Table 5
Number of cases of invasive pneumococcal disease by number of vaccine doses received, serotype, and patient age group.

Serotype age group (y)	Serotype number of cases by number of PCV7 doses received					Total number
	0 dose	1 dose	2 doses	3 doses	4 doses	
All PCV7 serotypes	63	2	0	0	0	65
0	12	0	0	0	0	12
1	23	1	0	0	0	24
2	12	1	0	0	0	13
3	7	0	0	0	0	7
4	6	0	0	0	0	6
≥5	3	0	0	0	0	3
All non-PCV7 serotypes	34	0	4	6	11	55
0	4	0	2	2	0	8
1	10	0	1	4	7	22
2	8	0	0	0	4	12
3	4	0	0	0	0	4
4	2	0	1	0	0	3
≥5	6	0	0	0	0	6
Not determined	175	2	5	6	3	191
0	33	0	0	2	0	35
1	77	1	3	4	2	87
2	21	0	0	0	1	22
3	18	0	2	0	0	20
4	10	0	0	0	0	10
≥5	16	1	0	0	0	17

PCV7, heptavalent pneumococcal conjugate vaccine.

the incidence of pediatric IHiD was rapidly increasing; the annual incidence for children less than 5 years of age in 2005 was 16.5 per 100,000 [17]. Sakata also evaluated the incidence of IHiD, in Kamikawa subprefucture, Hokkaido, Japan between 1996 and 2005. He mentioned that a marked increase in the annual incidence rate in children aged under 5 years was observed in 2005, reaching 56.8/100,000. Serotyping in 37 of 38 (97.4%) cases demonstrated Hib [18]. This incidence rate was similar to that in other developed countries in the pre-Hib conjugate vaccine era [19]. Our study revealed a 94.7% reduction in IHiD in children younger than 5 years of age in Chiba prefecture, Japan. This remarkable reduction was observed after the introduction of special funding from the Japanese government. Similar to previous investigations, we found that most children with IHiD were less than 2 years of age and that meningitis occurred more frequently in younger than in older children [19,20]. In this study, meningitis was the most important cause of long-term morbidity in IHiD patients, accounting for 87.5% (7/8) of the sequelae. Immediately before and in the early period of Hib conjugate vaccine usage, almost all IHiD in children was caused by Hib, and the majority had not been immunized with any dose of Hib vaccine. Hib vaccine efficacy is high, ranging from 93 to 100% after the 3-dose primary series, and IHiD after full vaccination is rare [21]. In this study period, there was only 1 patient who received the 3-dose primary series of Hib vaccine and subsequently developed Hib meningitis. Although rare, Hib disease still occurs despite high vaccination coverage and the low carriage prevalence in children, suggesting continued circulation and transmission of Hib [22]. Efforts should focus on maintaining high vaccination coverage based on appropriate timing of the primary series and booster doses to prevent Hib disease. In contrast, the number of the IHiD caused by NTHi gradually increased during this study period. NTHi has been the most common isolate among adults in virtually all published series [23–25]. Whether invasive strains of NTHi have distinct genotype and phenotype characteristics compared with noninvasive isolates remains largely unknown. Several strains of NTHi possess Hib-like encapsulated properties [26]. However, to date no single set of virulence determinants has been conclusively associated with invasive NTHi [27,28]. Although no vaccine is available for invasive disease caused by encapsulated non-type b and NTHi, serotyping of *H. influenzae* isolated from invasive diseases and analysis of the pathogenicity of NTHi are important for new vaccine development against all IHiD.

Using hospitalized population-based surveillance, we had already reported the annual incidence of IPD among Japanese children younger than 5 years of age (12.6–13.8 per 100,000) from 2003 to 2005, which was similar to those rates reported in a study conducted in Germany [29,30], but was much lower than the incidence reported in the pre-PCV era in the United States [31]. After that, the annual incidence of pediatric IPD increased, and this study determined the annual incidence of IPD among Japanese children younger than 5 years of age in the pre-PCV7 era. Compared with 6 years ago, there has been a 57.4% reduction in IPD. This remarkable reduction was observed after the introduction of special funding from the Japanese government. In this study, 60.5% of all IPD cases and 66.7% of the pneumococcal meningitis cases involved children under 2 years of age. Similar to previous investigations, we found that almost half of children with IPD were less than 2 years of age and that meningitis occurred more frequently in younger than in older children [32,33]. Analysis of current cases of IPD revealed that <5% (15/311) of IPD occurred in children younger than 6 months of age, before completion of the primary vaccination series. To have an impact on disease reduction in this subgroup of children, we must rely on herd immunity. Our data also indicated that 21.2% of the patients with IPD have various underlying diseases. These include malignancy, congenital anomaly syndrome and congenital heart disease. Prematurity has recently been associated with an increased relative risk for IPD [34]. The underlying disorders in our study were different from those in other studies. For instance, the most common underlying disorders in Bennett's and Levine's studies were sickle cell anemia and chronic pulmonary disorder, respectively [35,36]. These results may relate to racial differences. There were 2 fatal cases in our study. Both cases had severe underlying disease. Vaccination is one of the possible ways to prevent this type of infection. The introduction of PCV7 has dramatically decreased the incidence of IPD caused by vaccine serotypes of *S. pneumoniae* all over the world [37–39]. However, the non-vaccine serotype 19A has been the predominant agent of IPD among children and the second most common agent among older adults after PCV7 introduction in the US [40]. Serotype 19A, especially sequence type (ST) 320, was increasingly recognized in Korean children before

