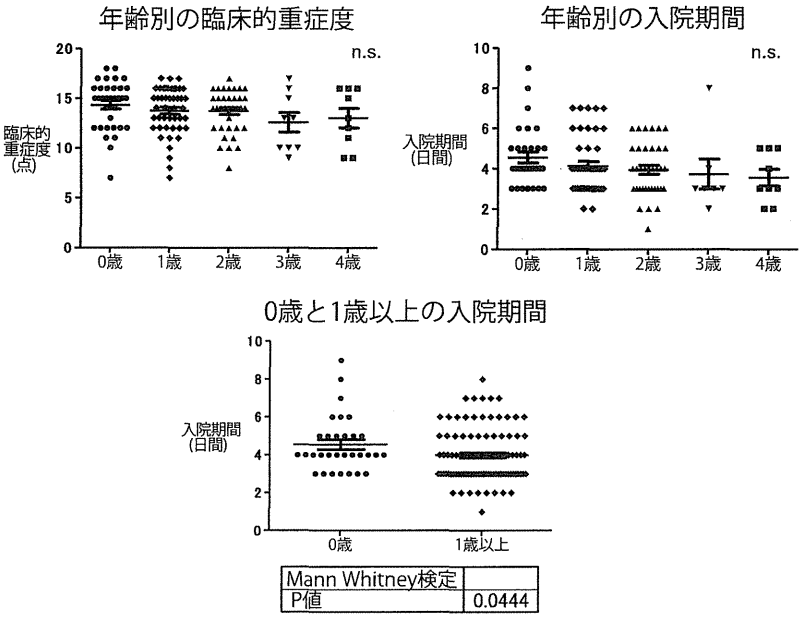

図4　年齢別の臨床的重症度と入院期間
n.s.：not significant

を比較したが，統計学的に有意差を認めなかった．年齢を0歳と1歳以上に分けて入院期間を比較すると，0歳では1歳以上より入院期間の平均値で0.57日長くなり，P＝0.0444で有意差を認めた．同様に遺伝子型別に臨床的重症度と入院期間を比較したが，有意差は認めなかった．

合併症

合併症は，調査票で記載漏れのない204例について検討した．脳炎・脳症，腎前性・腎後性腎不全などの重篤な合併症は認めなかった．胃腸炎関連けいれんと熱性けいれんを合併した症例は，それぞれ11名(5.4%)ずつ認めた．合併症症例で，特異的な遺伝子型は認めなかった．

考　察

日本では医療機関への受診のしやすさもあり，流行シーズンには昼夜を問わず，多数のロタウイルス胃腸炎患者がみられる．病初期には症状が強いため，輸液が必ずしも必要でない症例でも，家族の不安から輸液を行うことも多く，医療スタッフの負担も大きい．本調査でのロタウイルス胃腸炎の入院率は，1,000人・年あたり4.2であり，5歳になるまでに48人に1人がロタウイルス胃腸炎で入院する計算になる．この入院率は，他の先進諸国のロタウイルスワクチン導入前とほぼ同等であった．入院率は米国の1,000人・年あたり2.7が最も低く[8]，西ヨーロッパおよびオーストラリアでは3.7～13(中央値5.2)であった[9)~13)]．過去に日本でも入院率が調べられ，Nakagomiらは秋田県内の地域の中核病院で調査を行い1,000人・年あたり7.9～17.6であり[14]，Itoらは京都府の総合病院で調査を行い5.3であったとしている[15]．国内の入院率に関しては，地域の医療環境（外来で加療する症例が多い，入院施設のある病院までの距離が遠いなど）やロタウイルスの流行状況に影響されると考えられる．また本調査では，胃腸炎症例には可能な限りロタウイルスの迅速検査を行ったが，便が採取できなかった症例もある．そのため，実際の入院率は更に高いことが予想される．

検出されたロタウイルスの遺伝子型は，4シーズンを通してG3P [8] が主流で，次にG1P [8] が多くみられた．また4シーズンをまとめて，年齢別に遺伝子型を表したが，特に大きな偏りを認めなかった．遺伝子型の検出割合は地域やシーズンによって異なることが知られているが，三重県津市では各シーズンで遺伝子型の割合に大きな変動はみられなかった．世界的に多い遺伝子型はG1P [8] であり，全体の約半数を占める[16]．アジアでは2000～2009年の間で，G1P [8] が23.6%，G3P [8] が18.9%，G2P [4] が11.8%であった[17]．国内では，ロタウイルスG遺伝子型別の年ごとの推移をみた牛島の報告がある[18]．1980年代後半から2000年頃までは，G1が9割近くを占めていたが，その後一時的に減少し，G3が優占した．しかし数年後にはG1が再度増加し，G3は減少していった．ワクチンが導入された後，ロタウイルス胃腸炎の総数は著減したが，その検出割合に変化がみられるという報告がいくつかある．1価のロタウイルスワクチンを導入したブラジ

ルでは，接種開始2年後でG2P [4] の割合の増加がみられた[19]．2006年に1価のワクチン，2007年に5価のワクチンを導入したベルギーでもG2の割合が増加しているが，使用されたワクチンの大部分は1価ワクチンである[20]．今後，日本でもワクチン接種率の上昇とともに新たな遺伝子型の出現を含めた遺伝子型の変化の可能性がある．同地域での継続的な調査が今後重要と考えられる．

ロタウイルス胃腸炎の臨床的重症度を評価するスコアとして，Rennelsの重症度スコアやVesikariスコア，Clarkスコアがある[7][21]．我々は，研究協力者の臨床現場での負担にならないよう，Rennelsの重症度スコアを改変した独自の評価法を用いて重症度を評価した．年齢別，遺伝子型別に，臨床的重症度と入院期間を比較したところ，年齢の小さい方で臨床的重症度が高く，入院期間が長い傾向があったが，有意差を認めなかった．年齢を0歳と1歳以上に分けて入院期間を比較すると，0歳では入院期間が長くなり，有意差を認めた．乳児の方が，重症化しやすく，また臨床経験上，回復により多く時間を要する印象があり，調査結果と一致する．一方，遺伝子型の違いでは臨床的重症度に差を認めなかった．過去に遺伝子型の違いで臨床的重症度が異なるという報告は見当たらず，本調査では独自の評価基準を用いるため単純には比較できないが，同様の結果となった．

ロタウイルス胃腸炎の入院率を人口ベースで評価し，年齢，遺伝子型と臨床的重症度について比較検討した．入院率はワクチン導入前の先進諸外国と同様の疾病負荷があることが証明された．それらの国々ではロタウイルスワクチンを導入し，高い接種率を達成することによりロタウイルス胃腸炎の疾病負荷を大幅に減少させている．今後，わが国でもワクチンによる疾病負担軽減効果，遺伝子型の変化を評価していく必要があるため，引き続き調査を継続していく予定である．

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Active Surveillance for Hospitalizations due to Rotavirus Gastroenteritis among Children (<5 years) in Tsu City, Mie Prefecture, Japan

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A rotavirus vaccine was introduced in November 2011 in Japan. In order to evaluate the effectiveness of the vaccine, it was necessary to assess data on the burden of rotavirus disease and the circulating rotavirus strains before introducing the rotavirus vaccine. To obtain these data, we set up active surveillance for hospitalizations due to rotavirus gastroenteritis among children (<5 years) in Tsu city, Mie Prefecture, Japan. From November 1, 2007 through October 31, 2011, we enrolled children who were hospitalized with a diagnosis of rotavirus gastroenteritis. During this period, 205 children were hospitalized, and the incidence rate of hospitalization was 4.2 per 1,000 children (95% confidence interval, 3.7—4.8). Only 4.4% (9/205) of the hospitalized children were<6 months of age, 23.9% (49/205) were<1 year of age, and 61.5% (126/205) were<2 years of age. Of the 205 hospitalizations, 64% (131/205) cases underwent G and P typing using semi-nested PCR. The most dominant serotype (genotype) found during each season was G3P[8], which accounted for 62%—75% of cases. The second most dominant was G1P[8] (11%—28%). We assessed and compared clinical severity between each genotype by age but no significant differences were observed.

