3. 'Hokkaido measles-zero strategy'*

Unfortunately it has been well known that Japan is nowadays one of the disgraceful countries, which 'export' measles. In this point, I decided to accomplish to eliminate measles firstly from Hokkaido prefecture, where I was living, and created a plan, the 'Hokkaido measles-zero strategy', to eliminate measles from Hokkaido within 5 years in 2001 with very kind efforts of pediatricians in Hokkaido and those who were engaged in political and administrative activities. The result is shown in Fig. 4. The attempt, although initially looked successful until 2006, to my great regret, resulted in failure because of the fact that measles, which was transported from Tokyo to Hokkaido by a patient with measles during an incubation period, subsequently prevailed in Hokkaido in 2007. Secondary vaccine failure was a dominant reason for the outbreak. Since 2006 multiple (two) dose administration policy with combined measles-rubella vaccine has started in Japan. It is highly expected that measles will be eliminated from Japan in the near future.

## 3. Establishing the disease entity of influenza-associated encephalopathy

I encountered a peculiar case of encephalopathy, which occurred in a healthy 4-year-old boy with a fatal outcome in 32 h from the onset of fever to his death, during an influenza epidemic of 1994. I myself saw the patient at the Sapporo City General Hospital and felt instinctively at that time that the case was a distinct entity from previously known Reye syndrome or febrile convulsion (Fig. 5), and coworkers and I started a survey. We sent inquiries to 94 hospitals and institutes, in which pediatric age patients were treated in Hokkaido prefecture, asking if there were any hospitalized cases of acute onset brain dysfunction of unknown etiology during the current winter season (1994/95), irrespective of whether influenza was initially suspected or not. As a result, a total of 12 such cases were reported. As shown in Fig. 6, the peak incidence of the acute onset brain dysfunction was fairly coincident with the peak isolation of influenza virus from outpatients in the Sapporo City General Hospital, as well as the peak of cases of influenza-like illness reported weekly in Hokkaido. Since a very similar study result was again obtained in the 1995/96 winter season, we submitted the first report concerning 'influenza-associated encephalopathy' in 1996 to a scientific medical journal. However, unfortunately, the paper had not been accepted for publication by any journals published outside Japan. The paper was finally accepted for publication in 2000 in the journal "Pediatrics International" which is published by the Japan Pediatric Society. The survey on the 5 successive seasons (1994/95–1998/99) were described in the paper [3]. Subsequently, coworkers and I have reported that the vasogenic brain edema with generalized impairment of vascular endothelial cells caused by highly activated cytokines (i.e., 'cytokine storm') plays a central role in the pathophysiology of the disease entity (Fig. 7), through the journals which are published outside Japan [4,5]. Influenza-associated encephalopathy, for

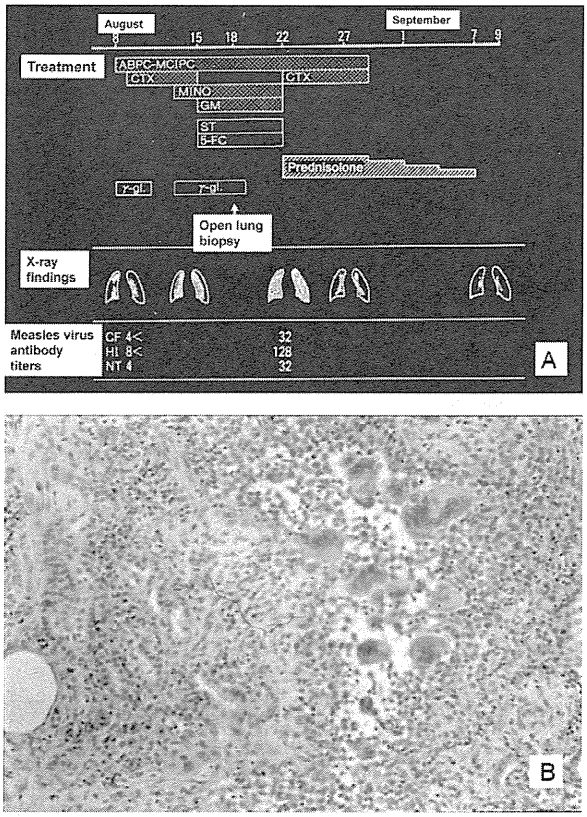


**Fig. 3.** Clinical course of a patient with measles pneumonia without immunodeficiency (A). Lung tissue of this patient (hematoxylin–eosin staining, B). No measles virus antigens were detected by immunofluorescent staining using the monoclonal antibodies in this case.

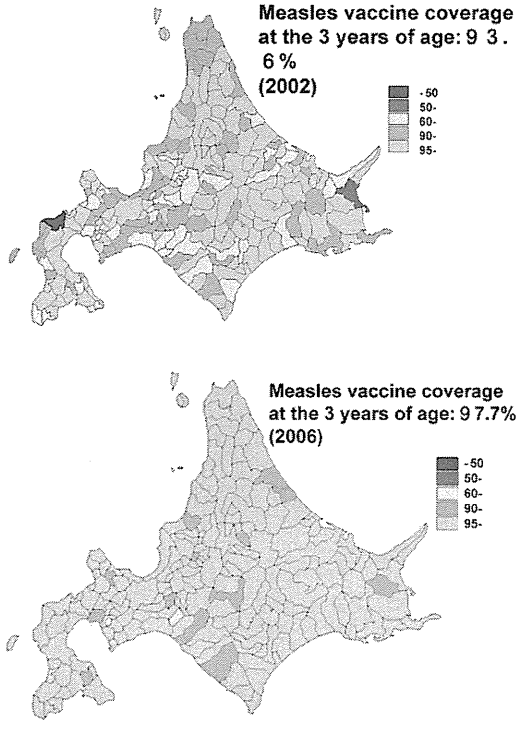


**Fig. 4.** Attempt to increase the measles vaccine coverage in Hokkaido ('Hokkaido measles-zero strategy'). The vaccine coverage increased from 93.6% in 2002 (upper panel) to 97.7% in 2006 (lower panel).

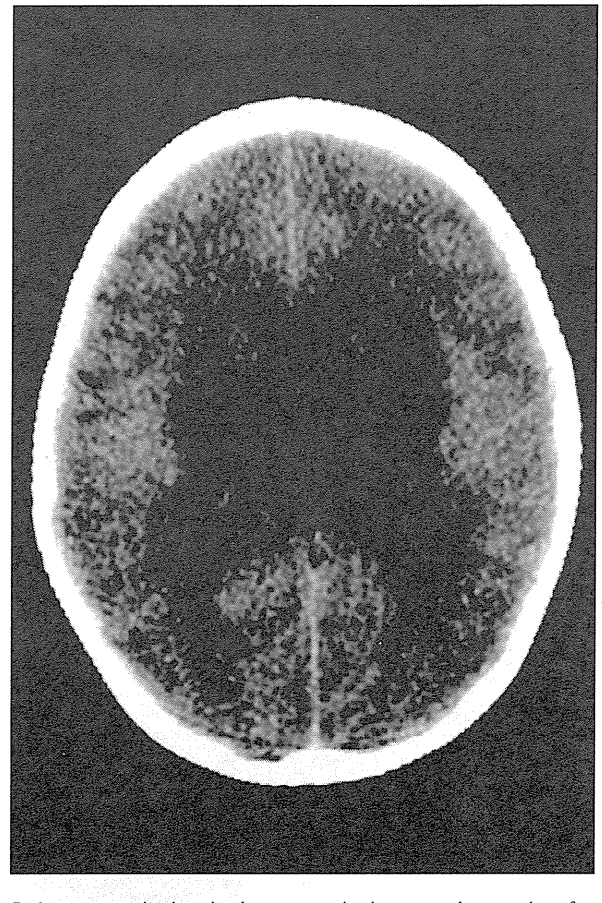

**Fig. 5.** A representative imaging by computerized tomography scanning of a case with influenza-associated encephalopathy which shows symmetrical low density areas in the bilateral thalami.