the introduction of PCV7 [41]. The serogroups 15 and 33 were also identified to be important in the US [42]. The 51% decline of IPD in Japanese children in this study is far below the 77% reduction observed in children under the age of 5 years in the regions covered by US surveillance conducted in 2005 [43]. In our study, immediately before and during the early period of PCV7 usage, more than 50% of IPD in children was caused by PCV7 serotype group disease. Chiba et al. [12] compared the serotypes of *S. pneumoniae* isolated from pediatric IPD between 2006 and 2011. They mentioned that coverage by PCV7 decreased from 71.8% in 2006 to 51.6% in 2011. Serotype 19A tended to increase and serotypes 15A and 22F also increased. Our study revealed that the rate of IPD caused by PCV7 serotypes decreased from 57.9% (11/19) in 2008 to 11.8% (2/17) in 2013. Although almost one-third of isolates were unavailable for serotyping, we have no expectation that the serotype distribution of the missing isolates was different than the available isolates.

Serotype replacement was recognized in the early stages of PCV7 introduction in Japan. In our study, 2 cases were breakthrough cases, caused by serotype 6B. Both of them received one dose of PCV7. We defined a breakthrough case as IPD in a child who had received ≧1 PCV7 dose and for which the pneumococcal isolate was a PCV7 serotype; a vaccine failure was defined as the subset of breakthrough infections in children who had completed the PCV7 vaccination schedule at least 2 weeks before the occurrence of IPD. The remaining cases in children who had received at least one shot of PCV7 were IPD caused by non-PCV7 serotypes. Park et al. [44] reported that IPD identified in vaccinated US children was primarily caused by disease resulting from non-PCV7 serotypes rather than failure of the PCV7. They also mentioned that incomplete vaccination and co-morbid conditions likely contribute to breakthrough vaccine-type pneumococcal infections. PCV13 added 6 serotypes to the original 7 serotypes in PCV7. PCV13 replaces PCV7 for routine administration in infants and children, and is now used in many countries. The Advisory Committee on Immunization Practices in the United States recommends, as the standard immunization schedule for PCV13, a 4-dose series at ages 2, 4, 6, and 12–15 months [45]. It is important that the standard immunization schedule for PCV13 be followed in all children. After the introduction of PCV13, several countries are reporting on the epidemiological effects on IPD in children [46,47]. PCV13 was introduced in November 2013 in Japan; we should continuously monitor serotype distribution in isolated strains from IPD patients.

In conclusion, the introduction of Hib conjugate and PCV7 vaccines was followed by a significant decrease in IHiD and IPD in young children in Chiba prefecture, Japan. However, NTHi and non-PCV7 serotype *S. pneumoniae* are gradually increasing as invasive pathogens after the introduction of these two conjugate vaccines. Nationwide surveillance to determine the incidences of IHiD and IPD and monitoring of *H. influenzae* and *S. pneumoniae* serotypes isolated from patients with invasive disease should be continuously performed in Japan.

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論 壇

肺炎球菌ワクチンの効果と血清型変化への対応

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抄　　録

7価結合型肺炎球菌ワクチン（PCV7）は，わが国では2010年2月に導入され，2013年4月に定期接種となった。その結果，5歳未満の侵襲性肺炎球菌感染症（IPD）の罹患率は，厚労科研「庵原・神谷班」調査によると2012年には半減した。しかし，2013年の減少傾向は緩徐となり，髄膜炎の罹患率はむしろやや増加した。その背景には，IPD原因菌の莢膜血清型の変化（serotype replacement）がある。2010年から2013年にかけて，IPD原因菌におけるPCV7タイプの割合は79%から5%に減少したが，19Aをはじめとする PCV13追加タイプは11%から48%に，非PCV13タイプも10%から47%に増加した。2013年11月に定期接種となったPCV13の普及により，19AによるIPDの減少が期待されるが，PCV7の4回接種終了者にもPCV13の補助的追加接種が望まれる。

キーワード：結合型肺炎球菌ワクチン，PCV7，PCV13，侵襲性肺炎球菌感染症，補助的追加接種

1．結合型肺炎球菌ワクチンの有効性

7価結合型肺炎球菌ワクチン（pneumococcal conjugate vaccine：PCV7，プレベナー®）は，わが国では2010年2月に任意接種として導入され，2011年から本格化した国の「子宮頸がん予防ワクチン等緊急接種促進事業」による公費補助により普及した。2013年4月には5歳未満の小児を対象に定期接種となり，11月にはPCV13に置きかえられた。

その結果，髄膜炎・菌血症などの小児侵襲性肺炎球菌感染症（invasive pneumococcal disease, IPD）は大きく減少した。髄膜炎以外のIPDの5歳未満人口10万あたりの罹患率は，10道県を対象とした厚労科研「庵原・神谷班」の全数調査によると，2012年には2008～10年の平均に比べて52%の減少がみられた（22.2→10.6）（**図1A**）[1]。しかし，2013年の減少傾向は緩徐であり，2012年に比べて8%の減少にとどまっている（10.6→9.7）。髄膜炎の罹患率は，菌血症等に比べて約10分の1であるが，2012年は2008～10年比べて71%減少したものの，2013年は2012年より38%増加した（**図1B**）。

鹿児島県の全数調査でも，2010年から2012年まで患者数は順調に減少してきた。しかしながら，2013年からは再び増加し，2014年は8月15日現在ですでに2013年の患者数に迫っている（**図2**）。

2．肺炎球菌の血清型の変化

このように当初顕著にみられたPCVの効果が停滞している背景には，PCV7に含まれない血清型の肺炎球菌によるIPDの増加，すなわちIPDの原因菌の莢膜血清型の変化（serotype replacement）がある。