IASR

Haemophilus influenzae b型菌（Hib）ワクチン導入前後の侵襲性感染症由来*H. influenzae* 分離株の解析：９県における検討

(IASR Vol. 34 p. 195-197: 2013年7月号)

わが国においては、*Haemophilus influenzae*莢膜ｂ型菌（Hib）に対するワクチンの任意接種が2008年12月より開始され、全国的公的補助が平成22（2010）年度補正予算により開始された（それ以前から公的補助を出していた自治体もある）。対象とした９県（福島、新潟、千葉、三重、岡山、高知、福岡、鹿児島、沖縄）における*H. influenzae* による侵襲性感染症症例の髄液、血液あるいは関節液由来の*H. influenzae* 菌株について、Hibワクチン導入前後において莢膜型別解析ならびに髄膜炎治療によく使用される抗菌薬について感受性試験を実施した。以下の３期間に分けて解析を実施した。期間１）Hibワクチン接種開始前（2007年６月～2008年11月）、期間２）Hibワクチン任意接種開始後（2008年12月～2010年12月）、期間３）Hibワクチンの任意接種に対する全国的公的補助開始後（2011年１月～2013年３月）。

解析方法

莢膜型別解析：インフルエンザ菌莢膜型別用免疫血清「生研」（デンカ生研）を用いた菌体凝集法により解析した。a, b, c, d, e, f株に対する抗血清のいずれの存在下においても凝集しない株をnon-typable *H.influenzae*（NTHi）とした[1]。

遺伝子解析による莢膜型別：一部の菌株については、抗血清による菌凝集法に加えて、ａ～ｆ型莢膜遺伝子の有無について、polymerase chain reaction (PCR)法による解析を行った。*H. influenzae* 莢膜型特異的遺伝子に対するPCR法は、Falla らの方法[2]をもとに、一部のプライマー配列を改良して実施した。

薬剤感受性試験：E-test（AB BIODISK）を用い、試験用培地にはHaemophilus Test Medium（HTM、ベクトン・ディッキンソン）を用いた。供試薬剤は、表２に示す。

結果と考察

分離菌株が国立感染症研究所に送付された*H. influenzae* による侵襲性感染症症例数（入院日が明らかな症例）と対象９県でのHib ワクチン出荷本数を５歳未満人口で割った割合の累計％（人口は平成22年度10月１日時点の推定値）の経時的変化から、全国的公的補助開始以降に症例数が減少していることが示唆された（図１）。

分離株の莢膜型について：Hibワクチン接種開始前ならびに任意接種開始後も全国的公的補助以前には、Hibが検討分離株の97％以上を占めていたが、全国的公的補助開始後に83％に減少し、一方、NTHiの割合が増加傾向を示した（表１）。NTHiは、２例の髄膜炎症例の髄液ならびに18例の菌血症症例の血液から分離された。対象９県においては、他の莢膜型菌の分離は無かった。ワクチン３回接種後にHib髄膜炎あるいは菌血症を発症した症例が１例ずつ報告された（参考までに、他県でHibワクチン３回接種後に髄膜炎を発症した患者の血液から分離された莢膜ｆ型菌の解析結果例を図２に示す）。

分離菌の薬剤感受性試験結果について：Clinical and Laboratory Standards Institute（CLSI）の微量液体希釈法の基準を参考値として、感性と考えられる株の割合を表２に示す。検討抗菌薬のいずれも、調査期間中感性株の割合に大きな変化は観察されなかった。一方、アンピシリン耐性株中のβ-lactamase非産生株の割合は、7.1％（期間１）、38.5％（期間２）、26.7％（期間３）と増加傾向を示したが、増加時期は、ワクチン普及時期と一致しなかった。

謝辞：本解析は、厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）、「新しく開発されたHib、肺炎球菌、ロタウイルス、HPV等の各ワクチンの有効性、安全性並びにその投与方法に関する基礎的・臨床的研究」班において実施したものです［班員の方々：菅　秀、庵原俊昭、浅田和豊、中野貴司、神谷　齋（国立病院機構三重病院）、細矢光亮、陶山和秀（福島県立医科大学）、石和田稔彦（千葉大学）、鳥谷部真一、内山　聖、大石智洋、齋藤昭彦（新潟大学）、小田　慈（岡山大学）、脇口　宏、佐藤哲也（高知大学）、岡田賢司（国立病院機構福岡病院）、西　順一郎（鹿児島大学）、安慶田英樹（沖縄県立南部医療センター）[3)]］。また、Hibワクチンの出荷本数は、サノフィパスツール社社内資料を出典としました。

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CASE REPORT

Fatal overwhelming postsplenectomy infection caused by *Streptococcus pneumoniae* in mothers within 1 year after delivery: case report

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Abstract Invasive pneumococcal disease (IPD) has a high mortality. Although preventive strategies including vaccination have been established for children, less attention has been devoted to pregnant and postnatal women and to mothers caring for their infants. As a significant number of women have either undergone splenectomy or are in a hyposplenic state and have not received adequate pneumococcal vaccinations, they can potentially develop overwhelming postsplenectomy infections. A 34-year-old woman with an 8-month-old baby, who underwent splenectomy at the age of 10 for benign pancreatic tumor, presented with fever and petechial eruption. Despite extensive treatment, she died 17 h after admission. A 40-year-old woman with a 11-month-old baby, who underwent splenectomy at 2 years of age for hemolytic anemia, was admitted for septic shock and disseminated intravascular coagulation. Despite extensive treatment, she died 2 h after admission. Blood cultures from both women were positive for *Streptococcus pneumoniae* and neither of them had been vaccinated against the bacterium. IPD rapidly progressed and developed to multiple organ dysfunction syndromes in mothers caring for their infants, particularly those who had undergone splenectomy or were in a hyposplenic state. Thus, routine pneumococcal vaccination is recommended for pregnant women. In addition, we suggest a thorough medical interview and checkup for splenectomy or hyposplenism in prenatal women.

Keywords Overwhelming postsplenectomy infection · *Streptococcus pneumoniae* · Breastfeeding

Introduction

Invasive pneumococcal disease (IPD) is defined by the isolation of *Streptococcus pneumoniae* from a normally sterile site (e.g., blood, cerebrospinal fluid, synovial fluid, pericardial fluid, pleural fluid, peritoneal fluid) and is associated with a high mortality rate [1]. Although IPD prevention strategies in children including vaccination have been established, much less attention has been devoted to pregnant and postnatal women and mothers caring for their infants [2]. In addition, patients who have undergone splenectomy or are in a hyposplenic state are highly susceptible unless adequately vaccinated and may develop an overwhelming postsplenectomy infection (OPSI) while caring for infants with extensive nasopharyngeal carriage of *S. pneumoniae* [3–5].

Among organisms that induce postsplenectomy fatal sepsis, *S. pneumoniae* is the most common, followed by *Haemophilus influenzae* type b and *Neisseria meningitidis* [6]. The condition of patients with OPSI may rapidly progress to coma and death, within a few days, because of the high incidence of septic shock and associated organ dysfunction, including disseminated intravascular coagulation (DIC) [7, 8]. Once OPSI develops, the mortality rates are high, ranging from 38 to 69 %, despite aggressive

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critical care [9] Cases of OPSI have been documented previously and have been well reviewed [10–12]. Therefore, currently, the indications for splenectomy are carefully assessed and vaccination is advised for all patients undergoing splenectomy to prevent OPSI.

Although the total number of patients with OPSI is unclear, a significantly large number of fertile women have undergone splenectomy for various reasons, including trauma, idiopathic thrombocytopenic purpura, tumors, or hyposplenism, but have not been vaccinated against *S. pneumoniae*. During the perinatal period, women become more susceptible to various infections, including those by *S. pneumoniae*; in particular, postpartum women are threefold more susceptible to pneumococcal infection when compared with nonpregnant women [13], and it is reasonable to speculate that mothers caring for their infants are also susceptible. In addition, they are more extensively exposed to *S. pneumoniae* because it frequently colonizes the nasopharyngeal space of infants.

Here we describe two alarming cases of fatal OPSI in mothers caring for their infants, which highlights the need for immediate action against this severe disease.

Case presentation

Case 1

A 34-year-old woman was referred to our hospital for fever, diarrhea, and petechial eruption. She had undergone splenectomy for a benign pancreatic tumor at the age of 10 but had not received pneumococcal vaccination. She gave birth 8 months before admission. Twelve hours before admission, she visited a local clinic complaining of high fever and was diagnosed with an upper respiratory tract infection; however, 2 h before admission, she developed purpura on her face and torso. On presentation, the patient's level of consciousness was slightly decreased with Glasgow Coma Scale score of 13/15. Her vital signs as recorded on admission were body temperature, 36.4 °C; blood pressure, 62/40 mmHg; heart rate, 130 beats/min; and respiration rate, 33 breaths/min. The patient was critically ill, and a myriad of petechiae were observed on her face and torso. Laboratory tests revealed increased white blood cell count (13,700 cells/μl), moderately increased C-reactive protein concentration (11.38 mg/dl), extremely low platelet count (37,000/μl), and high levels of D-dimer (40.5 μg/ml), indicating inflammation and severe coagulopathy. Blood gas analysis revealed severe metabolic acidosis: pH, 7.268; $PaCO_2$, 19.1 mmHg; PaO_2, 45 mmHg; HCO_3, 8.5 mmol/l; and base excess (BE), −16.7. Urinary specific antigen test for *S. pneumoniae* was positive, but chest radiography revealed no abnormalities. The patient was intubated and admitted to the intensive care unit for further treatment. After being diagnosed with septic shock and DIC, she was extensively treated with fluid resuscitation, early administration of meropenem (MEPM) and human recombinant thrombomodulin, continuous hemodiafiltration, and hemoperfusion using a polymyxin B immobilized fiber column. However, she died 17 h post admission of multiple organ dysfunction syndrome (MODS). Her blood culture was positive for *S. pneumoniae*.