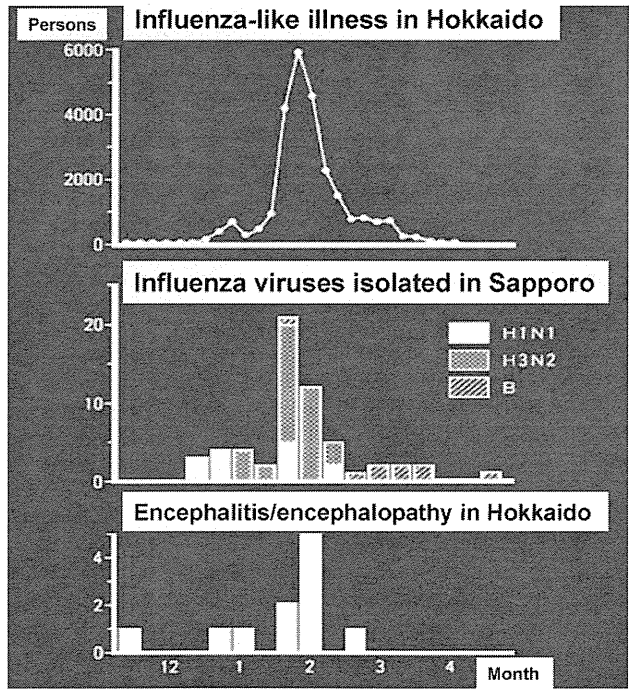


**Fig. 6.** Influenza epidemic, virus isolation, and encephalopathy during the 1994/95 season in Sapporo city, Hokkaido prefecture. Sapporo city is a Prefectural capital of Hokkaido prefecture.

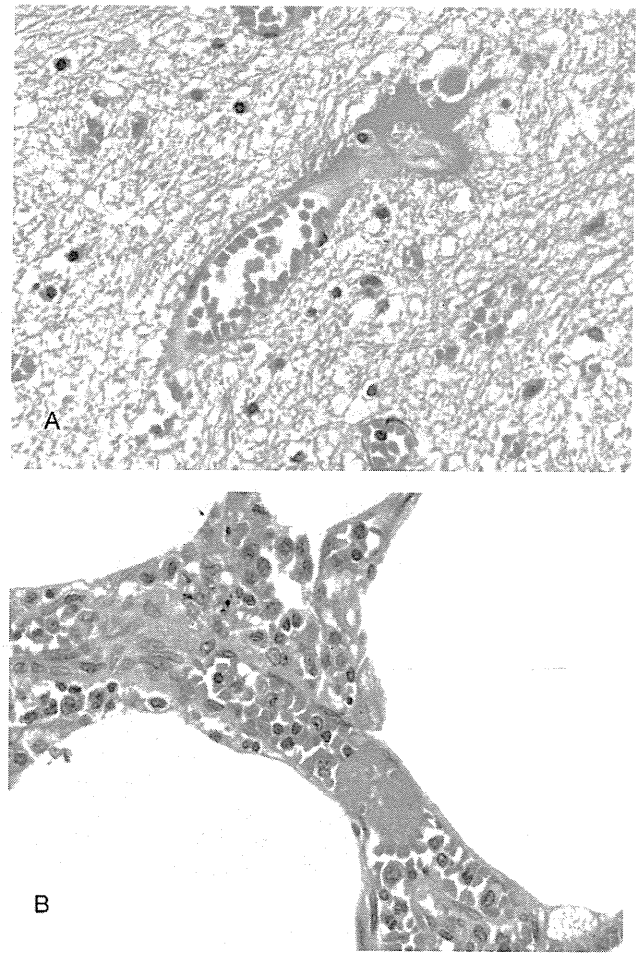


**Fig. 7.** Postmortem histological findings of influenza-associated encephalopathy. A; Brain (medulla) tissue showing vasogenic edema with hydropic degeneration, hyalinization of vessels, and extravasation of plasma proteins (hematoxylin–eosin staining). B; Lung tissue showing interstitial pneumonia and microvascular thrombi (hematoxylin–eosin staining).

which, initially even Japanese pediatricians outside Hokkaido prefecture had suspiciously said "Isn't it 'a local disease' in Hokkaido?", has now acquired its distinct disease entity worldwide [6]. To prevent children from suffering from this devastating disease condition, influenza vaccination must further be promoted. At the same time, development of more efficacious influenza vaccine is highly expected.

**Table 1**
Number of the reported cases of bacterial meningitis, which occurred in children less than 16 years old in Hokkaido prefecture during recent four years of period.

| Year | *Hib* | *S. pn* | GBS | *E. coli* | Others | Total |
|---|---|---|---|---|---|---|
| 2007 | 11 (6/5) | 6 (4/2) | 2 (1/1) | 1 (1/0) | 1 (0/1) | 21 (12/9) |
| 2008 | 13 (7/6) | 1 (1/0) | 2 (1/1) | 1 (1/0) | 1 (1/0) | 18 (11/7) |
| 2009 | 12 (8/4) | 4 (1/3) | 1 (1/0) | 2 (1/1) | | 19 (11/8) |
| 2010 | 11 (5/6) | 4 (1/3) | 0 | 1 (0/1) | | 16 (6/10) |
| Total | 47 (26/21) | 15 (7/8) | 5 (3/2) | 5 (3/2) | 2 (1/1) | 74 (40/34) |

Numbers in parentheses are (male/female). *Hib*, *Haemophilus influenzae* type b; *S. pn*, *Streptococcus pneumoniae*; GBS, group B Streptococcus; *E. coli*, *Escherichia coli*.

## 4. Introduction of the *H. influenzae* type b conjugate vaccine and the pediatric 7-valent pneumococcal conjugate vaccine in Japan

The *H. influenzae* type b conjugate vaccine and the pediatric 7-valent pneumococcal conjugate vaccine were introduced in Japan in 2008 and 2010, respectively. I worked as a member of some groups, which accumulated the basic data on the occurrence of bacterial meningitis in Japan and urged the government to introduce those vaccines. Number of cases of bacterial meningitis, which occurred in children in Hokkaido during recent four years of period, are shown in Table 1. To accumulate this kind of data is sober and important in evaluating the efficacy of the *H. influenzae* type b conjugate vaccine and the pediatric 7-valent pneumococcal conjugate vaccine. I honestly hope that the figures in Table 1 will be rewritten as zero in the future.

## Acknowledgements

I express many thanks to all of my colleagues not only for their contributions to the works which are cited, but also for those which are not cited in this paper.

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ORIGINAL ARTICLE

# Anti-polyribosylribitol phosphate antibody in pediatric patients with *Haemophilus influenzae* type b invasive disease

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**Abstract** *Haemophilus influenzae* type b conjugate vaccine was recently introduced to Japan for voluntary immunizations. *H. influenzae* type b remains a leading cause of pediatric invasive diseases in Japan. The purposes of this study were to verify the suitability of the *H. influenzae* type b conjugate vaccine for immunizing children with a history of invasive *H. influenzae* type b disease and to determine whether *H. influenzae* type b conjugate vaccine is immunogenic in these children. The subjects comprised 64 children with a history of invasive *H. influenzae* type b disease. Serum samples from 64 patients with *H. influenzae* type b systemic infection in the acute and convalescent phases were analyzed. Serum anti-polyribosylribitol phosphate antibody responses of patients <2 years old were poorer than those observed in patients ≥2 years old. Nineteen of the 64 patients received a single dose of *H. influenzae* serotype b conjugate vaccine, and then follow-up serum was taken and analyzed. Eighteen of 19 patients had ≥1 μg/mL of anti-polyribosylribitol phosphate antibody titer after the first dose of *H. influenzae* type b conjugate vaccine. *H. influenzae* type b conjugate vaccine is immunogenic in children with invasive *H. influenzae* type b disease. Children <4 years old, and particularly <2 years old, with invasive *H. influenzae* type b disease should receive subsequent immunization with a *H. influenzae* type b conjugate vaccine.