2000年にPCV7が導入された米国では，その数年後にはPCV7に含まれない莢膜型である19Aや7FによるIPDの増加がみられた[2]。わが国でも，**図3**に示すように，2010年から2013年にかけてIPD原因菌におけるPCV7タイプの割合が79%から5%に大幅に減少した一方で，PCV7に含まれないタイプの株が急増した。

鹿児島県のIPD原因菌の血清型（**図4**）も，PCV7導入前はほとんどがPCV7タイプ（交叉免疫のある6Aを含む）だったが，2010～12年には59%（16/27），

Effectiveness of pneumococcal conjugate vaccine and serotype replacement
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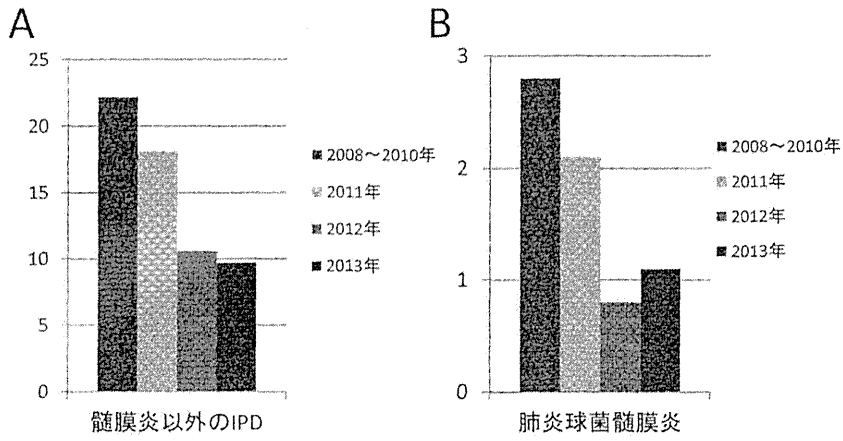


図1　10道県のIPD罹患率の推移

「庵原・神谷班」調査。5歳未満人口10万人あたりの患者数（北海道，福島，新潟，千葉，三重，岡山，高知，福岡，鹿児島，沖縄）。

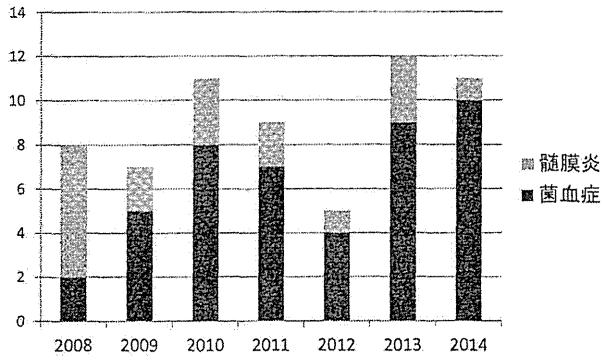


図2　鹿児島県の5歳未満IPD患者数の推移

（2014年は8月20日現在）

2013~14年には8％（2/24）と減少している。一方，PCV13に追加されたタイプ（交差免疫のある6Cを含む）は，2004~09年にはみられなかったが，2010年以後増加し，特に19Aの増加が顕著であった。さらに，PCV13に含まれないタイプも2012年以後増加している。

PCV7導入後の莢膜血清型の変化は，非ワクチンタイプの株が単に選択されただけではない。世界に広がっている19A株のMLST（multi locus sequence type）解析から，特定の遺伝子型の株に異なる莢膜型の遺伝子群が存在することが明らかになり，特定のクローンが形質転換によって莢膜遺伝子群の組換えを行った可能性が指摘されている[3]。肺炎球菌はDNAの取り込みによる形質転換を起こしやすい性質を持つが，莢膜血清型を変化させ，PCV7の圧力を積極的に回避（vaccine escape）しているという点で興味深い。

このような血清型変化を受けて，2013年11月にPCV13（プレベナー13®）が定期接種として導入されたが，2014年8月現在ではまだ明らかなPCV13追加タイプによるIPDの減少は確認されていない。