Case 2

A 40-year-old woman was transferred to our hospital for purpura, acute kidney dysfunction, DIC, and shock. She had undergone splenectomy at the age of 2 years for hemolytic anemia but had not received a pneumococcal vaccination. Moreover, she gave birth 11 months before admission. Four days before admission, she complained of general fatigue, but did not take any medications. Thereafter, 1 day before admission, she developed high fever, diarrhea, and vomiting. However, her symptoms rapidly progressed, and she was admitted to a community hospital with facial purpura and dysarthria. On examination, the patient's level of consciousness was slightly decreased with Glasgow Coma Scale score of 14/15. Her vital signs as recorded on admission were body temperature, 40.7 °C; blood pressure, 107/74 mmHg; heart rate, 127 beats/min; and respiration rate, 35 breaths/min. The patient was critically ill, and a myriad of petechiae were observed on her face. Laboratory tests revealed high white blood cell count (23,100 cells/μl), moderate levels of C-reactive protein (8.11 mg/dl), extremely low platelet count (81,000/μl), and high levels of D-dimer (96.9 μg/ml). Blood gas analysis revealed moderate metabolic acidosis: pH, 7.393; $PaCO_2$, 21.9 mmHg; PaO_2, 73.5 mmHg; HCO_3, 13.0 mmol/l; and BE, −9.7. Moreover, urinary specific antigen test for *S. pneumoniae* was positive. After a diagnosis of septic shock and DIC, the patient was immediately treated with fluid resuscitation and MEPM. However, 1 h after arrival, she suddenly developed ventricular fibrillation. Despite cardiopulmonary resuscitation with electrical defibrillation, she died 2 h after arrival. Her blood culture was positive for *S. pneumoniae*.

Analysis of *S. pneumoniae* strains isolated from the two cases

Strains of *S. pneumoniae* isolated from these two cases were analyzed at the National Institute of Infectious Disease (Tokyo, Japan). The isolated *S. pneumoniae* was serotyped using the Quellung reaction with pneumococcal antisera (Statens Serum Institut, Copenhagen, Denmark). Molecular

typing of the isolate was performed using multilocus sequence typing as described by Enright and Spratt [14].

In both these cases, the serotypes of the isolates were 6B, and the sequence types (ST) were ST2983. Both strains were susceptible to meropenem, panipenem, cefotaxime, cefditoren, ciprofloxacin, and vancomycin and were resistant to erythromycin alone.

Discussion

Here we present two fatal cases of OPSI in mothers caring for their infants. These fatalities should sound the alarm for an effective prevention strategy by vaccination and post-splenectomy screening for *S. pneumoniae* in pregnant women, particularly for those who have undergone splenectomy or have hyposplenism.

S. pneumoniae transmission typically occurs via respiratory droplets, as the human nasopharynx is the only known reservoir of *S. pneumoniae*. Nasopharyngeal carriage is required for the pathogenesis of *S. pneumoniae*-associated invasive infections such as septicemia, meningitis, and arthritis. More than 70 % of infants have colonized *S. pneumoniae* infection by the age of 1 year [15].

In the present cases, both mothers had delivered their infants. Infection can spread from an infant to its mother during routine child care, as they may be surrounded by other babies who are highly infected with *S. pneumoniae*. Although a detailed examination of *S. pneumoniae* DNA from the infants for nasopharyngeal carriage was not performed, environmental factors seemed to have played an important role in development of infection in these cases.

IPD is marked by high mortality even if the patients are aggressively treated following surviving sepsis campaign guidelines [16]. Severe coagulopathy is frequently progressive despite therapy with heparin, packed red blood cells, platelets, cryoprecipitates, and fresh frozen plasma [9]. Therefore, IPD prevention strategies, including vaccination and screening, are fundamental for those who have undergone splenectomy or have hyposplenism.

In both the cases reported here, sequence types were identified as ST2983, which was well recognized as causative organisms of IPD and pneumonia, and with nasopharyngeal carriage in children younger than 5 years.

The pneumococcal serotypes were identified as 6B, a mucinous type associated with high mortality [17], which is included in a panel of 23-valent pneumococcal polysaccharide vaccine (PPV-23), 13-valent pneumococcal conjugate vaccine (PCV-13), and PCV-7. Although a controversy exists on the efficacy of PPV-23 in the population at large, the PPV vaccine has been regarded as a first-line preventive therapy for patients who have undergone splenectomy and have hyposplenism; in fact, vaccination followed by repeated revaccination is recommended [18]. Moreover, previous studies have suggested that such preventive strategies effectively reduce the risk of serious pneumococcal infection in such populations [19, 20]. In addition, PCV-13 is also an effective measure for preventing infection [21].

Ideally, routine administration of PPV-23 or PCV-13 is preferable in pregnant women to protect them against IPD. Vaccination for *H. influenza*e type b (Hib) and meningococcal C might also be necessary against OPSI [22]. The vaccination should be administered before the mothers are exposed to babies who are highly colonized or infected with *S. pneumoniae*.

Screening for splenectomy and hyposplenism is an ongoing challenge in such populations. Without a spleen registry, such as the Victorian Spleen Registry adopted in Australia, we cannot provide relevant information or direct significant vaccination programs in such patients [18]. We recommend a thorough medical interview during prenatal checkups, particularly in pregnant women who underwent splenectomy or have hyposplenism, and vaccinate them as soon as inadequate vaccination is identified. Through these preventive strategies, we believe that the risk of IPD, including OPSI, will be decreased.

In conclusion, IPD rapidly develops into sepsis and MODS. Therefore, preventive strategies, including vaccination and screening, are extremely important. Routine administration of pneumococcal vaccine is preferable; nevertheless, we recommend a thorough medical interview during prenatal checkups, particularly in pregnant women who underwent splenectomy or have hyposplenism without adequate vaccination.

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Conflict of interest The authors declare that they have no competing interests.

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Molecular epidemiology and serogroup 6 capsular gene evolution of pneumococcal carriage in a Japanese birth cohort study

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Antibiotic resistance in *Streptococcus pneumoniae* is a major concern worldwide. However, it is unclear whether resistance is associated with only a few highly prevalent clones or numerous and diverse clones. We monitored 349 healthy children and obtained nasopharyngeal cultures at five time points coinciding with health check-ups (4, 7, 10, 18 and 36 months) between 2008 and 2012. A total of 497 *S. pneumoniae* isolates from 257 healthy children were characterized using capsular serotyping, multilocus sequence typing and antibiotic resistance genotyping (*ermB*, *mefA/E* and *pbp* mutations). Among these isolates, 25 serotypes and 66 sequence types (STs) were found, including 24 new STs with 11 new alleles. Although resistance was present in a variety of ST clones, most of the clones (57/66, 86.4 %) had one specific resistant or susceptible genotype. Of 233 phenotypically penicillin-non-susceptible isolates, 196 (84.1 %) belonged to only six clones, comprising ST90^{6B}, ST236^{19F}, ST242^{23F}, ST3787^{6A}, ST1437^{23F} and ST338^{23A} and their variants. We concluded that drug-resistant *S. pneumoniae* is associated with a limited number of highly prevalent clones that are capable of adapting to the community setting. Furthermore, we analysed the capsular gene evolution in serogroup 6. The strain ST2924^{6D} was probably the result of recombination of a 3563 bp fragment of the capsule locus acquired by an ST2924^{6C} strain from an ST90^{6B} or ST2924^{6B} strain. Compared with previous studies, our results showed a different recombination site (*wciN* and *wzx*) and a different *cps* profile (8-7-11), indicating that serogroup 6 strains have multiple sites for *cps* recombination as a mechanism of vaccine escape.

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INTRODUCTION

Streptococcus pneumoniae is a leading cause of invasive and non-invasive bacterial infection in children worldwide. A seven-valent pneumococcal conjugate vaccine (PCV7), which includes capsular polysaccharide antigens of seven serotypes – 4, 6B, 9V, 14, 18C, 19F and 23F – has been available since 2000. A significant decrease both in colonization and in invasive diseases due to these serotypes has been observed in populations that have adopted widespread use of PCV7. However, the World Health Organization estimated that pneumococcal diseases still caused 476 000 deaths among children <5 years of age in 2008 (WHO, 2012). In Japan, PCV7 was introduced for voluntary immunization in February 2010, and has been provided as routine vaccinations since April 2013. The vaccination schedules are standardized 3 + 1 doses in all areas of Japan. Therefore, the impact of PCV7 in Japan is likely to be small until a larger proportion of the population is immunized.