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**Keywords** *Haemophilus influenzae* · Vaccine · Child · Polyribosylribitol phosphate

## Introduction

*Haemophilus influenzae* is one of the leading causes of pediatric infectious disease, and *H. influenzae* type b (Hib) strains are known to constitute a major cause of invasive infections such as meningitis, sepsis and epiglottitis in children. More than 100 countries have introduced Hib vaccines as a part of routine immunization programs. As a consequence, the prevalence of infectious diseases caused by Hib has decreased dramatically [1, 2]. Hib vaccine is regarded as highly safe, and is widely used [3]. Hib vaccine has only recently been introduced into the voluntary immunization schedule in Japan, and Hib remains a leading cause of pediatric invasive infections, particularly meningitis, in Japan [4]. Most invasive Hib disease occurs in children <5 years old, with a peak incidence between 7 and 23 months old [5]. Hib is an encapsulated bacteria, with the capsule composed of polyribosylribitol phosphate (PRP). PRP antibody is an important protective antibody against invasive Hib disease. Children <2 years old may not develop protective antibodies to PRP after episodes of invasive Hib disease [6]. Furthermore, a subpopulation of children who have recovered from invasive Hib disease may also be at risk of developing a second episode of invasive Hib disease [7]. Strategies aimed at preventing a second episode of Hib disease in children with a history of Hib disease have included immunization with Hib conjugate vaccine.

The purposes of this study were to verify the suitability of the Hib conjugate vaccine for immunizing children with a history of invasive Hib disease and to determine whether

the Hib conjugate vaccine is immunogenic in these children.

## Patients and methods

Subjects comprised 64 children with a history of invasive Hib disease. The children were admitted to either the Department of Pediatrics at Chiba University Hospital or to 25 other hospitals located in various areas throughout Japan between 1997 and 2009. Diagnoses included meningitis ($n = 38$), epiglottitis ($n = 13$), sepsis ($n = 4$), cellulitis ($n = 3$), arthritis ($n = 3$), pneumonia ($n = 1$), endocarditis ($n = 1$), and osteomyelitis ($n = 1$). Five of the 64 children had a history of recurrent invasive Hib disease. These 5 children did not suffer from congenital immunodeficiency or congenital anomalies, for example cerebrospinal fluid fistula and Mondini anomaly. Serum samples from 64 children with invasive Hib disease in the acute phase and convalescent phase (2–3 weeks after admission) were analyzed. The number of serum samples in the acute phase obtained <24 h, 1–2 days, and 3–5 days after onset of symptoms were 16, 38, and 10, respectively. Nineteen of the 64 children received a single dose of Hib conjugate vaccine. Serum for analysis was taken just before vaccination and at follow-up (4–8 weeks after vaccination). Serum samples were transported to our laboratory and stored at −20°C until needed. Informed consent was obtained from the parents and permission from the health care provider of each child was obtained. All study protocols were approved by the Chiba University Institutional Review Board for Clinical Investigations. Anti-PRP antibody titers were analyzed using a Bindazyme anti-haemophilus B enzyme immunoassay kit (The Binding Site, Birmingham, UK). This is the only commercially available EIA kit for measurement of anti-PRP antibody. Schauer et al. measured anti-PRP antibody in serum samples of 386 age-stratified subjects using this EIA kit. They reported that in all unimmunized infants below 1 year of age the concentration of anti-PRP antibodies was <1.0 μg/mL [8]. To date, there has been no comparative data between standard radioantigen-binding assay and this EIA kit. Statistical analyses were performed using SPSS software (SPSS, IL, USA). Fisher's exact test was used to compare the proportion of children in the convalescent phase of infection with ≥1.0 μg/mL of anti-PRP antibody. Geometric mean titers (GMTs) were calculated for pre and post-immunization titers. Titers <0.1 μg/mL (the low cutoff of assay sensitivity) were considered equal to 0.1 μg/mL for the purposes of data analysis. Pre and post-immunization GMTs were compared using a paired $t$ test on log-transformed data.

## Results

Anti-PRP antibody titers were <0.15 μg/mL for 40 of the 64 children with invasive Hib disease in the acute phase, and <1 μg/mL for 63 of the 64 children. Anti-PRP antibody titer for one 3-year-old child with endocarditis was ≥1 μg/mL in the acute phase (1.13 μg/mL). Table 1 shows immune responses after Hib invasive disease according to age. All 5 children ≥4 years old responded with ≥1 μg/mL of anti-PRP antibody titer after invasive Hib disease. Anti-PRP antibody titers were <0.15 μg/mL for 19 of the 59 children <4 years old with invasive Hib disease in the convalescent phase, and <1 μg/mL for 42 of the 59 children.

Three of 5 children with recurrent Hib invasive diseases did not respond with anti-PRP antibody titer ≥1 μg/mL after a second episode of invasive Hib disease. Anti-PRP antibody responses of children <2 years old were poorer than those of patients ≥2 years old. Anti-PRP antibody responses of children with meningitis were poorer than those of children with epiglottitis. Nineteen of the 64 children had been given one dose of Hib conjugate vaccine.

**Table 1** Immune response after Hib invasive disease according to age group

| Diagnosis | 0 Year | 1 Year | 2 Years | 3 Years | 4 Years | ≥5 Years | Total |
|---|---|---|---|---|---|---|---|
| Meningitis | 14 (1) | 13 (0) | 4 (0) | 3 (0) | 1 (1) | 3 (3) | 38 (5) |
| Epiglottitis | 0 | 1 (1) | 6 (6) | 5 (4) | 1 (1) | 0 | 13 (12) |
| Sepsis | 3 (0) | 1 (0) | 0 | 0 | 0 | 0 | 4 (0) |
| Cellulitis | 1 (0) | 1 (1) | 1 (1) | 0 | 0 | 0 | 3 (2) |
| Arthritis | 0 | 2 (0) | 1 (1) | 0 | 0 | 0 | 3 (1) |
| Endocarditis | 0 | 0 | 0 | 1 (1) | 0 | 0 | 1 (1) |
| Pneumonia | 0 | 0 | 0 | 1 (0) | 0 | 0 | 1 (0) |
| Osteomyelitis | 0 | 0 | 1 (1) | 0 | 0 | 0 | 1 (1) |
| Total | 18 (1) | 18 (2) | 13 (9) | 10 (5) | 2 (2) | 3 (3) | 64 (22) |

Numbers in parentheses are the number of children with anti-PRP antibody ≥1.0 μg/mL in the convalescent phase

**Table 2** Characteristics and antibody responses of children with Hib invasive disease

| Diagnosis | Age at diagnosis (months) | Age at vaccine (months) | Pre-GMT (μg/mL) | Post-GMT (μg/mL) |
|---|---|---|---|---|
| Meningitis | 5 | 10 | <0.1 | 1.31 |
| Meningitis[a] | 5 | 20 | 0.82 | 8.90 |
| Meningitis | 6 | 8 | <0.1 | 9.14 |
| Meningitis | 6 | 33 | <0.1 | 9.42 |
| Meningitis | 7 | 15 | <0.1 | 3.20 |
| Meningitis | 7 | 15 | 0.35 | 4.83 |
| Meningitis | 8 | 34 | <0.1 | 9.50 |
| Sepsis | 10 | 41 | 0.35 | 0.45 |
| Meningitis | 12 | 14 | <0.1 | 1.68 |
| Meningitis | 12 | 29 | <0.1 | 14.0 |
| Meningitis | 13 | 53 | <0.1 | 9.22 |
| Meningitis | 14 | 19 | <0.1 | 8.92 |
| Meningitis | 15 | 24 | 0.27 | 16.05 |
| Meningitis[a] | 16 | 29 | <0.1 | 8.64 |
| Sepsis[a] | 17 | 39 | 0.47 | 15.90 |
| Meningitis | 19 | 23 | 0.86 | 8.80 |
| Meningitis | 24 | 36 | 0.1 | 10.18 |
| Meningitis[a] | 29 | 30 | 3.82 | 6.15 |
| Pneumonia | 41 | 43 | <0.1 | 7.36 |
| GMT | | | 0.198 | 6.20[b] |

[a] Second episode of Hib invasive disease

[b] $P < 0.001$, Pre-GMT versus Post-GMT

Eighteen of the 19 children had anti-PRP antibody titer ≥1 μg/mL after administration of Hib conjugate vaccine (Table 2). No serious adverse reactions to the vaccine occurred in any child who received Hib vaccine.