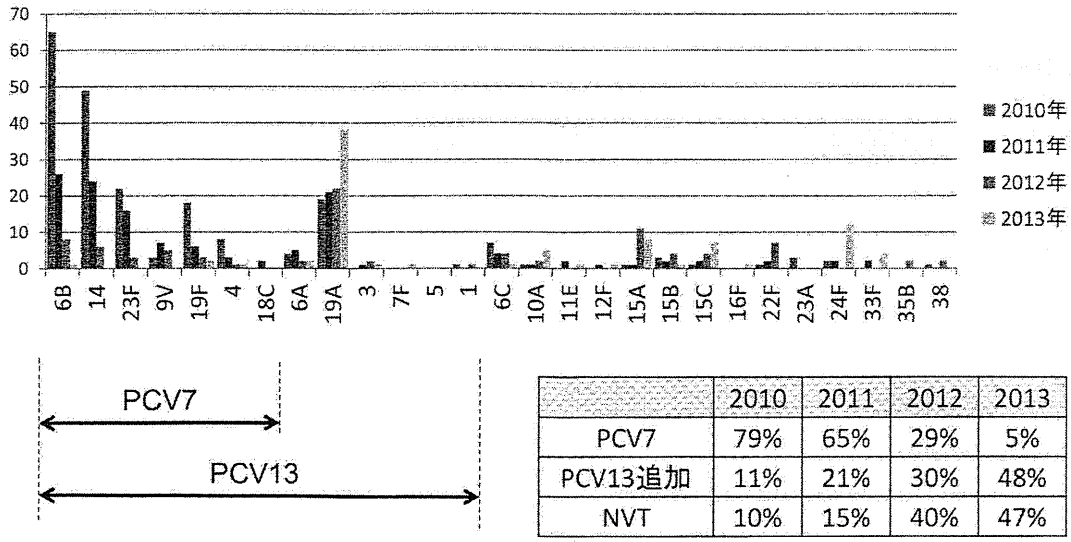
3．PCV13の補助的追加接種の必要性

米国では2010年にPCV13を導入し，PCV7のスケジュール途中の場合はそのままPCV13に置き換え，さらにPCV7を4回接種終了した児にも補助的追加接種（supplemental dose）を推奨した。その結果，2002年以後停滞していた米国の5歳未満人口10万人当たりのIPD罹患率は，2011年には早くも減少傾向がみられた[4]。さらに，米国8主要小児病院での調査でも，PCV13の追加6血清型によるIPDの減少，特に19AによるIPDが58%減少したことが報告されている[5]。

補助的追加接種は米国だけでなく多くの国で推奨されているが，わが国では残念ながら定期接種としては認められなかった。「庵原・神谷班」の2013年の調査結果によると，IPD患者の年齢分布は，PCV7が終了している年齢である1歳6カ月以上が46%を占めており[1]，その血清型はPCV13の追加6種が約43%，とくに19Aが38%を占めていた（国立病院機構三重病院・菅 秀 先生私信）（**図5**）。したがって，PCV7の4回接種終了児も19AによるIPDのリスクが存在するため，任意接種ではあるが1回の補助的追加接種が望まれる。また，PCV7の追加接種を済ませていない児に対するPCV13の追加接種を徹底することも重要である。

4．おわりに

Hibワクチンの普及でHib感染症はほぼみられなくなったのに対して，標的とする莢膜血清型の種類がきわめて多いPCVの有効性には限界が伴う。PCV13の普及により，血清型19AによるIPDの減少がわが国でも期待されるが，非PCV13タイプによるIPDにも注意



	2010	2011	2012	2013
PCV7	79%	65%	29%	5%
PCV13追加	11%	21%	30%	48%
NVT	10%	15%	40%	47%

図3　10道県のIPD由来株の血清型の頻度と推移

参考文献[1]より作図。図内の表は，各期におけるワクチンタイプの血清型の占める割合を示す。NVT：非PCV13タイプ

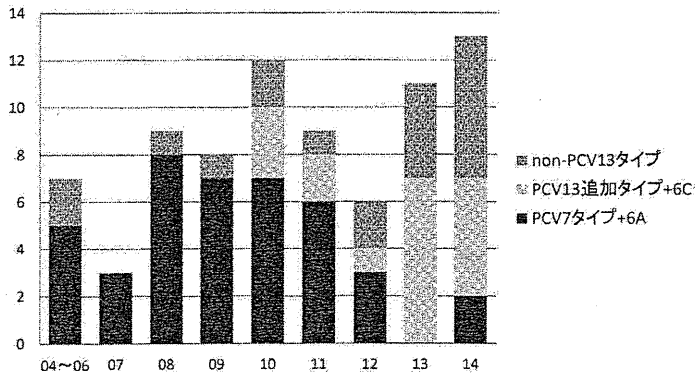


図4　鹿児島県の小児IPDの原因菌血清型の年次推移

（2014年は8月20日現在）

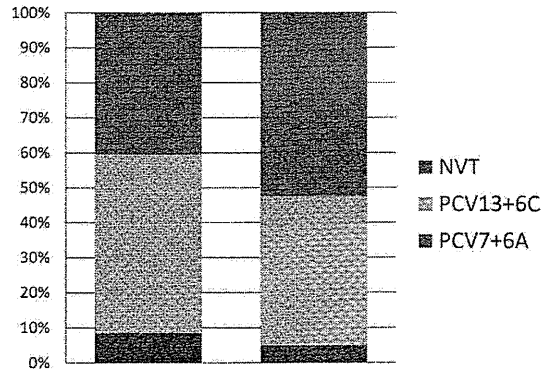


図5　10道県のIPD原因菌の血清型（2013）

「庵原・神谷班」調査より作図。NVT：非PCV13タイプ

が必要である。PCVの有効性と限界について，医療従事者から保護者への正しい情報提供が重要である。

今後，付着因子などの共通タンパク抗原を標的としたワクチンの開発が期待されるが，感染防御効果は抗莢膜多糖体抗体がもっともすぐれている。したがって，今後も莢膜多糖体結合型ワクチンが予防戦略の中心であることは変わらないと考えられ，ワクチンに含める血清型の種類の増加や選択が重要になると思われる。

利益相反自己申告

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Presentation

Global Control of Pneumococcal Infections by Pneumococcal Vaccines

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Abstract: *Streptococcus pneumoniae* is a major worldwide cause of morbidity and mortality. Pneumococcal carriage is considered to be an important source of horizontal spread of this pathogen within the community. Pneumococcal conjugate vaccine (PCV) is capable of inducing serotype-specific antibodies in sera of infants, and has been suggested to reduce nasopharyngeal carriage of vaccine-type pneumococci in children. PCV is generally immunogenic for pediatric patients with invasive pneumococcal disease, with an exception for the infecting serotypes. Based on evidences from the clinical trials of PCV, the health impact of childhood pneumococcal pneumonia appears to be high in developing countries where most of global childhood pneumonia deaths occur. PCV vaccination may prevent hundreds of deaths per 100,000 children vaccinated in developing countries, while PCV vaccination is expected to prevent less than 10 deaths per 100,000 children vaccinated in the developed countries. Therefore, the WHO has proposed a strategy to reduce the incidence of severe pneumonia by 75% in child less than 5 years of age compared to 2010 levels by 2025.
Key words: *Streptococcus pneumoniae*, Bacterial colonization, Invasive pneumococcal disease, Pneumococcal conjugate vaccine, Serotype-specific IgG, Opsonization index, Childhood pneumonia, WHO