The rate of penicillin-non-susceptible *S. pneumoniae*, defined as a penicillin MIC of $\geqslant 0.12$ μg ml^{-1}, in Japan has remained high (76.9 % in 2010) among paediatric patients including invasive and non-invasive diseases (Tajima *et al.*, 2013). In another report, even in healthy carriage 46.3 % of the *S. pneumoniae* isolates were non-susceptible to penicillin (Otsuka *et al.*, 2013). Penicillin-non-susceptible *S. pneumoniae* usually harbours *pbp* mutations for penicillin resistance, and may have macrolide-resistant genes. In order to design

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Abbreviations: gPISP, genetically penicillin-intermediate *S. pneumoniae*; gPRSP, genetically penicillin-resistant *S. pneumoniae*; gPSSP, genetically penicillin-susceptible *S. pneumoniae*; MLST, multilocus sequence typing; PCV7, 7-valent pneumococcal conjugate vaccine; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the *wchA* (partial), *wciN*, *wciO*, *wciP*, *wzy* and *wzx* (partial) genes of serogroup 6 isolates are AB795221–AB795237.

Two supplementary figures are available with the online version of this paper.

measures to reduce the resistance rate, an understanding of the distribution of resistant clones is critical.

Ninety-four different capsular serotypes of *S. pneumoniae* have been identified to date (van der Linden *et al.*, 2013). The capsular polysaccharide of *S. pneumoniae* enables the bacterium to escape phagocytic killing and allows its survival *in vivo* (AlonsoDeVelasco et al., 1995; Dhingra *et al.*, 1977) and plays an important role during the early phase of colonization of the upper airways (Magee & Yother, 2001). In the post-PCV7 era, serotype replacement and capsular switching has led to a critical problem, i.e. a rapid increase in the incidence of invasive diseases with non-PCV7 serotypes (e.g. 19A, 15A, 23A, 35B and 6C) (Carvalho *et al.*, 2009; Gertz *et al.*, 2010; Kaplan *et al.*, 2010; van Gils *et al.*, 2010).

More recently, *S. pneumoniae* has been classified by multilocus sequence typing (MLST) (Enright & Spratt, 1998). Each capsular serotype may include multiple sequence types (STs) with specific features, such as invasiveness and antibiotic resistance. For example, ST199 and ST320 are the major STs in the 19A serotype with multidrug resistance, whilst other STs are not associated with multidrug resistance (Ardanuy *et al.*, 2009; Moore *et al.*, 2008; Pillai *et al.*, 2009; Song *et al.*, 2009). These variations make it difficult to predict the risk of penicillin-non-susceptible *S. pneumoniae* infection based on serotype alone.

Although there are several reports on the relationship between MLST and resistant phenotypes/genotypes of *S. pneumoniae*, few studies have analysed healthy children (Lambertsen *et al.*, 2010; Sadowy *et al.*, 2007; Sakai *et al.*, 2011; Sogstad *et al.*, 2006). Therefore, it is still unclear whether resistance is associated with only a few highly prevalent clones or with numerous and diverse clones in children, the main reservoir of *S. pneumoniae* in a population.

Among the 94 *S. pneumoniae* serotypes, 6A, 6B and 6C isolates are commonly found in healthy colonization and invasive pneumococcal disease, particularly resistant 6B strains (Otsuka *et al.*, 2013; van der Linden *et al.*, 2013). Genetic studies have revealed the near identity of the capsular loci of serotypes 6A and 6B, with only a single nucleotide difference in the *wciP* gene ($wciP_{\alpha}$ and $wciP_{\beta}$) (Bratcher *et al.*, 2011; Mavroidi *et al.*, 2004). Serotype 6C is thought to have emerged by the introduction of the $wciN_{\beta}$ gene, replacing $wciN_{\alpha}$ in the capsular locus of serotype 6A. Recently, serotype 6D was identified in Fiji (Jin *et al.*, 2009), Korea (Bratcher *et al.*, 2010), Japan (Chang *et al.*, 2010) and other countries (McEllistrem & Nahm, 2012). Some of these serotype 6D isolates were resistant to penicillin.

It has been suggested that serotype 6D emerged by two proposed mechanisms: replacement and recombination. The first is replacement of the $wciP_{\alpha}$ gene with the $wciP_{\beta}$ gene in the capsular locus of serotype 6B. The second proposed mechanism is recombination between serotype 6B and 6C *cps* loci including the *wciN* and *wciP* genes (Bratcher *et al.*, 2011; Song *et al.*, 2011). Therefore, serogroup 6 currently consists of four serotypes: serotype 6A ($wciN_{\alpha}$ and $wciP_{\alpha}$), 6B ($wciN_{\alpha}$ and $wciP_{\beta}$), 6C ($wciN_{\beta}$ and $wciP_{\alpha}$) and 6D ($wciN_{\beta}$ and $wciP_{\beta}$). These serotypes are highly diverse based on the observation that each serotype includes multiple STs (van der Linden *et al.*, 2013). Bratcher *et al.* (2011) also showed evidence that the recombination event of serotype 6D may have occurred only once and spread worldwide, using '*cps* profile' analysis, which assigns a specific number based on sequencing of serogroup 6-specific genes (*wciP*, *wzy* and *wzx*) (Mavroidi *et al.*, 2004). Their *cps* profiles were 5-1-1 and its variant. However, the evolution of serotype 6D is still controversial, because there is no direct evidence to date.

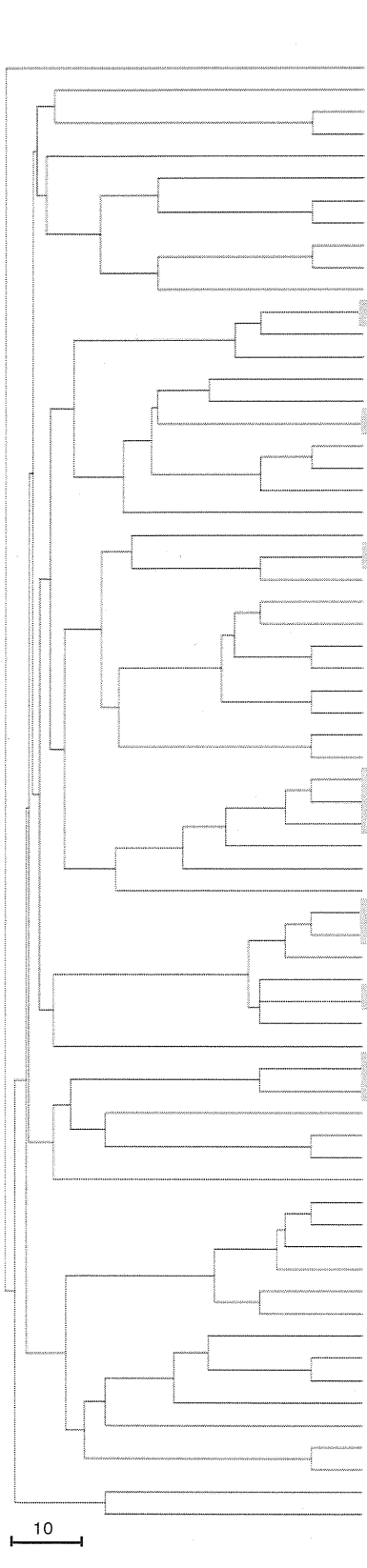
The aim of this study was to identify the prevalence of resistant clones in a birth cohort on Sado Island. We evaluated and characterized *S. pneumoniae* isolates from healthy children using MLST determination and antibiotic resistance genotyping. Furthermore, we focused on a newly discovered serotype 6D as a new clone. We proposed an explanation for the evolution of serotype 6D using sequences of capsular genes.

METHODS

***S. pneumoniae* isolates.** The study was conducted as a part of the SADO Study (Otsuka *et al.*, 2013). In the prospective birth cohort study, we monitored 349 children as healthy subjects, and nasopharyngeal cultures were obtained from each of the healthy subjects at five time points coinciding with health check-ups (4, 7, 10, 18 and 36 months, participation rate: 91.7–97.7 %) between 2008 and 2012. A total of 551 *S. pneumoniae* isolates were detected from 1654 samples. The serotype and antibiotic susceptibility of these isolates were determined previously (Otsuka *et al.*, 2013). Isolates that showed negative reactions for all pooled sera were considered to be non-typable. Because of the Japanese vaccination programme, no subject in this study had been administered PCV7 by the 18-month health check-up, whilst 106/328 subjects (32.3 %) were administered PCV7 by the 36-month health check-up. Of the 106 vaccinees, 93 received one vaccine dose and 13 received two vaccine doses (Otsuka *et al.*, 2013). The total population of Sado Island was 64 120, with 2167 (3.4 %) aged <5 years, at the midpoint of the study (January 2010).

MLST. We performed MLST as described previously (Enright & Spratt, 1998). Briefly, internal fragments of each of the seven housekeeping genes *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl* were amplified by PCR and sequenced, and their 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 our own MLST data (http://spneumoniae.mlst.net/sql/uniquetree.asp).