## Discussion

The most important factor for susceptibility to Hib is young age. This is explained by the inability of children <24 months old to produce PRP antibodies in sufficiently large amounts to protect against the disease [9, 10]. Anti-PRP antibody titers of 0.15 and 1 μg/mL have been established as the minimum levels required to achieve protection and long-term protection, respectively [11]. In our study, 19 (29.7%) of the 64 children had antibody levels <0.15 μg/mL after invasive Hib disease and 42 (65.6%) of the 64 children had <1 μg/mL antibody. In particular, 15 (41.7%) of 36 children <2 years old had <0.15 μg/mL antibody after invasive Hib disease and 33 (91.7%) of these 36 children had <1 μg/mL antibody, confirming previous observations that young children typically do not develop protective levels of antibodies to invasive Hib disease. Similarly, Walter et al. [12] reported that only 1 of 10 children ≥12 months old and none of 13 children <12 months old had significant antibody responses after recovering from invasive Hib disease. Furthermore, 9 (39.1%) of 23 children 2–4 years old with invasive Hib disease in our study did not have ≥1 μg/mL antibody and 4 (80.0%) of 5 children with recurrent invasive Hib diseases likewise did not achieve ≥1 μg/mL after a second episode of invasive Hib disease. Interestingly, the proportion of children with ≥1 μg/mL anti-PRP antibody in the convalescent phase was significantly higher for the 13 children with epiglottitis than for the 38 children with meningitis. Johnson et al. compared levels of anti-PRP antibody in a larger group of children with either epiglottitis or meningitis. According to their results, children with epiglottitis respond more vigorously in convalescence than those with meningitis, a finding that cannot be explained by age alone. They suggested that the poor convalescent-phase response was not a general feature of children with Hib meningitis, but was instead attributable to a sub-group of poor responders [13]. Host factors related to lower antibody responses with invasive Hib disease have yet to be determined and further studies are warranted.

The Hib conjugate vaccine is currently indicated for voluntary immunization of children at 2–59 months old in Japan. In this study we also measured the immunogenicity of the Hib conjugate vaccine (tetanus toxoid conjugate) in children with previous invasive Hib disease. Hib conjugate vaccine induced an immunogenic response in 18 of the 19 children tested. The mean age at vaccination was 27.1 months (range, 8–53 months). In a study similar to ours, Kaplan et al. [14] reported that 15 of 17 children responded with ≥1 μg/mL anti-PRP antibody after a single dose of Hib conjugate vaccine and all children responded

with ≥1 μg/mL anti-PRP antibody after two doses of Hib conjugate vaccine. Conversely, Walter et al. reported that only 9 of 19 children <15 months old responded with ≥1 μg/mL anti-PRP antibody after a single dose of vaccine. They suggested that a two-dose regimen should be considered for children <15 months old who are recovering from an episode of invasive Hib disease [12]. Hib conjugate vaccine is immunogenic in children with no anti-PRP response to invasive Hib disease, because children are most at risk of developing a second episode of Hib invasive disease within 6 months of the initial illness [7]. Indeed, our study included 5 children who experienced recurrent episodes of invasive Hib disease. Hib conjugate vaccine should optimally be used promptly after recovery from invasive Hib disease in any child <4 years old, particularly in those <2 years old, in Japan.

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# Incidence of childhood pneumonia and serotype and sequence-type distribution in *Streptococcus pneumoniae* isolates in Japan

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## SUMMARY

The 7-valent pneumococcal conjugate vaccine (PCV7) is reported to decrease the incidence of community-acquired pneumonia (CAP) in children. To determine the annual incidence of CAP before the introduction of PCV7, we counted the number of children hospitalized with CAP between 2008 and 2009 in Chiba City, Japan. We investigated serotype and multilocus sequence typing (MLST) for *Streptococcus pneumoniae* isolates in CAP cases. The annual incidence of hospitalized CAP in children aged <5 years was 17·6 episodes/1000 child-years. In 626 episodes, *S. pneumoniae* was dominant in 14·7% and 0·8% of sputum and blood samples, respectively. The most common serotypes were 6B, 23F and 19F. The coverage rates of PCV7 were 66·7% and 80% in sputum samples and blood samples, respectively. MLST analysis revealed 37 sequence types. Furthermore, 54·1% of the sputum isolates and 40% of the blood isolate were related to international multidrug-resistant clones.

**Key words**: Antibiotic resistance, community-acquired pneumonia, immunization (vaccination), incidence, *Streptococcus pneumoniae* (pneumococcus).

## INTRODUCTION

*Streptococcus pneumoniae* is a frequent aetiological cause of community-acquired pneumonia (CAP) in children. The 7-valent pneumococcal conjugate vaccine (PCV7), introduced in the USA and Europe, has reduced the incidence of invasive pneumococcal disease (IPD) caused by vaccine serotypes (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F) and of carriage of these serotypes [1–4]. Several reports indicate that PCVs are effective against pneumonia [5–7]. Black *et al.* [5] reported that PCV7 reduces the incidence of first episode of clinically diagnosed pneumonia by 6·0%. The rate of all-cause pneumonia hospitalizations in children in the USA aged <2 years decreased by about 35% after the vaccine was licensed [6]. In these studies, bacteraemic pneumococcal pneumonia constituted a minority of the total amount of observed clinical pneumonia. These results indicate that PCV not only prevents invasive pneumococcal pneumonia but also reduces the incidence of all-cause pneumonia. However, little is known about the rate of pneumonia attributable to *S. pneumoniae* and their serotypes.

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PCV7 was introduced in Japan in February 2010. Surveillance of the population-based incidence of CAP and molecular characterization of isolates causing CAP are fundamental to understanding the impact of PCV7 and to assessing whether the genetic structure of the pneumococcal population changes after implementation of an immunization programme. However population-based studies of CAP in Japanese children are rare.

To estimate bacterial pathogens and to better manage lower respiratory tract infections in children, we examined microbiological specimens of washed sputum according to the Japanese Guidelines for the Management of Respiratory Infectious Diseases [8–13]. Here, we surveyed the incidence of CAP in hospitalized children to obtain baseline data before the introduction of PCV7. We also determined the isolation rate of *S. pneumoniae* in children with CAP using washed sputum and blood samples to estimate the effect of PCV7 on CAP. The isolates were characterized by serotyping and by multilocus sequence typing (MLST).

## METHODS

### Incidence of CAP and of CAP with pneumococcal bacteraemia in Chiba City

We determined the annual incidence of hospitalized CAP and CAP with pneumococcal bacteraemia in children aged <16 years in Chiba City as follows. We retrospectively counted the total number of patients admitted to 18 hospitals with paediatric wards in and around Chiba City serving the catchment population between 1 April 2008 and 31 March 2009. A questionnaire was sent to 18 hospitals and information was obtained from the clinical records of all of them. We defined CAP as pneumonia that occurred in patients who had not been hospitalized within the past 2 weeks. Acute lower respiratory infection was diagnosed by clinicians at each hospital based on clinical symptoms of one or more of: fever, rapid or difficult breathing, cough and crackle in lung fields on auscultation. Radiographs were taken before admission and the diagnosis of CAP was confirmed by clinicians based on positive radiograph findings at the time of occurrence. Patients with CAP who did not require hospitalization were excluded from this study. The catchment area comprised 944 557 inhabitants, including 140 345 and 42 606 children aged <16 and <5 years, respectively [14].