Pneumococcal Diseases and Pneumococcal Conjugate Vaccine

Streptococcus pneumoniae is a major worldwide cause of morbidity and mortality resulting from pneumonia, bacteremia, and meningitis [1]. An important feature is that pneumococcal diseases will not occur without preceding nasopharyngeal (NP) colonization with homologous strain [2]. Pneumococcal carriage is considered to be an important source of horizontal spread of this pathogen within the community. Crowding in the hospital or day-care center, increases horizontal spread of pneumococcal strains. The rates of NP colonization of *S. pneumoniae* were found to be 20 to 40% in healthy children in Japan [3] and Thailand (Oishi K, et al. unpublished data). In contrast the rate of NP colonization of *S. pneumoniae* was reported to be high (approximately 90%) in Gambia, Africa [4].

Antibodies to pneumococcal capsular polysaccharide (CPS) and complement provide protection against pneumococcal strains with homologous or cross-reactive capsular serotypes [5]. The seven-valent pneumococcal conjugate vaccine (PCV7) is capable of inducing serotype-specific antibodies in sera of infants, and has been suggested to reduce nasopharyngeal carriage of vaccine-type (VT) pneumococci in toddlers, possibly by preventing acquisition rather than by eradicating pneumococci from the NP [6, 7].

The introduction in 2000 of PCV7 for children in the United States younger than 2 years and children aged 2–4 years in a high-risk category was effective, dramatically reducing the incidence of invasive pneumococcal disease (IPD) [8, 9].

In Japan, PCV7 was licensed in October 2009, the Japanese government began to subsidize it for children less than 5 years of age in November 2010. PCV7 for children under 5 years of age was subsequently included in the routine immunization schedule at public expense in April 2013. According to "Research report on evidence of and measures for improvement of usefulness of vaccination" (Ihara-Kamiya Research Project that started in 2007), incidence of IPD per 100,000 population under the age of five decreased significantly owing to the immunization program. Namely, meningitis decreased from 2.8 in 2008–

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2010 to 0.8 in 2012 (decrease by 71%), and non-meningitis IPD from 22.2 to 10.6 (decrease by 52%) (http://www.nih.go.jp/niid/ja/iasr-vol34/3343-iasr-397.html).

Vaccine-induced protective immunity is currently estimated by measuring the concentrations of serotype-specific immunoglobulin G (IgG) using enzyme-linked immunosorbent assay [10] and the opsonization index (OI) using a multiplex opsonophagocytic assay [11]. We recently determined the geometric mean concentration (GMC) of serotype-specific IgG and the geometric mean titers (GMT) of OIs among 17 pediatric patients with IPD using paired sera obtained at the onset of IPD and after PCV doses following the resolution of IPD. The GMCs of serotype-specific IgG for all PCV7 serotypes other than serotype 6B were significantly increased after the last PCV7 dose compared with those at the time of IPD onset (Table 1), as were the GMTs of OIs for all PCV7 serotypes (Table 2). These data suggest that PCV7 is generally immunogenic for pediatric patients with IPD, with an exception for the infecting serotypes [12].

Impact of Childhood Pneumonia and Pneumococcal Conjugate Vaccine Worldwide

Determining the cause of pneumonia in young children is difficult, but nearly all studies undertaken in the developing world have identified *S. pneumoniae* as the most frequent bacterial cause of severe pneumonia [13]. In 2003, the World Health Organization (WHO) estimated that up to 1 million children die each year from pneumococcal disease, primarily pneumococcal pneumonia [14]. Currently, the WHO provisionally estimates that pneumococcal infections are responsible for 1.6 million deaths each year, including approximately 716,000 deaths among children < 5 years of age [15]. Therefore, the health impact of childhood pneumococcal pneumonia appears to be high in developing countries, especially those with high child mortality rates, where > 90% of global childhood pneumonia deaths occur [16].

Several clinical trials of PCV have been conducted in African countries. PCV9 reduced the incidence of IPD caused by vaccine serotype in human immunodeficiency syndrome (HIV)-negative children by 83% and that of radiological pneumonia by 20% [17]. Another study repor-

Table 1. Comparison of serotype-specific IgG concentrations between the time of onset of invasive pneumococcal disease (IPD) and after PCV7 vaccination in 17 children following the resolution of IPD.

serotype	serotype specific IgG concentrations (μg/ml)		P-value
	at the first blood sampling	at the second blood sampling	first vs. second
4	0.46 (0.26–0.81)*	4.08 (3.23–5.16)	< 0.01
6B	0.97 (0.58–1.62)	1.47 (0.82–2.65)	0.266
9V	0.34 (0.19–0.61)	3.97 (2.91–5.42)	< 0.01
14	1.76 (0.92–3.36)	6.30 (3.63–10.94)	< 0.01
18C	0.41 (0.22–0.76)	3.63 (2.69–4.91)	< 0.01
19F	1.23 (0.80–1.89)	3.51 (2.48–4.96)	< 0.01
23F	0.69 (0.40–1.21)	2.66 (1.52–4.67)	< 0.01

*Numbers in parentheses, 95% CI

Table 2. Comparison of serotype-specific opsonization index (OI) between the time of onset of invasive pneumococcal disease (IPD) and after PCV7 vaccination in 17 children following the resolution of IPD.