Resistant genes. Molecular typing for resistance was determined by PCR. Macrolide-resistant genes (*ermB* and *mefA/E*), and mutations for penicillin resistance (*pbp1a*, *pbp2x* and *pbp2b* mutations) in *S. pneumoniae* were detected using previously reported primers (Nagai *et al.*, 2001; Sutcliffe *et al.*, 1996; Tait-Kamradt *et al.*, 1997). *S. pneumoniae* isolates were classified into resistant genotypes by the presence of *pbp1a*, *pbp2x* and *pbp2b* mutations: genetically penicillin-susceptible *S. pneumoniae* (gPSSP, no mutation), genetically penicillin-intermediate *S. pneumoniae* (gPISP, with one or two mutations)



ST	Serotype	Allelic profile	Isolation age (month)					Resistance genotype
			4M	7M	10M	18M	36M	
393	38	10-43-41-18-13-49-6	-	-	-	-	1	gPSSP
63	15A	2-5-36-12-17-21-14	-	2	1	1	2	gPISPax+E
3111	19A	61-60-67-16-10-104-14	1	1	2	3	2	gPISPx+ME
7780	19A	61-60-67-1-10-104-14	-	1	-	-	-	gPISPx+ME
282	6A	30-4-2-4-4-1-1	-	1	1	1	-	gPRSP+M
2224	6A, 6B	7-12-7-1-116-14-29	-	-	1	2	-	gPISPx+M
5236	10A	7-12-1-1-10-1-11	-	1	2	1	4 (3)	gPISPx+E
7158	22F	7-12-1-1-10-1-14	1	-	-	-	-	gPSSP
2331	19A	10-16-150-1-17-1-29	-	-	-	1	-	gPSSP+E
7796	19A	10-16-150-1-**334**-1-29	-	-	-	3	-	gPISPx
3116	34, NT	10-8-6-1-9-1-279	-	1	-	-	5 (1)	gPSSP
90	6B	5-6-1-2-6-3-4	3	13	13	12	9 (5)	gPRSP+E (98%), gPISPax+ME (2%)
2983	6B	5-6-1-2-6-1-271	3	6	5	3	3 (1)	gPISPax+M
7783	6B	5-6-1-2-2-2-271	-	-	1	-	-	gPISPx+M
3594	18B,18C	10-13-34-16-6-1-31	2	-	-	-	3 (1)	gPSSP
7791	10A	15-13-**356**-16-6-1-14	-	1	-	-	-	gPISPxb+M
3787	6A, 6B	8-8-19-16-6-1-68	5	4	13	9	7 (2)	gPISPax (82%), gPISPx (18%)
6185	11D	5-8-4-16-6-104-31	-	1	-	-	-	gPSSP
7153	11A/E	5-8-4-16-6-439-31	-	-	2	1	3 (1)	gPSSP (67%), gPISPx (33%)
7785	11A/E	5-60-4-16-6-1-31	-	-	1	-	1	gPISPx
7388	34	5-5-7-16-9-1-14	1	1	-	1	3 (2)	gPSSP+E
180	3	7-15-2-10-6-1-22	2	1	-	-	6 (1)	gPISPx+E
1437	23F, 19F	1-32-6-6-6-1-14	1	5	2	6	2	gPRSP+ME
2924	6C, 6D, 6B	1-5-2-6-6-1-14	1	2	3	8	6 (3)	gPISPx+E
1092	6B	2-13-2-1-6-19-14	-	-	-	1	-	gPISPx+E
6413	6B	2-13-2-5-6-124-14	-	-	2	1	-	gPRSP+M
7813	6B	2-13-2-5-6-**470**-410	-	-	-	-	1 (1)	gPISPax+M
7834	6B	2-13-2-5-6-58-410	-	1	1	1	-	gPISPax+M
2923	6C	2-13-2-5-6-121-29	-	1	5	9	5 (2)	gPISPx+M
7798	6C	2-**356**-2-5-6-121-29	-	-	-	1	-	gPISPax+M
7781	6C	7-60-8-5-6-14-14	-	1	-	-	-	gPISPx+E
7788	6C	7-**356**-8-5-6-14-14	-	1	2	1	1	gPISPax+M
338	23A, 19F	7-13-8-6-1-6-8	3	1	-	3	5	gPISPxb+E (83%), others (17%)
5246	23A	7-13-8-6-1-138-8	1	1	1	1	-	gPISPxb+E
7814	23A	7-13-**357**-6-1-6-8	1	1	2	2	2	gPISPxb+E
5241	6C	7-9-8-6-1-6-384	1	-	-	1	-	gPISPxb
7799	6C	7-13-2-6-6-6-14	-	-	-	1	-	gPISPx+E
7782	6C	7-9-4-16-1-20-384	1	0	2	0	2 (1)	gPISPxb+M (80%), gPISPx+E (20%)
236	19F, 6D	15-16-19-15-6-20-26	3	1	9	22	10 (1)	gPRSP+M (96%), others (4%)
1428	19F	15-16-19-15-6-20-102	1	1	1	1	-	gPRSP+M
7978	19F	15-16-19-15-1-20-26	-	-	-	1	-	gPRSP+M
257	19F	22-16-19-15-6-20-14	-	1	1	1	-	gPISPx+E
1464	19F	4-16-19-15-6-20-106	-	-	-	1	-	gPRSP+ME
7979	19F	8-16-19-15-6-20-102	-	1	-	-	-	gPISPax+M
447	37	29-33-19-1-36-22-31	1	-	1	1	3 (1)	gPSSP
242	23F	15-29-4-21-30-1-14	8	8	10	13	7 (2)	gPRSP+E (83%), gPISPx+M (17%)
7784	23F	15-29-4-**209**-30-20-14	1	-	1	-	1 (1)	gPRSP+E
433	22F	1-1-4-4-18-58-17	-	1	-	1	3 (1)	gPISPx+E (60%), gPISPax (40%)
2922	14	1-5-4-5-5-20-8	1	6	8	5	1	gPISPax+E
7795	14	1-5-4-5-5-20-**515**	-	-	1	2	-	gPISPax+E
558	35B	18-12-4-44-14-77-97	1	-	-	-	1	gPRSP+M
199	15B, 15C, 19F	8-13-14-4-17-4-14	3	6	2	7	12 (3)	gPISPx+E (57%), others (43%)
7792	15C	8-13-14-4-17-4-**514**	-	-	1	1	-	gPISPx+E
7793	15B, 15C	8-13-14-4-30-4-14	-	1	1	2	-	gPISPx+M
7794	15C	8-**357**-14-4-17-4-14	-	-	1	-	-	gPISPx+M
7787	15C	8-13-14-4-1-**439**-31	-	-	1	-	-	gPISPax+M
7797	15B, 15C	8-13-14-4-1-**467**-14	-	1	-	1	-	gPISPax+M
343	14	8-8-4-15-39-12-14	-	-	-	1	-	gPRSP+E
1106	NT	8-29-4-15-17-12-31	-	-	-	2	4 (1)	gPISPxb+E
3288	NT	8-29-4-15-172-12-31	-	1	1	1	-	gPSSP+M
7786	NT	8-29-9-15-77-155-31	-	-	1	-	-	gPISPx+E
344	NT	8-37-9-29-2-12-53	-	-	-	1	-	gPISPx+E
7494	NT	8-5-2-15-2-11-505	-	-	2	5	-	gPISPxb+M
7789	NT	8-5-2-27-2-11-505	-	-	1	-	-	gPISPxb+M
717	33F	5-35-29-1-45-39-18	3	3	-	3	1	gPSSP+M
7800	31	1-2-29-16-15-155-18	-	-	-	1	1 (1)	gPSSP
		Total	49	79	105	147	117(35)	

10

Fig. 1. Genetic tree of MLST results and resistant genes. We identified 25 serotypes and 66 STs among the 497 isolates including 24 new STs and 11 new alleles. Bar, genetic linkage distance. No subject in this study had been administered PCV7 by the age of 18 months. The number of isolates detected from PCV7 vaccinees is shown in parentheses in the (continued)

Fig. 1. (continued) 36 months (36M) column. Bold underlining indicates new STs and new alleles. Serotypes are listed in order of decreasing prevalence where the ST included isolates of more than two serotypes. Serotypes detected from PCV7 vaccinees are underlined. Shading of STs and serotypes indicates major resistant clones and their variants. NT, non-typable; x, *pbp2x* mutation; b, *pbp2b* mutation; M, *mefA/E* gene; E, *ermB* gene.

and genetically penicillin-resistant *S. pneumoniae* (gPRSP, with all mutations). After all PCR experiments, the combined penicillin and macrolide-resistant genotype was assigned.

Nucleotide sequence. Nucleotide sequences of the *cps* loci from the *wchA* to the *wzx* gene of serogroup 6 isolates were determined using primers reported previously (Song *et al.*, 2011). *cps* profiling analysis for the *wciP*, *wzy* and *wzx* genes was performed as described previously (Mavroidi *et al.*, 2004). Serotype 6A (isolates SP664, SP700, SP1095 and SP1097), 6B (SP598, SP658, SP711, SP806 and SP1107), 6C (SP391, SP412, SP622, SP829, SP1081 and SP1085) and 6D isolates (SP687 and SP1090) were used.

Informed consent was obtained from the parents/guardians of all healthy subjects prior to participation in the study. This study protocol was approved by the Ethics Committee at Sado General Hospital and was registered at the UMIN Clinical Trials Registry, Japan (trial no. UMIN000004928).