### Rate of *S. pneumoniae* isolated from sputum and blood

We surveyed children who were admitted to six major hospitals in Chiba City. These six hospitals covered 75% of hospitalized children who were diagnosed with CAP within the city during 2005. [15]. Written informed consent was obtained from the parents or guardians of the patients before collecting samples, in accordance with the guidelines of the Institutional Review Board of Chiba University. Demographic and clinical data were collected by paediatricians. Upon admission, blood samples were collected and sputum samples were obtained using a tongue depressor with a light as follows. The tongue was depressed to induce the cough reflex and then sputum was collected using a swab or aspirated into a 1-ml disposable syringe. Sputum samples were washed three times in sterilized saline as described previously [9]. A small portion of washed sputum was homogenized and smeared onto glass slides for Gram staining. Stained smears were judged valid according to Geckler's classification based on the number of leucocytes or alveolar macrophages and squamous or ciliated epithelial cells per low-power field (100x). Smears with Geckler's groups of 4–5 containing >25 leucocytes or macrophages and <25 squamous or ciliated epithelial cells in the low-power microscopic field (100x) were considered adequate. Washed sputum and blood samples were cultured at the microbiology laboratory of each hospital. Pathogens accounting for >50% of the colonies in culture or presenting $>1 \times 10^7$c.f.u./ml of washed sputum were regarded as 'dominant'. *S. pneumoniae* isolates dominant in sputum samples and/or isolates from blood samples were initially stored at −80 °C at each hospital and then sent to Chiba University Hospital and the Department of Bacteriology of the National Institute of Infectious Diseases.

### Antimicrobial susceptibility

Antimicrobial susceptibility was tested *in vitro* using broth dilution according to the Clinical and Laboratory Standards Institute guidelines (CLSI M100-S18). Although the CLSI published new breakpoints for penicillin therapy in 2008 (CLSI M100-S18), we used the previously published breakpoints. *S. pneumoniae* was interpreted as susceptible (PSSP), intermediate (PISP), and resistant (PRSP) if the minimum inhibitory concentration (MIC) of penicillin G was ≤0·06, 0·12–1 and ≥2 μg/ml, respectively.

### Serotyping

Serotypes were determined by the Quellung reaction using antiserum purchased from Statens Serum Institut, Copenhagen, Denmark. We serotyped 6C and 6D using an in-house antiserum and confirmed the results by genetic characterization as described previously [16].

### MLST

We performed MLST as described previously [17]. Briefly, internal fragments of each of the seven housekeeping genes, *aroE, gdh, gki, recP, spi, xpt* and *ddl* were amplified by Polymerase chain reaction (PCR) and their sequence types (STs) were determined by reference to the MLST database (http://spneumoniae.mlst.net/). New alleles and allelic profiles were submitted to the database for assignment. The relatedness of isolates and known similar strains in the database were determined by constructing a neighbour-joining tree using the online program, Draw Tree Using Own MLST Data. Relationships among the isolates were determined using eBURST v. 3 software and strains were assigned to clonal complexes (CCs) using the definition of a stringent group, in which all STs share six of seven identical alleles with at least one of the other STs within the group [single-locus variants (SLV)]. We compared STs with those of 43 pneumococcal clones in the Pneumococcal Molecular Epidemiology Network (PMEN; http://www.sph.emory.edu/PMEN/).

### Statistical analysis

Data were analysed using PASW Statistics 18 (SPSS Japan Inc., Japan). Associations between underlying diseases and the number of hospitalizations or the results of sputum culture were tested using Fisher's exact test. Correlations between age and isolation rates of pneumococcus and coverage rates of PCV7 were analysed using Pearson's $\chi^2$ test. A $P$ value of $<0{\cdot}05$ was considered statistically significant.

## RESULTS

### Annual incidence of CAP

During the study period, CAP caused 860 episodes of children being hospitalized. The incidences of CAP in children aged $<16$ and $<5$ years were 6·13 and 17·6/1000 child-years, respectively.

### Annual incidence of CAP with pneumococcal bacteraemia

Five patients were diagnosed with pneumococcal bacteraemia combined with CAP. The incidences of CAP with pneumococcal bacteraemia in children aged $<16$ and $<5$ years were 3·56 and 11·7 episodes/100 000 child-years, respectively.

### Isolation of *S. pneumoniae* from sputum and blood culture

A total of 579 children with 626 episodes were admitted to six major hospitals with a diagnosis of CAP. This corresponded to 73% of all CAP episodes occurring in Chiba City during the study period. We obtained sputum and blood samples representing 502 (80·2%) and 544 (86·9%) of the 626 episodes. *S. pneumoniae* was identified and culture-dominant in 175 (27·9%) and 92 (14·7%) sputum samples, respectively. Of five patients with blood samples that were positive for *S. pneumoniae*, one also had a positive sputum culture. The serotypes and STs of the blood and sputum isolates from this patient were identical (serotype 6B, ST90).

Figure 1 shows the age distribution of children with CAP and the results of *S. pneumoniae* identified in sputum and blood cultures. The median age of children with CAP episodes was 1 year. Patients with CAP included 331 (52·9%) children aged $<2$ years and 230 (36·7%) aged 2–4 years. The median age of children with pneumococcus-positive episodes was also 1 year. *S. pneumoniae* was isolated from the blood of one 2-year-old and four 1-year-old patients. The detection rate of pneumococcus from sputum was the highest in children aged 2–4 years (18·7%), followed by those aged $<2$ (11·5%) and 5–15 (16·9%) years [statistically not significant, $\chi^2(2)=5{\cdot}92$, $P=0{\cdot}052$].

Of the 579 patients, 215 had one or more underlying diseases, 174 had bronchial asthma, 24 were premature, ten had congenital heart diseases, seven had chromosome anomalies, five had cerebral palsy, and 18 had other diseases. Thirty-seven patients were hospitalized more than once. Multiple hospitalizations were significantly associated with bronchial asthma ($P<0{\cdot}001$), congenital heart disease ($P=0{\cdot}021$) and cerebral palsy ($P=0{\cdot}035$) (Table 1). Underlying diseases and the detection of *S. pneumoniae* from sputum were not significantly related. No one had sequelae with CAP episodes.

Table 1. *Risk of multiple hospitalizations with community-acquired pneumonia based on underlying diseases*

| Underlying disease | No. of multiple hospitalizations/ no. with factors (%) | No. of multiple hospitalization/ no. without factors (%) | *P* value* | Relative risk (95% CI) |
|---|---|---|---|---|
| Bronchial asthma | 21/174 (12%) | 16/405 (4%) | <0·001 | 3·06 (1·63–5·71) |
| Premature birth | | | | |
| <37 weeks† | 3/24 (13%) | 34/555 (6%) | 0·193 | 1·07 (0·92–1·25) |
| <30 weeks | 1/8 (13%) | 36/571 (6%) | 0·412 | 1·98 (0·31–12·74) |
| Congenital heart disease | 3/10 (30%) | 34/569 (6%) | 0·021 | 5·02 (1·85–13·67) |
| Chromosomal anomaly | 1/7 (14%) | 36/572 (6%) | 0·372 | 2·27 (0·36–14·32) |
| Cerebral palsy | 2/5 (40%) | 35/574 (6%) | 0·035 | 6·56 (2·14–20·12) |
| Other‡ | 1/18 (6%) | 36/561 (6%) | 1·000 | 0·87 (0·13–5·97) |

CI, Confidence interval.
* Fisher's exact test.
† Including preterm birth <30 weeks.
‡ Epilepsy (2), neutropenia (2), achondroplasia (1), acute myeloid leukaemia (during consolidation therapy) (1), bronchiectasis (1), congenital diaphragmatic hernia (1), cretinism (1), gastro-oesophageal reflux disease (1), Kawasaki disease (1), mitochondrial diseases (1), periodic fever syndrome (1), polycystic kidney disease (1), post-cleft lip and palate repair (1), Sotos syndrome (1), tracheal stenosis (1), malnutrition (1).