serotype	serotype specific OI (Log_{10} OI)		P-value
	at the first blood sampling	at the second blood sampling	first vs. second
4	0.63 (0.42–0.96)*	3.54 (3.36–3.70)	< 0.01
6B	0.53 (0.36–0.79)	1.64 (0.94–2.60)	< 0.01
9V	0.80 (0.43–1.46)	3.60 (3.34–3.81)	< 0.01
14	0.78 (0.43–1.38)	3.71 (3.54–3.90)	< 0.01
18C	0.93 (0.57–1.51)	3.53 (3.29–3.69)	< 0.01
19F	0.65 (0.41–1.01)	3.13 (2.85–3.38)	< 0.01
23F	0.56 (0.37–0.85)	3.04 (2.21–4.06)	< 0.01

*Numbers in parentheses, 95% CI

ted that PCV9 efficacy was 37% against first episode of radiological pneumonia [18]. Furthermore, PCV9 reduced the incidence of pneumonia-associated with any of respiratory viruses in children by 31% [19]. This finding also suggests that *S. pneumoniae* plays a major role in the development of pneumonia-associated with respiratory viruses, and viruses contribute to the pathogenesis of bacterial pneumonia. These effects of PCV against childhood pneumonia were found in the clinical trials in African countries, but not in developing countries in Asia.

Based on the accumulated evidences, the impact of PCV vaccination on childhood illness and mortality in the developing countries appears to be much greater than that in industrialized countries. PCV vaccination is expected to prevent about 700 deaths per 100,000 children vaccinated in developing countries, such as Gambia, while in the United States, PCV vaccination is expected to prevent 6 deaths per 100,000 children vaccinated [20]. The authors also demonstrated that analysis of expected health impact of the Global Alliance for Vaccines and Immunization (GAVI) eligible countries illustrated the values of accelerated PCV may prevent 3.7 millions child deaths. According to this idea, the WHO has proposed a strategy to reduce mortality from pneumonia in children less than 5 years of age to fewer than 3 per 1000 births and to reduce the incidence of severe pneumonia by 75% in child less than 5 years of age compared to 2010 levels by 2025 [21].

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Comparison of the immunogenicity and safety of polysaccharide and protein-conjugated pneumococcal vaccines among the elderly aged 80 years or older in Japan: An open-labeled randomized study

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ABSTRACT

An open-labeled randomized study was conducted to compare the immunogenicity and safety of polysaccharide (PPV23) or protein-conjugated pneumococcal vaccine (PCV7) among the elderly aged 80 years or older. A total of 105 nursing home residents were enrolled in this study. We analyzed the geometric mean concentration (GMC) of serotype-specific immunoglobulin G (IgG) and the geometric mean titer (GMT) of the opsonization index (OI) for serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. The GMCs of serotype-specific IgG and the GMTs of the OI significantly increased one month after vaccination in both groups for all seven serotypes evaluated. In the PCV7 group, study subjects with serotypes 4, 9V, 18C, and 23F exhibited statistically significant elevations in both serotype-specific IgGs and OIs compared to those of the PPV23 group. Both vaccines were tolerated without any severe adverse events, and no differences in systemic adverse events were observed between the two groups, although adverse reactions such as redness and localized swelling were more common in the PCV7 group. Our data demonstrated that the GMCs of serotype-specific IgG and the GMTs of the OI were higher in the PCV7 group compared to those in the PPV23 group. Our study also confirmed the safety of both the PCV7 and PPV23 vaccines in elderly people aged 80 years or older.

1. Introduction

Streptococcus pneumoniae infection is a major cause of mortality and morbidity worldwide among the elderly. The 23-valent pneumococcal polysaccharide vaccine (PPV23) is widely recommended for administration to those who are at a high risk of *S. pneumoniae* infection, such as elderly people and splenectomy patients [1]. However, owing to the purified free polysaccharides that comprise its surface capsule, PPV23 does not elicit T cell-dependent immune responses and is a poor inducer of immunologic memory. Furthermore, vaccine-induced antibody titers may achieve insufficient levels and decrease annually, particularly 5 years after vaccination [2].

The conjugation of the capsular polysaccharide to a diphtheria protein stimulates not only B-cell immune response but also T cell-dependent immune responses and enhanced memory response at the time of boosting [3]. Therefore, pneumococcal conjugate vaccines produce superior immune responses, particularly in infants. For this reason, the heptavalent pneumococcal conjugate vaccine (PCV7) was licensed in 2000 in the United States and in 2009 in Japan. PCV7 also produces better immune responses than PPV23 in groups at higher risk of developing invasive pneumococcal diseases

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and pneumococcal pneumonia, such as individuals with HIV [4] or chronic obstructive pulmonary disease [5].

In healthy elderly people 50–80 years old, Goldblatt et al. [6] reported that PCV7 produced superior immunogenicity compared with PPV23. In recent years, the increasing number of elderly people over 80 years old hospitalized for pneumococcal pneumonia has been reported [7]. While pneumococcal vaccination is strongly recommended for this population, no data are currently available for comparison of the immunogenicity and safety between PCV7 and PPV23 for this age group. Therefore, we performed this prospective study to clarify these unknown aspects.

2. Materials and methods

2.1. Study subjects

The present study was a randomized, open-label study designed to compare the immunogenicity and safety of PCV7 (Prevenar; Pfizer) with those of PPV23 (Pneumovax; MSD). Data were collected between April 2011 and December 2012 from participants who were 80 years or older and had never received pneumococcal vaccinations. None of the participants had any documented history of pneumococcal infection. They were selected from five different nursing homes around Tokyo and were randomly assigned to either the PPV23 group or the PCV7 group using the sealed envelope system with a 1:1 allocation ratio. A total of 105 participants were enrolled in this study, and all participants provided written informed consent.