RESULTS

MLST

Of the 551 isolates obtained during the SADO Study, 497 (462 isolates obtained from non-PCV7 vaccinees and 35 isolates obtained from PCV7 vaccinees) were available for both serotyping and MLST. Among the 497 isolates recovered from the 257 subjects, 25 serotypes and 66 STs were found, including 24 new STs (ST7780–ST7789, ST7791–ST7800, ST7813, ST7814, ST7978 and ST7979) with 11 new alleles (Fig. 1).

Of the 66 STs, nine STs included isolates of two serotypes. Five of these STs had isolates that were different serotypes that belonged to the same serogroups [ST2224 (6A and 6B), ST3594 (18B and 18C), ST3787 (6A and 6B), ST7793 (15B and 15C) and ST7797 (15B and 15C)]. Four of the nine STs included isolates that belonged to different serogroups [ST3116 (34 and non-typable), ST1437 (23F and 19F), ST338 (23A and 19F) and ST236 (19F and 6D)]. ST2924 (6B, 6C and 6D) and ST199 (15B, 15C and 19F) included isolates of three serotypes. Serotype 19F and other serogroups consisted of the STs that included two different serogroups (Fig. 1, Table 1). The serotype and ST prevalence showed no difference among samples isolated before and after PCV7 introduction (Fig. 1).

Conversely, of the 25 serotypes, ten contained one ST, five contained two STs, five contained three STs, and two contained four to six STs. The remaining serotypes (6C, 19F and 6B) had eight, nine and ten STs, respectively (Table 1). Based on the observation that serogroup 6 isolates included multiple STs, we concluded that serogroup 6 isolates are highly diverse.

Resistant genotypes and phenotypes

Of the 497 isolates, 52 (10.5 %) were gPSSP, 130 (26.2 %) were gPISP *pbp2x*, 104 (20.9 %) were gPISP *pbp1a*+ *pbp2x*, 45 (9.1 %) were gPISP *pbp2x*+*pbp2b* and 166 (33.4 %) were gPRSP (Table 2). There was a large gap between the rates of penicillin-non-susceptible genotypes (89.5 %, 445/497) and phenotype (46.9 %, 233/497). In particular, gPISP *pbp2x* isolates were almost all phenotypically penicillin susceptible.

Regarding macrolide-resistant genes, 28 (5.6 %) had both *ermB* and *mefA/E*, 243 (48.9 %) had *ermB*, 151 (30.4 %) had *mefA/E*, and 75 (15.1 %) had no macrolide-resistant genes. Combined genetic classification of the *pbp* mutation and macrolide-resistant gene showed 17 resistant genotypes (Table 2). All of the gPRSP isolates had at least one of the macrolide-resistant genes.

Each ST had a specific resistant genotype(s). Of the 66 STs detected in this study, 57 STs (86.4 %) had only one specific resistant genotype (Fig. 1). For example, all 21 of the ST2922 isolates in this study had the same resistant genotype (gPISP *pbp1a*+*pbp2x* with *ermB*). Six STs (9.1 %) had two resistant genotypes, and only three STs (4.5 %) had more than three resistant genotypes (Fig. 1). Of 233 phenotypically penicillin-nonsusceptible isolates, 196 (84.1 %) belonged to only six clones, comprising ST90^{6B}, ST3787^{6A}, ST1437^{23F}, ST338^{23A}, ST236^{19F} and ST242^{23F} and their variants. Furthermore, these clones showed macrolide resistance with resistance gene(s), except for the ST3787^{6A} clone. ST338^{23A} is the only clone that is not included in the 13-valent PCV serotypes, which includes PCV7 serotypes plus serotypes 1, 3, 5, 6A, 7F and 19A. Nine STs contained 26 isolates that showed a susceptible genotype for both penicillin and macrolide, and all of them were non-PCV7 serotypes except for 18C (three isolates).

Serogroup 6 capsular loci

In order to evaluate differences in the capsular loci of serogroup 6, DNA sequence comparison was carried out for the *cps* locus from the *wchA* to the *wzx* gene of serotype 6A, 6B, 6C and 6D isolates. The differences of the sequences in the *cps* loci (Figs S1 and S2, available in JMM Online) were consistent with the results reported previously by Song *et al.* (2011). The nucleotide position of nt 1 in this study corresponded to nt 5545 in a previous report by Bratcher *et al.* (2011). For example, deletions in the *wciN* (*wciN*$_\beta$) gene were found in 6C and 6D isolates corresponding to the sequences between nt 1866 and 2226 (361 bp) in ST90^{6B}, and nt 1866 and 2227 (362 bp) in ST2924^{6B} isolates. Also, there were insertions of about 300 bp between the *wciN*$_\alpha$ and

Table 1. Relationship between capsular serotype and MLST

Underlining indicates ST including different serotype isolates that belonged to the same serogroups. Bold type indicates ST including different serotype isolates that belonged to the different serogroups.

Serogroup	Serotype	No. isolates	No. MLST	MLST
3		9	1	180
6	6A	42	3	282, 2224, 3787
	6B	82	10	90, 1092, 2224, 2924, 2983, 3787, 6413, 7783, 7813, 7834
	6C	51	8	2923, 2924, 5241, 7781, 7782, 7788, 7798, 7799
	6D	4	2	**236**, 2924
10	10A	9	2	5236, 7791
11	11A/E	8	2	7153, 7785
	11D	1	1	6185
14		25	3	343, 2922, 7795
15	15A	6	1	63
	15B	16	3	**199**, 7793, 7797
	15C	18	6	**199**, 7787, 7792, 7793, 7794, 7797
18	18B	2	1	3594
	18C	3	1	3594
19	19A	14	4	2331, 3111, 7780, 7796
	19F	62	9	**199**, **236**, 257, **338**, 1428, **1437**, 1464, 7978, 7979
22	22F	6	2	433, 7158
23	23A	23	3	**338**, 5246, 7814
	23F	64	3	242, **1437**, 7784
31		2	1	7800
33	33F	10	1	717
34		11	2	**3116**, 7388
35	35B	2	1	558
37		6	1	447
38		1	1	393
NT		20	7	344, 1106, **3116**, 3288, 7494, 7786, 7789

wciO genes (indel sequence) between nt 2227 and 2535 (309 bp) in ST90^{6B}, and nt 2228 and 2536 (309 bp) in ST2924^{6B} isolates. ST2924^{6C} and ST2924^{6D} isolates had a partial indel sequence in the *wciN*$_{\beta}$ gene (Fig. 2).

To clarify how the ST2924^{6D} strain emerged, the sequences of the ST90^{6B}, ST2924^{6B}, ST2924^{6C} and ST2924^{6D} isolates were compared (Fig. 2, Fig. S2). The sequences from nt 1 to nt 1872 and nt 5539 to nt 6573 of the two ST2924^{6D} isolates were identical to the sequences of the corresponding regions of the ST2924^{6C} isolates. In contrast, the sequences from nt 1936 to nt 5498 of the ST2924^{6D} isolates were identical to the corresponding sequences of the ST90^{6B} and ST2924^{6B} isolates. These results are consistent with a recombination event in which a 3563 bp fragment of the capsule locus was acquired by the ST2924^{6C} strain from the ST90^{6B} or ST2924^{6B} strain resulting in the ST2924^{6D} strain (Fig. 2). The *cps* profile for two ST2924^{6D} isolates was 8-7-11. These results showed a different recombination site and *cps* profile from a previous study (Bratcher *et al.*, 2011).

DISCUSSION

Here, we have reported a genotypic surveillance of *S. pneumoniae* among healthy children and capsular gene evolution in serogroup 6 on Sado Island. We found 25 serotypes showing a total of 66 STs. Interestingly, the serotypes with multiple STs were highly prevalent serotypes, including 6B, 19F and 6C. It is possible that the more diverse serotypes were able to adapt to the community setting and spread in healthy children through their increased capacity to undergo recombination compared with other serotypes. Alternatively, the prevalent serotypes that had already spread in the community may be undergoing recombination at a rate that is not different from the other serotypes but is detected as a result of the increased prevalence of those serotypes.

S. pneumoniae has a variety of drug-resistant and -susceptible genotypes (17 genotypes) in this setting. Most of the STs (86.4 %) had one specific resistant genotype, indicating that factors besides drug resistance play an important role in the survival and circulation of *S. pneumoniae* strains in the community. In this study, six clones and their variants accounted for 84.1 % (196/233) of phenotypically penicillin-non-susceptible isolates. Furthermore, most clones showed phenotypic and genotypic macrolide resistance. We conclude that drug-resistant *S. pneumoniae* is associated with a limited number of highly prevalent clones that are capable of adapting to the community setting. We will continue

Table 2. Resistant phenotype and genotype of 497 *S. pneumoniae* isolates

PCR-based genotype*	*n* (%)	Susceptibility to penicillin G			*mefA/E* + *ermB*	Macrolide-resistant genes		
		Susceptible	Intermediate	Resistance		*ermB*	*mefA/E*	None
gPSSP (none)	52 (10.5 %)	52	0	0	0	18	8	26
gPISP (2x)	130† (26.2 %)	127	2	0	10	68	38	14
gPISP (1a+2x)	104 (20.9 %)	73	31	0	1	36	34	33
gPISP (2x+2b)	45 (9.1 %)	9	36	0	0	28	15	2
gPRSP (1a+2x+2b)	166 (33.4 %)	2	130	34	17	93	56	0
Total	497 (100 %)	263 (52.9 %)	199 (40.0 %)	34 (6.8 %)	28 (5.6 %)	243 (48.9 %)	151 (30.4 %)	75 (15.1 %)

*1a, *pbp1a*; 2x, *pbp2x*; 2b, *pbp2b*.
†One isolate was not available for the susceptibility test.

to survey and identify changes in *S. pneumoniae* clones in Japan.