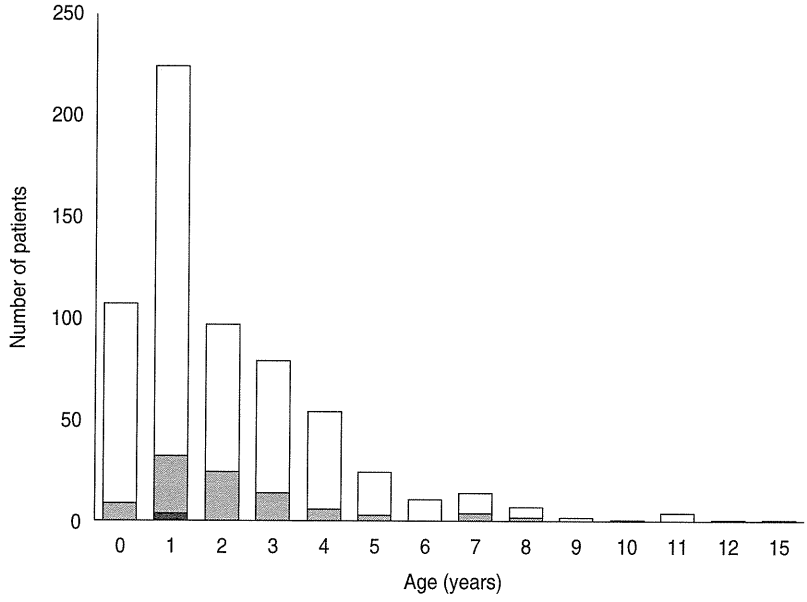



**Fig. 1.** Age distribution and identification of *Streptococcus pneumoniae* in children hospitalized with community-acquired pneumonia. □, *S. pneumoniae* dominantly isolated from sputum; ■, *S. pneumoniae* isolated from blood ($n=626$).

**Antimicrobial susceptibility and serotype distribution**

Antimicrobial susceptibility and serotypes were tested against 63/92 cultured sputum isolates in which *S. pneumoniae* was the dominant organism and against five blood isolates. Table 2 shows the susceptibility of the *S. pneumoniae* isolates to penicillin G and their serotype distribution. The rates of PISP and PRSP in sputum isolates were 54% and 22%, respectively, and 40% and 40%, respectively, in the blood isolates. Resistance rates in sputum isolates against cefotaxime (MIC $\geqslant 2\,\mu$g/ml), erythromycin (MIC $\geqslant 1\,\mu$g/ml) and clindamycin (MIC $\geqslant 1\,\mu$g/ml) were 4·8%, 92% and 60%, respectively, and 20%, 100% and 60%, respectively, in blood isolates. All isolates were susceptible to meropenem and vancomycin.

Of the 17 identified serotypes, the most frequent in the sputum isolates were 6B (28·6%), 23F (17·5%), and 19F (15·9%) and those in the blood isolates were 6B (60%), 19F (20%), and 19A (20%). Serotype 6C

Table 2. *Serotype distribution and susceptibility of* Streptococcus pneumoniae *isolated from samples obtained from children with community-acquired pneumonia in Japan*

| | | | No. of isolates | | | |
|---|---|---|---|---|---|---|
| Sample | Coverage rate | Serotype | PSSP | PISP | PRSP | All |
| Sputum | 7-valent (66·7%) | 6B | 3 | 9 | 6 | 18 |
| | | 23F | | 9 | 2 | 11 |
| | | 19F | | 5 | 5 | 10 |
| | | 14 | | 2 | | 2 |
| | | 9V | 1 | | | 1 |
| | 10-valent (71·4%) | 1 | 2 | | | 2 |
| | | 7F | 1 | | | 1 |
| | 13-valent (81. 0%) | 6A | | 3 | 1 | 4 |
| | | 3 | 1 | | | 1 |
| | | 19A | 1 | | | 1 |
| | Others | 6C | 1 | 2 | | 3 |
| | | 23A | | 2 | | 2 |
| | | 35B | | 2 | | 2 |
| | | 38 | 2 | | | 2 |
| | | 15B | 1 | | | 1 |
| | | 22F | 1 | | | 1 |
| | | 24B | 1 | | | 1 |
| | | Total | 15 | 34 | 14 | 63 |
| Blood | 7-valent (80%) | 6B | | 2 | 1 | 3 |
| | | 19F | | | 1 | 1 |
| | 13-valent (100%) | 19A | 1 | | | 1 |
| | | Total | 1 | 2 | 2 | 5 |

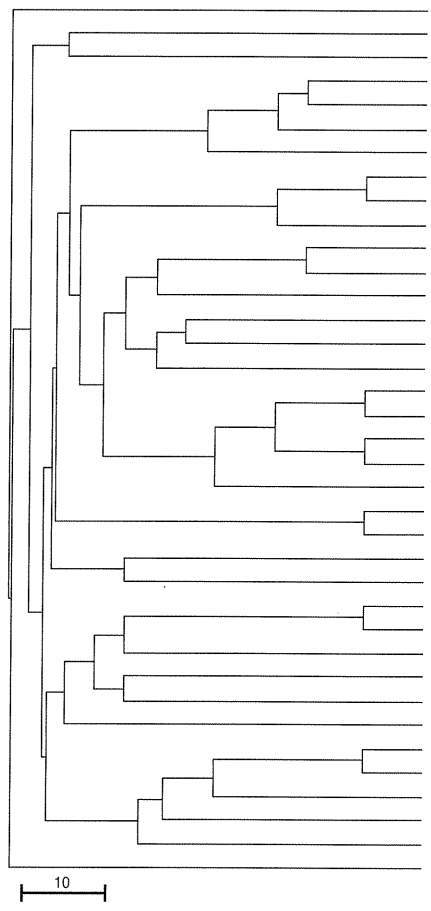
PSSP, Penicillin-susceptible *S. pneumoniae*; PISP, penicillin-intermediate *S. pneumoniae*; PRSP, penicillin-resistant *S. pneumoniae*.

was identified in three sputum isolates. Serotype 6D was not found. The coverage rates of PCV7 were 66·7% and 80·0% in sputum and blood isolates, respectively. The coverage rates in sputum isolates based on age were 65·2%, 73·5% (70·2% in those aged $<5$ years) and 33·3% in children aged $<2$, 2–4 and 5–15 years [statistically not significant, $\chi^2(2)=3{\cdot}74$, $p=0{\cdot}154$]. Ten-valent (PCV7 plus additional serotypes 1, 5, 7F), 13-valent (PCV7 plus additional serotypes 1, 3, 5, 6A, 7F. 19A) and investigational 15-valent (PCV7 plus additional serotypes 1, 3, 5, 6A, 7F, 19A, 22F, 33F) PCVs would potentially increase the coverage rates of sputum isolates by 4·7%, 14·3% and 15·8%, respectively. The 13-valent and investigational 15-valent PCVs covered all of the blood isolates.

The serotypes of PSSP in sputum isolates widely varied whereas the 14 PRSP sputum isolates fell into only the following serotypes: 6B (42·9%), 19F (35·7%), 23F (14·3%) and 6A (7·1%). The PISP sputum isolates were represented by eight serotypes with 6B (26·5%), 23F (26·5%), 19F (14·7%) and 6A (8·8%) being the most prevalent. The serotypes of PRSP in blood isolates were also 6B (50%) and 19F (50%), whereas that of PISP isolates was only 6B (100%). The PCV7 and PCV13 coverage rates for PRSP were 92·9% and 100% in sputum, respectively, and 100% in blood isolates.