In addition, subjects were excluded if they had a history of any streptococcal vaccination, a history of anaphylactic reaction to diphtheria toxin, or symptoms of fever on the day of vaccination.

We set the sample size on the basis of a study by Goldblatt et al. [6] on the comparison of immunogenicity between PCV7 and PPV23 among adults aged 50–80 years. They assigned 33–60 subjects to a subgroup of one arm and showed higher geometric mean concentrations (GMCs) of serotype-specific IgG response in several serotypes.

This study was reviewed and approved by the Research Ethics Committee of Keio University School of Medicine (2010-231-2) and by the Research Ethics Committee of Kitasato University Kitasato Institute Hospital (1108-02). This trial was registered with the UMIN Clinical Trials Registry (UMIN000006132).

2.2. Vaccines

The PCV7 used in this study is currently licensed only for pediatric use in Japan. PCV7 contains polysaccharides of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, which are conjugated to the protein carrier CRM197, a nontoxic variant of the diphtheria toxin. Each serotype-specific polysaccharide is conjugated separately prior to formulation as a multivalent vaccine. The vaccine contains aluminum phosphate as an adjuvant.

PPV23 contains a mixture of purified capsular polysaccharides from 23 different serotypes of *S. pneumoniae*: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. This vaccine is adjuvant-free.

Each participant received 0.5 mL of either PPV23 or PCV7 via subcutaneous injection. PPV23 and PCV7 were dispensed and administered by members who were not blinded and not involved in subsequent data analysis.

2.3. Samples

Blood samples (10 mL) were drawn from all the subjects on the day of vaccination and approximately one month after vaccination. Sera were separated by centrifugation (3500 rpm, 15 min, 4 °C) and stored at −80 °C.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Anti-pneumococcal immunoglobulin G (IgG) antibodies were measured by World Health Organization (WHO)-approved ELISA, using standard reference serum (89-SF or 007sp) and C-polysaccharide and 22F polysaccharide absorption, as previously reported [8,9]. The levels of serotype-specific IgGs for seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) were determined in both vaccination groups according to the WHO protocol (a detailed version of the protocol is available at http://www.vaccine.uab.edu/ELISAProtocol(89SF).pdf). These serotypes are covered by PCV7.

2.5. Multiplexed opsonophagocytic killing assay

A multiplexed opsonophagocytic killing assay for seven serotypes, 4, 6B, 9V, 14, 18C, 19F, and 23F, based on antibiotic-resistant strain target bacteria, was performed at the Research Institute for Microbial Diseases, Osaka University, as previously described [10]. The quality control serum used in each assay was prepared from the pooled sera of adults vaccinated with PPV23 or PCV7. The opsonization index (OI) was defined as the serum dilution capable of killing 50% of the bacteria, which was determined by using opsotiter3 software according to the WHO protocol (a detailed version of this protocol is available at www.vaccine.uab.edu/UAB-MOPA.pdf) [11]. Laboratory analysis, ELISA, and a multiplexed opsonophagocytic killing assay were performed by members who were blinded to vaccine allocation.

2.6. Adverse reactions

All patients were observed daily by medical staff to monitor body temperature and any local or systemic reactions, starting from the day of vaccination to day 7. Injections were graded based on the occurrence of several possible adverse events as follows: grade I (the reaction was present but easily tolerated), grade II (the reaction interfered with normal activity), and grade III (the reaction was severe or incapacitating).

2.7. Statistical analysis

Average antibody concentrations and the increases from baseline were expressed as geometric means. Differences in the GMCs of serotype-specific IgG and the geometric mean titers (GMTs) of the OI were assessed by the Wilcoxon matched-pairs signed-ranks test. For multiple comparisons, we calculated Bonferroni-adjusted P values. The frequencies of adverse reactions were compared between vaccinations by the Fisher exact test. Differences with $P < 0.05$ were considered to be statistically significant. Data analysis was performed by members who were blinded to vaccine allocation.

3. Results

3.1. Participant characteristics

Overall, 623 eligible participants were reviewed in the 5 nursing homes (Fig. 1). One hundred and five participants were enrolled in this study after they provided written informed consent. Five subjects were subsequently dropped from the study prior to vaccination (2 subjects were hospitalized, 2 subjects left the nursing home, and 1 subject died). Consequently, 100 subjects were vaccinated (Table 1); of these, 49 received PPV23 and 51 received PCV7. The mean ages at enrollment were 88.3 years for the PPV23 group and 87.7 years for the PCV7 group, with 45 subjects in their

Table 1
Participants characteristics.