Capsule switch events probably occur through genetic transformation with different serotypes of *S. pneumonie* co-infecting the nasopharynx simultaneously in which the capsule locus is exchanged between strains (Temime *et al.*, 2008). For example, the multidrug-resistant ST320^{19A} strain was genetically derived from the multidrug-resistant ST320^{19F} strain (Ardanuy *et al.*, 2009; Moore *et al.*, 2008; Pillai *et al.*, 2009; Song *et al.*, 2009).

Our data regarding serogroup 6 (Fig. 2) provide direct evidence that the ST2924^{6D} strain was genetically derived from a recombination of ST2924^{6C} and ST90^{6B} strains known as a 'capsule switch', and not by a point mutation in 6B strains. The recombination probably occurred on Sado Island, based on several lines of evidence: (i) ST2924^{6C}

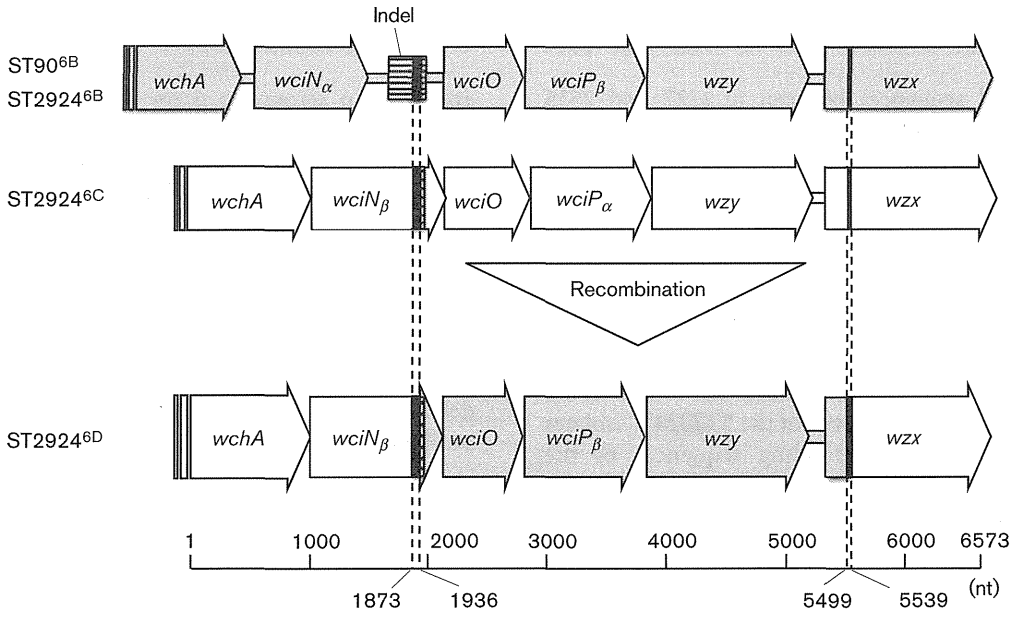


Fig. 2. Recombination of the capsular biosynthetic loci in ST90^{6B}, ST2924^{6B}, ST2924^{6C} and ST2924^{6D}. The capsule locus resides between genes *wchA* and *wzx*. The genes in the serogroup 6 loci vary in size. The position of nt 1 in this figure corresponds to nt 5545 in the previous report by Bratcher *et al.* (2011). The genes of the capsular biosynthetic locus of ST90^{6B} and ST2924^{6B} isolates are shown as shaded arrows and those of ST2924^{6C} isolate as open arrows. A filled box indicates a putative site of recombination. The hatched box indicates the indel sequence. The length of the nucleotide sequence and the putative recombination sites are indicated under the capsular biosynthetic locus of ST2924^{6D} isolate.

and ST90^{6B} strains have spread throughout Sado Island; (ii) one subject had the ST90^{6B} strain (SP598) before having the ST2924^{6D} strain (SP1090); (iii) there is limited movement of people (particularly children) between Sado Island and the Japanese mainland; and (iv) our study is the only report of serotype 6D in Japan. Another possible explanation is that the ST2924^{6D} strain was derived from a recombination of the ST2924^{6C} and ST2924^{6B} strains. The sequence from the *wchA* to the *wzx* gene of the ST2924^{6B} isolates was identical to that of the ST90^{6B} isolates but different from those of strains ST2924^{6C} and ST2924^{6D}. The ST2924^{6B} strain seems to have emerged by a recombination of the ST2924^{6C} and ST90^{6B} strains by exchange of the entire capsule locus. The ST2924^{6D} strain then emerged, if all recombination events occurred on Sado Island.

Bratcher *et al.* (2011) suggested that serotype 6D *cps* may have resulted from a recombination once between serotypes 6B and 6C at a location between the *wciN* and *wciP* loci and then spread worldwide, because *cps* profiles among their serotype 6D isolates were almost identical (5-1-1 and a single-base mutation of 5-1-1). However, our results showed a different recombination site (*wciN* and *wzx*) and a different *cps* profile (8-7-11), indicating that serotype 6D *cps* can also emerge through recombination between serotypes 6B and 6C involving *wciN* and *wzx*. These results suggest that serogroup 6 strains have multiple sites for *cps* recombination.

In conclusion, our results indicate that prevention of infection by drug-resistant clones could effectively reduce the prevalence of pneumococcal drug resistance. The serotypes in the 13-valent PCV include the drug-resistant clones. By contrast, our results also show that recombination in two capsular loci of seroproup 6 isolates is a mechanism of vaccine escape by *S. pneumoniae*.

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Original Article

Changes in *Streptococcus pneumoniae* Serotypes in the Nasopharynx of Japanese Children after Inoculation with a Heptavalent Pneumococcal Conjugate Vaccine

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SUMMARY: In this study, we prospectively investigated changes in *Streptococcus pneumoniae* serotypes among Japanese children after heptavalent pneumococcal conjugate vaccine (PCV7) inoculation. We acquired nasopharyngeal swabs from the children at each routine PCV7 inoculation and again at least 2 months after the last PCV7 inoculation. We defined 2 periods with regard to each culture: the inoculation period as "the period of pre- or incomplete vaccination" and post-inoculation as "the period of post- or completed vaccination." The prevalence of vaccine-type (VT) pneumococci was significantly reduced from 9.5% in the inoculation-period cultures to 2.9% in the post-inoculation cultures ($P < 0.01$). There was no statistical difference in the prevalence of non-vaccine-type pneumococci between the inoculation-period and post-inoculation cultures (24.1% versus 23.4%). The protection of PCV7 against nasopharyngeal colonization was inferred from the decrease in VT carriage post-inoculation. The decrease in VT carriage may be conducive to reducing VT transmission within the study area.

INTRODUCTION

Streptococcus pneumoniae is part of the commensal flora of the nasopharynx, but it is also a clinically important pathogen that causes invasive pneumococcal diseases, such as sepsis, meningitis, and pneumonia, in children (1). In the United States, the introduction of the heptavalent pneumococcal conjugate vaccine (PCV7) resulted in a decreased incidence of invasive pneumococcal diseases through a reduction in the prevalence of vaccine-type (VT) (serotypes included in PCV7) pneumococci among vaccinated children (2,3). It also had indirect effects on pneumococcal transmission through herd immunity (4).

PCV7 was introduced in Japan in 2010 and has been administered under the National Health Insurance System since 2011. The standard PCV7 schedules in Japan are as follows: 3 doses between the ages of 2 months and 7 months, followed by 1 booster dose after the age of 12 months (3 + 1-dose); 2 doses between the ages of 7 months and 11 months, followed by 1 booster dose after the age of 12 months (2 + 1-dose); 2 doses between the ages of 12 months and 23 months (2-dose); and a single dose after the age of 24 months (1-dose).

The present study is the first to prospectively investigate changes in *S. pneumoniae* serotypes in the nasopharynx of Japanese children inoculated with PCV7.