## MLST

MLST was performed on 61/92 sputum isolates in which *S. pneumoniae* was the dominant organism and on five blood isolates. Of the 66 isolates, 37 STs were found including nine new STs (ST5830–5834 and ST5494–5497) with four new alleles. A dendrogram was constructed (Fig. 2) and eBURST analysis revealed six CCs and 25 singletons containing 23 and 43 isolates, respectively. Furthermore, 54·1% and 40% of the sputum and blood isolates had STs identical to 11 international PMEN clones or their SLVs. Eight multidrug-resistant PMEN clones




| Sequence Type* | Serotype | Allelic profile*† | No. of isolates | |
|---|---|---|---|---|
| | | | Sputum | Blood |
| ST393 | 38 | 10-43-41-18-13-49-6 | 2 | 0 |
| ST558 | 35B | 18-12-4-44-14-77-97 | 2 | 0 |
| ST433 | 22F | 1-1-4-1-18-58-17 | 1 | 0 |
| ST90 | 6B | 5-6-1-2-6-3-4 | 9 | 1 |
| **ST5830** | 6B | 5-6-1-2-6-1-42 | 1 | 0 |
| **ST5497** | 6B | 5-6-1-2-**242**-1-271 | 0 | 1 |
| **ST5834** | 6B | 7-28-1-2-6-1-5 | 1 | 0 |
| ST236 | 19F | 15-16-19-15-6-20-26 | 6 | 1 |
| ST2993 | 19F | 15-16-4-15-6-20-26 | 1 | 0 |
| **ST5495** | 19F | 22-16-19-15-6-20-**401** | 1 | 0 |
| ST1437 | 23F | 1-32-6-6-6-1-14 | 4 | 0 |
| ST2924 | 6B | 1-5-2-6-6-1-14 | 1 | 0 |
| ST855 | 6A | 10-32-19-1-6-14-14 | 1 | 0 |
| ST191 | 7F | 8-9-2-1-6-1-17 | 1 | 0 |
| ST280 | 9V | 15-17-4-16-6-1-17 | 1 | 0 |
| ST180 | 3 | 7-15-2-10-6-1-22 | 1 | 0 |
| ST2923 | 6B | 2-13-2-5-6-121-29 | 1 | 0 |
| **ST5831** | 6B | 2-13-2-5-6-124-29 | 1 | 0 |
| ST902 | 6B | 2-13-2-5-6-121-121 | 1 | 1 |
| **ST5494** | 6B | 2-13-2-5-6-121-**400** | 1 | 0 |
| ST5232 | 6B | 2-29-4-1-6-121-121 | 1 | 0 |
| ST81 | 23F | 4-4-2-4-4-1-1 | 1 | 0 |
| ST282 | 6A | 30-4-2-4-4-1-1 | 1 | 0 |
| ST2331 | 19A | 10-16-150-1-17-1-29 | 1 | 0 |
| ST2224 | 6B | 7-12-7-1-116-14-29 | 1 | 0 |
| ST242 | 23F | 15-29-4-21-30-1-14 | 5 | 0 |
| ST3563 | 23F | 15-29-4-21-17-1-14 | 1 | 0 |
| ST179 | 19F | 7-14-40-12-1-1-14 | 1 | 0 |
| ST199 | 15B | 8-13-14-4-17-4-14 | 1 | 0 |
| ST343 | 14 | 8-8-4-15-39-12-14 | 1 | 0 |
| ST3111 | 19A | 61-60-67-16-10-104-14 | 0 | 1 |
| ST338 | 23A | 7-13-8-6-1-6-8 | 1 | 0 |
| ST5246 | 23A | 7-13-8-6-1-138-8 | 1 | 0 |
| **ST5833** | 6A | 7-13-1-6-6-6-121 | 2 | 0 |
| **ST5496** | 24B | 7-**257**-9-6-25-6-14 | 1 | 0 |
| **ST5832** | 6C | 7-9-4-16-1-6-384 | 3 | 0 |
| ST306 | 1 | 12-8-13-5-16-4-20 | 2 | 0 |

**Fig. 2.** Genetic relatedness, multilocus sequence-typing profile, and serotypes in 37 sequence types of 66 *Streptococcus pneumoniae* isolates from children with community-acquired pneumonia in Japan. Scale bar indicates genetic linkage distance. PMEN, Pneumococcal Molecular Epidemiology Network. * New sequence types and alleles in bold. † In the order: *aroE-gdh-gki-recP-spi-xpt-ddl*.

comprised Spain$^{6B}$-2, Taiwan$^{19F}$-14, Taiwan$^{23F}$-15, Spain$^{23F}$-1, Utah$^{35B}$-24, Colombia$^{23F}$-26, Portugal$^{19F}$-21, Greece$^{6B}$-22 and three susceptible PMEN clones comprised Sweden$^{1}$-28, Netherlands$^{15B}$-37 and Netherlands$^{7F}$-39. Isolates related to the eight multidrug-resistant PMEN clones comprised 49·1% and 40% of sputum and blood isolates, respectively. Table 3 shows the numbers and antimicrobial susceptibility of isolates with STs identical to multidrug-resistant PMEN clones or their SLVs. Sputum isolates related to multidrug-resistant PMEN clones comprised 62·5% and 71·4% of PISP and PRSP clones, respectively.

## DISCUSSION

The annual incidence of CAP in children aged <5 years who were hospitalized with this condition was 17·6 episodes/1000 child-years and that of CAP with pneumococcal bacteraemia in those aged <5 years was 11·7 episodes/100 000 child-years. In 626 episodes, *S. pneumoniae* was dominant in 14·7% and 0·8% of sputum and blood samples, respectively.

Our findings of the incidence of pneumococcal bacteraemia in children hospitalized with CAP were equivalent to those of our previous survey [15, 18]. The incidence of pneumonia in children aged <5 years that required hospitalization before the introduction of PCV7 in the USA and European countries was 2·25–6·55/1000 child-years [19–21]. The annual incidence of paediatric CAP was higher in the present study than in these reports and similar to that of a study in Germany that included outpatients (13·7–16·9/1000 child-years in children aged <5 years) [22]. The reasons for the variation in incidence

Table 3. *Antimicrobial susceptibility of isolates with sequence types identical to multidrug-resistant PMEN clones or their single locus variants*

| Sequence type | Sero-type | Related PMEN clone | No. | $MIC_{50}$ (μg/ml)/(MIC range) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | PcG | ABPC | CDTR | CTX | MEPM | PAPM | EM | CLDM | VCM |
| ST90 | 6B | Spain6B-2 (ST90) | 10 | 1·0 (0·25–2·0) | 1·0 (0·25–2·0) | 0·50 (0·12–0·50) | 0·50 (0·12–0·50) | 0·50 (0·50) | 0·03 (≤0·008–0·06) | ≥8 (≥8) | ≥8 (≥8) | 0·25 (0·25–0·50) |
| ST236, ST2993 | 19F | Taiwan19F-14 (ST236) | 8 | 2·0 (1·0–2·0) | 2·0 (1·0–4·0) | 0·50 (0·25–2·0) | 0·50 (0·25–4·0) | 0·25 (0·25) | 0·06 (0·03–0·06) | 4 (2–≥8) | ≤0·12 (≤0·12) | 0·25 (0·25–0·50) |
| ST242, ST3563 | 23F | Taiwan23F-15 (ST242) | 6 | 1·00 (1·0–2·0) | 2·0 (1·00–2·0) | 0·50 (0·50–2·0) | 0·50 (0·25–2·00) | 0·25 (0·12–0·25) | 0·03 (0·03–0·06) | ≥8 (4–≥8) | ≥8 (4–≥8) | 0·25 (0·25–0·50) |
| ST81, ST282 | 23F 6A | Spain23F-1 (ST81) | 2 | 2·0 (2·0) | 2·0 (2·0–4·0) | 0·50 (0·50) | 0·5 (0·5–1·0) | 0·50 (0·50) | 0·06 (0·06–0·12) | 2 (2–≥8) | ≤0·12 (≤0·12–≥8) | 0·25 (0·25–0·50) |
| ST558 | 35B | Utah35B-24 (ST377) | 2 | 1·0 (1·0) | 2·0 (2·0–4·0) | 0·25 (0·25–0·50) | 0·50 (0·50) | 0·25 (0·25) | 0·03 (0·03–0·06) | ≤0·12 (≤0·12–≥8) | ≤0·12 (≤0·12) | 0·50 (0·25–0·50) |
| ST338, ST5246 | 23A | Colombia23F-26 (ST338) | 2 | 0·25 (0·25–0·5) | 0·5 (0·5–1·0) | 0·25 (0·25) | 0·25 (0·25–0·50) | 0·03 (0·03) | ≤0·008 (≤0·008) | 4 (4) | 0·5 (0·5–≥8) | 0·25 (0·25–0·50) |
| ST179 | 19F | Portugal19F-21 (ST177) | 1 | 1·0 | 2·0 | 0·5 | 0·5 | 0·03 | 0·25 | ≥8 | ≥8 | 0·25 |
| ST5830 | 6B | Greece6B-22 (ST273) | 1 | 1·0 | 1·0 | 0·5 | 0·5 | 0·06 | 0·03 | ≥8 | ≤0·12 | 0·5 |
| Other | | | 34 | 0·12 (≤0·015–2·0) | 0·50 (≤0·03–4·0) | 0·25 (≤0·03–0·50) | 0·25 (≤0·03–0·50) | 0·06 (≤0·008–0·25) | ≤0·008 (≤0·008–0·06) | ≥8 (≤0·12–≥8) | ≥8 (≤0·12–≥8) | 0·25 (0·25–0·50) |

ABPC, Ampicillin; CDTR, cefditoren; CLDM, clindamycin; CTX, cefotaxime; EM, erythromycin; MEPM, meropenem; MIC, minimum inhibitory concentration; PAPM, panipenem; PcG, penicillin G; PMEN, Pneumococcal Molecular Epidemiology Network; VCM, vancomycin.

rates in countries might include differences in access to healthcare, willingness to hospitalize patients and the costs of admission. Free access to any hospital or clinic is guaranteed in Japan, and the costs of almost all medical care for children up to age 6 years in Chiba City are compensated by local government. Thus, most children with CAP in Chiba City, and infants in particular, were treated in hospital.

Another possible reason for the higher incidence is differences in the definition of pneumonia. Pneumonia is usually diagnosed in Japan based on clinical signs and chest radiographic findings confirmed by clinicians, not radiologists. A World Health Organization (WHO) working group developed a method for standardizing the interpretation of chest X-rays of children for epidemiological purposes [23]. Pneumonia was diagnosed by clinicians at each hospital in the present study and included not only WHO-confirmed end-point pneumonia but also other radiographic findings. The incidence of CAP with end-point pneumonia according to WHO standards has yet to be determined.

Identifying the aetiology of childhood CAP is difficult because of the lack of accurate, non-invasive tests. The diagnostic yields of sputum culture are limited by potential contamination from the upper respiratory tract. However, the reliability of microbiological sputum tests can be improved by washing. Bartlett & Finegold [24] showed that washing sputum decreases the number of contaminants by 100- to 1000-fold and does not result in a qualitative and quantitative loss of bacteria recovered in percutaneous transtracheal aspirates. Combining quantitative culture with washing sputum specimens enhances the value of findings. We combine a washing technique with semi-quantitative culture to evaluate pathogenic bacteria [8, 9]. We applied this method with serology to determine the aetiology of CAP in 596 hospitalized children between 1990 and 1991 [25] and identified pathogens in 64·4% of them. Evidence of bacterial, *Mycoplasma pneumoniae* and viral (mostly respiratory syncytial virus) infection was found in 28·8%, 14·9% and 29·9%, respectively, of these children. Two major bacterial pathogens were *Haemophilus influenzae* (19·6%) and *S. pneumonia* (8·6%). The major pathogens defined in this study were consistent with a study of 1700 Japanese paediatric patients with CAP using real-time reverse transcription–PCR [26]. Moreover, the clinical responses to antibiotics administered based on the results of sputum culture are good [10, 11].

Of the 626 episodes in six hospitals examined in the present study, *S. pneumoniae* was identified as the causative pathogen in 96 (15·3%) episodes. Five and 92 were identified from blood and sputum cultures, respectively, including one that tested positive in both cultures. The rate of infection with pneumococcus (15·3%) was similar to that described in a study from Turkey (17·1%) that used washing and quantitative sputum cultures [27], and with a study from Italy (17·8%) that used serological assays with paired sera [28]. However, the findings were relatively lower than those in a study from the USA using pneumolysin-based PCR (44%) [29], even when all *S. pneumoniae* isolates identified in sputum (27·9%) were taken into account. One of the limitations of the present study is the absence of information about previous antibiotic use. The low rate of *S. pneumoniae* detection herein compared with PCR might be related to previous use of antibiotics, which is frequent in Japan.

We could not identify a relationship between underlying disease and the detection of *S. pneumoniae*. However, patients with asthma, congenital heart disease, and cerebral palsy had multiple hospitalizations for CAP. The only vaccines for the prevention of bacterial pneumonia (excluding pertussis) are *H. influenzae* type b and pneumococcal vaccines. Therefore, such patients should be recommended for immunization with these vaccines, both of which are elective in Japan.

The incidence of *S. pneumoniae* that is not susceptible to penicillin has rapidly increased in Japan since around 1990 [30]. The rate of PRSP in the present study was as high as that in previous studies of IPD [18] and acute otitis media (AOM) [31] in Japanese children. The frequency of STs related to multiresistant PMEN clones was also high in the present study. The spread of these clones might be responsible for the high rate of resistant strains developing in Japanese children.

The most prevalent serotype in sputum isolates of children with CAP was 6B, followed by 19F and 23F. The high prevalence of these serotypes was the same as that in a report describing IPD in Japanese children [32]. The overall PCV7 coverage rates in sputum and blood were 66·7% and 80%, respectively. These rates in children aged $<5$ years were 70·2% and 80%, respectively. PCV7 coverage of bacteraemic pneumonia was equal to that of IPD in the USA and Europe before the introduction of PCVs [33]. However, serotype 14, which is the most common serotype in the USA

and Europe preceding PCVs [34], and serotype 1, which has been predominant in complicating pneumonia before and after PCVs became available [35], were undetectable in our blood culture. Information about non-bacteraemic pneumonia serotypes is limited. The coverage rates of PCV7 in patients aged <5 years with respiratory infections determined using oropharyngeal swab samples in Vietnam, hypopharyngeal aspirates in China and nasopharyngeal isolates in Switzerland were 88·7% [36], 76·3% [37], and about 70% (<2 years) [38], respectively, with serotype 19F being the most frequent.

PCV7 coverage rates for PRSP were 92·9% and 100% in sputum and blood isolates, respectively. We hope that PCV7 will reduce the incidence of respiratory tract infections, especially those caused by strains that are less susceptible to penicillin.

Serotypes have changed in countries where PCV7 has been introduced as routine immunization and the emergence of serotype 19A with multidrug resistance has become a problem [1, 2, 39–41]. ST199 and ST320 are the major STs found in these countries. Here, we found only serotype 19A *S. pneumoniae* with ST2331. One isolate had ST199 but its serotype was 15B.

The incidence of serotype 6C, which was distinguished from serotype 6A in 2007 [42], also increased after the introduction of PCV7 [43]. Serotype 6C was isolated from <2% of children with IPD and from 9·5% of samples from the nasopharyngeal mucosa of healthy children in Japan [16]. We identified three 6C isolates from sputum (4·8%) with the new sequence type ST5832 in our patients with CAP.

Some limitations of this study should be considered. This study covered only a 1-year period and therefore does not account for annual variations in either the incidence of disease or the detected serotypes. In addition, information about previous antibiotic administration was not available.

PCV7 was introduced as an elective vaccine in Japan in February 2010. New PCVs, especially 13-valent and the investigational 15-valent types, would potentially increase the coverage rate of sputum isolates. Switching to these new PCVs should be considered with the increase of non-vaccine serotype. Continued surveillance to detect changes in the incidence of CAP caused by pneumococci, their antimicrobial resistance, serotypes and genotypes are crucial for evaluating the impact of PCV7 and to effectively prevent pneumococcal infections.

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## DECLARATION OF INTEREST

None.

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