MATERIALS AND METHODS

Study population: During the study period (July 2010 through November 2012), 137 healthy children were enrolled from 3 institutions in Saitama Prefecture and Tokyo: Hasuda Issinkai Hospital, Yashio Ekimae Internal Medicine, and Pediatric Clinic and Tokyo Women's Medical University Medical Center East. Parents were informed about the study when they brought their children for their first routine PCV7 inoculation, and

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written informed consent was obtained from those who agreed to participate. All subjects were aged between 2 months and 6 years, and none had, at registration, a prior history of immunization with a pneumococcal vaccine or an obvious infection. This study (22026) was approved by the Ethical Review Board of Toho University.

Specimen collection and testing: Pediatricians acquired nasopharyngeal swabs (Amies charcoal transport medium) from the children at each routine PCV7 inoculation and again at least 2 months after the last PCV7 inoculation. The samples were plated on agar medium containing 5% sheep blood and incubated overnight at 35°C in 5% CO_2. Pneumococci were isolated and identified on the basis of colony morphology and optochin susceptibility. Colonies that were difficult to identify with the optochin susceptibility test were confirmed by means of polymerase chain reaction analysis of *lytA* gene (5).

Characteristics of the study population: The following information was collected from the medical records of each participating child and/or through a questionnaire completed by their parents or guardians: age (<2 or 2–6 years), sex, day-care attendance (yes/no), presence of older/younger siblings (yes/no), and antibiotic treatment within the preceding 3 months (yes/no).

Serogrouping and serotyping: Serogrouping and serotyping of *S. pneumoniae* isolates from the children were performed using the quellung reaction at the Department of Bacteriology I, National Institute of Infectious Diseases. Isolates that had negative reactions to all pooled sera and omni serum were considered to be nontypeable. Isolates were classified as either VT pneumococci (4, 6B, 9V, 14, 18C, 19F, and 23F) or non-vaccine-type (NVT) pneumococci (all others).

Definition of "inoculation-period" and "post-inoculation" cultures: Pediatricians used flexible, sterile, dry nasopharyngeal cotton-wool swabs to transnasally acquire nasopharyngeal samples from the subjects during each PCV7 inoculation, which was performed according to the standard PCV7 schedules described above. A total of 233 culture samples created from these swab samples were defined as "inoculation-period" cultures (i.e., the period of pre- or incomplete vaccination) (Fig. 1). In addition, 2 months or more after the last PCV7 inoculation, nasopharyngeal swab samples were acquired again and cultured (defined as "post-inoculation" cultures, i.e., the period of completed vaccination) (Fig. 1).

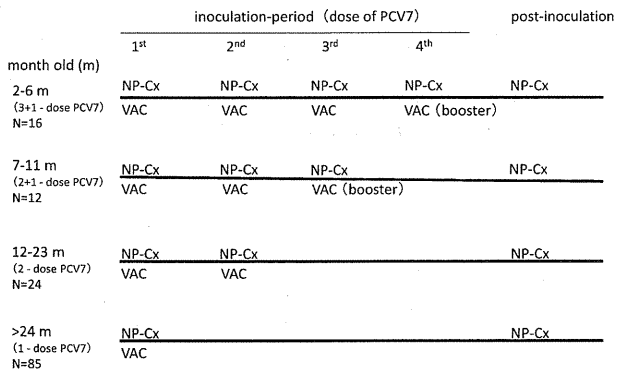

Fig. 1. Study procedures in 137 children receiving 3 + 1-dose of PCV7, 2 + 1-dose of PCV7, 2-dose of PCV7 or 1-dose of PCV7. NP-Cx, nasopharyngeal sample obtained for culture; VAC, vaccination given.

Statistical analysis: McNemar's test was used to analyze the differences between the prevalence of VT and NVT pneumococci in the inoculation-period and post-inoculation cultures. A 2-sided P of <0.05 was considered statistically significant. STATA ver. 12 (STATA Corporation, College Station, Tex., USA) was used for all data analyses.

RESULTS

The demographic characteristics of the study participants at their first routine PCV7 inoculation are shown in Table 1.

S. pneumoniae was isolated from 32 of the 137 (23%) children during the first PCV7 vaccination in the inoculation period. The pneumococci obtained at the second, third, and fourth vaccinations during the inoculation period were isolated from 11 of 52 (21%), 7 of 28 (25%), and 5 of 16 (31%) samples, respectively, and the pneumococci obtained at post-inoculation were isolated from 35 of 137 (26%) samples. The serotype-specific prevalence of pneumococcal isolates is shown in Table 2. The percentages of VT and NVT pneumococci at the first PCV7 vaccination in the inoculation-period cultures were 30% and 70%, respectively. The corresponding figures for the post-inoculation cultures were 11% and 89%, respectively. The most prevalent serotype was 6C in the first PCV7 vaccination in both the inoculation-period (15%) and post-inoculation (22%) cultures. Among the VT pneumococci isolates, serotype 6B was isolated from both the inoculation-period and post-inoculation cultures, but serotypes 14, 19F, and 23F were not isolated from the post-inoculation cultures. Among the NVT pneumococci isolates, serotypes

Table 1. Characteristics of the 137 subjects at 1st visit

Characteristic	No. (%) of children
Age	
<2 years old	52 (38)
2–6 years old	85 (62)
Sex	
Male	77 (56)
Female	60 (44)
Day care attendance	
Yes	45 (33)
No	92 (67)
Presence of older siblings	
Yes	60 (44)
No	77 (56)
Presence of younger siblings	
Yes	32 (23)
No	102 (74)
Not known	3 (3)
Antibiotic treatment within the preceding 3 months	
Yes	55 (40)
No	81 (59)
Not known	1 (1)

Table 2. Pneumococcus serotypes in inoculation-period and post-inoculation cultures

Serotype	Inoculation period								Post-inoculation	
	1st		2nd		3rd		4th			
	No.	Column %	No.	Column %	No.	Column %	No.	Column %	No.	Column %
VT										
6B	4	12.12	2	16.67	2	25.00	0	—	4	11.11
14	3	9.09	0	—	0	—	0	—	0	—
19F	1	3.03	0	—	0	—	0	—	0	—
23F	2	6.06	0	—	0	—	0	—	0	—
total	10	30.30	2	16.67	2	25.00	0	—	4	11.11
NVT										
3	0	—	0	—	0	—	0	—	1	2.78
6A	1	3.03	1	8.33	0	—	0	—	1	2.78
6C	5	15.15	4	33.33	3	37.50	1	20.00	8	22.22
10A	0	—	0	—	0	—	0	—	1	2.78
15A	1	3.03	1	8.33	0	—	2	40.00	4	11.11
15B	2	6.06	0	—	0	—	1	20.00	3	8.33
15C	3	9.09	1	8.33	1	12.50	0	—	3	8.33
19A	3	9.09	0	—	0	—	0	—	2	5.56
22F	2	6.06	1	8.33	0	—	0	—	3	8.33
23A	0	—	0	—	0	—	0	—	2	5.56
33F	3	9.09	0	—	0	—	0	—	0	—
34	0	—	0	—	0	—	0	—	2	5.56
35B	2	6.06	1	8.33	0	—	0	—	1	2.78
38	1	3.03	0	—	0	—	0	—	0	—
UT	0	—	1	8.33	2	25.00	1	20.00	1	2.78
total	23	69.70	10	83.33	6	75.00	5	100.00	32	88.89
Total	33*	100.00	12*	100.00	8*	100.00	5	100.00	36*	100.00

*There are overlaps in the total numbers because some different serotypes were detected in several subjects.
PCV7, heptavalent pneumococcal conjugated vaccine; VT, vaccine-type; NVT, non-vaccine-type.

Table 3. Number of pneumococcal carriages (VT/NVT pneumococci) detected in inoculation-period and post-inoculation cultures

S. pneumoniae (VT or NVT)		No. of children	
Inoculation period (at 1st-4th dose)	Post-inoculation (>2 mo after last inoculation)	VT	NVT
negative	negative	123	86
positive	negative	10	19
negative	positive	1	18
positive	positive	3	14
	total	137	137

VT, vaccine-type; NVT, non-vaccine-type.

33F and 38 were absent from the post-inoculation cultures, but serotypes 3, 10A, 23A, and 34 were newly isolated from the same cultures.

The mean duration (±SD) from the last PCV7 inoculation to the last sampling was 139.4 (±79.3) days.

The numbers of pneumococcal carriages, categorized as VT or NVT, detected during each period are shown in Table 3. The prevalence of VT pneumococci was significantly reduced from 9.5% in the inoculation-period cultures to 2.9% in the post-inoculation cultures ($P < 0.01$). There was no statistical difference in the prevalence of NVT pneumococci between the inoculation-period and post-inoculation cultures (24.1% versus 23.4%).

DISCUSSION

To the best of our knowledge, this is the first study in Japan to prospectively investigate changes in *S. pneumoniae* serotypes in nasopharynx samples of children following PCV7 inoculation. Reports from other countries have indicated that the prevalence of VT pneumococci is individually reduced after PCV7 administration (6,7). Millar et al. reported that VT pneumococcal carriage was lower among adults and unvaccinated children living with others who received the PCV7 vaccine (8). In the United States, the decreased nasopharyngeal carriage of VT strains among PCV7-immunized children led to decreased transmission to nonimmunized