Characteristics	PPV23[a] ($n=49$)	PCV7[b] ($n=51$)	P value
Age, years	88.3 ± 1.4	87.7 ± 1.5	0.29
Male, %	12 (24.5)	11 (21.6)	
Female, %	37 (75.5)	40 (78.4)	
Height, cm	145.1 ± 3.9	146.5 ± 2.2	0.24
Weight, kg	45.9 ± 2.4	46.0 ± 2.1	0.47
Hypertension, %	34 (69.4)	32 (62.7)	
Diabetes mellitus, %	15 (30.6)	18 (35.2)	
Old cerebral infarction, %	17 (34.7)	14 (27.5)	
Dementia, %	15 (30.6)	13 (25.5)	
Dyslipidemia, %	14 (28.6)	12 (23.5)	
Neck of femur fracture, %	12 (24.5)	13 (25.5)	
Congestive heart failure, %	12 (24.5)	10 (19.6)	
Vertebral compression fracture, %	11 (22.4)	9 (17.6)	
Cataract, %	6 (12.2)	5 (9.8)	
Chronic obstructive pulmonary disease, %	6 (12.2)	3 (5.9)	
Old myocardial infarction, %	5 (10.2)	5 (9.8)	
Malignancy, %	4 (8.1)	5 (9.8)	
Benign prostatic hyperplasia, %	5 (10.2)	3 (5.9)	
White blood cells, counts/μl	5796 ± 398	6101 ± 431	0.15
Hemoglobin, g/dl	11.8 ± 0.4	12.2 ± 0.4	0.09
Platelets, $\times 10^4$ counts/μl	23.6 ± 2.2	22.8 ± 2.0	0.28
Albumin, g/dl	3.7 ± 0.1	3.7 ± 0.1	0.35
AST, IU/l	20.7 ± 2.0	21.1 ± 2.9	0.40
ALT, IU/l	12.9 ± 2.0	13.5 ± 2.5	0.35
BUN, mg/dl	17.6 ± 1.0	16.5 ± 1.3	0.10
Creatinine, mg/dl	0.68 ± 0.5	0.70 ± 0.5	0.25

Data are presented as mean ± SD (standard deviation) unless otherwise indicated.
[a] 23-valent pneumococcal polysaccharide vaccine.
[b] 7-valent pneumococcal conjugate vaccine.

90s and 3 subjects who were 101 years old. The majority (77%) of the subjects were female. There were no significant differences in major co-morbidities between the PPV23 group and the PCV7 group. No other significant differences in laboratory data were observed between the two groups. All the participants from both groups received routine immunization against seasonal influenza.

3.2. Immunogenicity: levels of serotype-specific IgG

Data for the GMCs of serotype-specific IgG responses before and one month after vaccination with PPV23 or PCV7 are summarized in Table 2 and presented graphically in Fig. 2. The original data on serotype-specific IgG are also shown in Supplementary Table 1. No significant differences of baseline serotype-specific IgG GMCs were observed between the two groups for all serotypes measured. In both groups, significant increases in IgG GMCs were observed from baseline to one month following the initial dose for all seven serotypes evaluated. The GMCs of serotype-specific IgGs for serotypes 4, 9V, 18C, and 23F of the study subjects were significantly more elevated in the PCV7 group than in the PPV23 group.

3.3. Immunogenicity: OI

Data for the GMTs of serotype-specific OIs before and one month after vaccination with PPV23 or PCV7 are summarized in Table 3 and presented graphically in Fig. 3. The original data on serotype-specific OIs are also shown in Supplementary Table 2. No significant differences in the baseline GMTs of serotype-specific OIs were observed between the two groups for all serotypes measured. In both groups, significant increases in the GMTs of OIs were observed from baseline to one month following the initial dose for all seven serotypes evaluated. The GMTs of serotype-specific OIs for serotypes 4, 9V, 18C, and 23F of the study subjects were significantly elevated in the PCV7 group compared to the PPV23 group.

3.4. Safety

Both vaccines were tolerated without any severe adverse events. No differences were observed in systemic side effects between the two groups; however, local side effects such as redness and localized swelling were more commonly observed in the PCV7 group (Table 4). No participants required unscheduled medical examinations within the first 7 days after vaccination.

4. Discussion

The current study is the first to demonstrate pneumococcal vaccine responses in pneumococcus vaccine-naïve elderly people (at or over 80 years of age) by evaluating serotype-specific IgG antibodies and serotype-specific OIs between PPV23 and PCV7. Our major findings are that both PPV23 and PCV7 elicited increases in IgG and OI, and that PCV7 is more potent than PPV23 in terms of its immunogenicity against four out of seven serotypes included in PCV7. We also demonstrated the safety of these preparations in these elderly individuals, with no serious adverse effects observed in either group.

We believe that there are several important strengths of this study. One of them is that not only serotype-specific IgG levels but also serotype-specific OIs were evaluated. Due to technical difficulties with OI assays, OI measurements have been reported in only a limited number of clinical studies to date. However, we were able to evaluate functional antibodies, which are superior surrogate markers for protection against pneumococcal pneumonia and bacteremia, by utilizing the latest generation of ELISA methodology [12].

Another important strength of this study is the age distribution of the participants, considering the current inevitable tendency toward increasing longevity in humans. Since the host response induced by vaccinations varies depending on the age of the recipient, the development of safe and effective vaccinations for the elderly is clinically important.

In our study, antibodies against serotypes 4, 9V, 18C, and 23F were significantly elevated in the study subjects. These data were consistent with a previous study indicating that serotype-specific IgG levels of 4, 6B, 9V, 14, 18C, and 23F, and serotype-specific OIs of 4, 9V, 14, 18C, and 23F were significantly elevated in the PCV7 group consisting of elderly people more than 70 years old [2]. In addition, in accordance with our data, they reported that serotype 6B and 19F did not show superior immunogenicity compared with other serotypes in elderly people.

In several studies, 1.0-mL doses of PCV7 were administered [5,13]. However, we used half this dosage in our clinical study to minimize potential adverse effects. In fact, both PPV23 and PCV7 were tolerated by the participants and were associated with few local reactions or systemic adverse effects. No severe adverse effects were observed in either group. A higher frequency of local reactions was observed in the PCV7 group compared with the PPV23 group, although we were unable to determine if this increase was caused by the conjugation specifically. According to a dose-range study of pneumococcal conjugate vaccine reported by Lode et al. [14], both serotype-specific IgG and OI displayed increases in dose-dependent manners, although local reactions for the double dose were not statistically higher than for the single dose. Based on this notion, in our study, 1.0-mL injections rather than

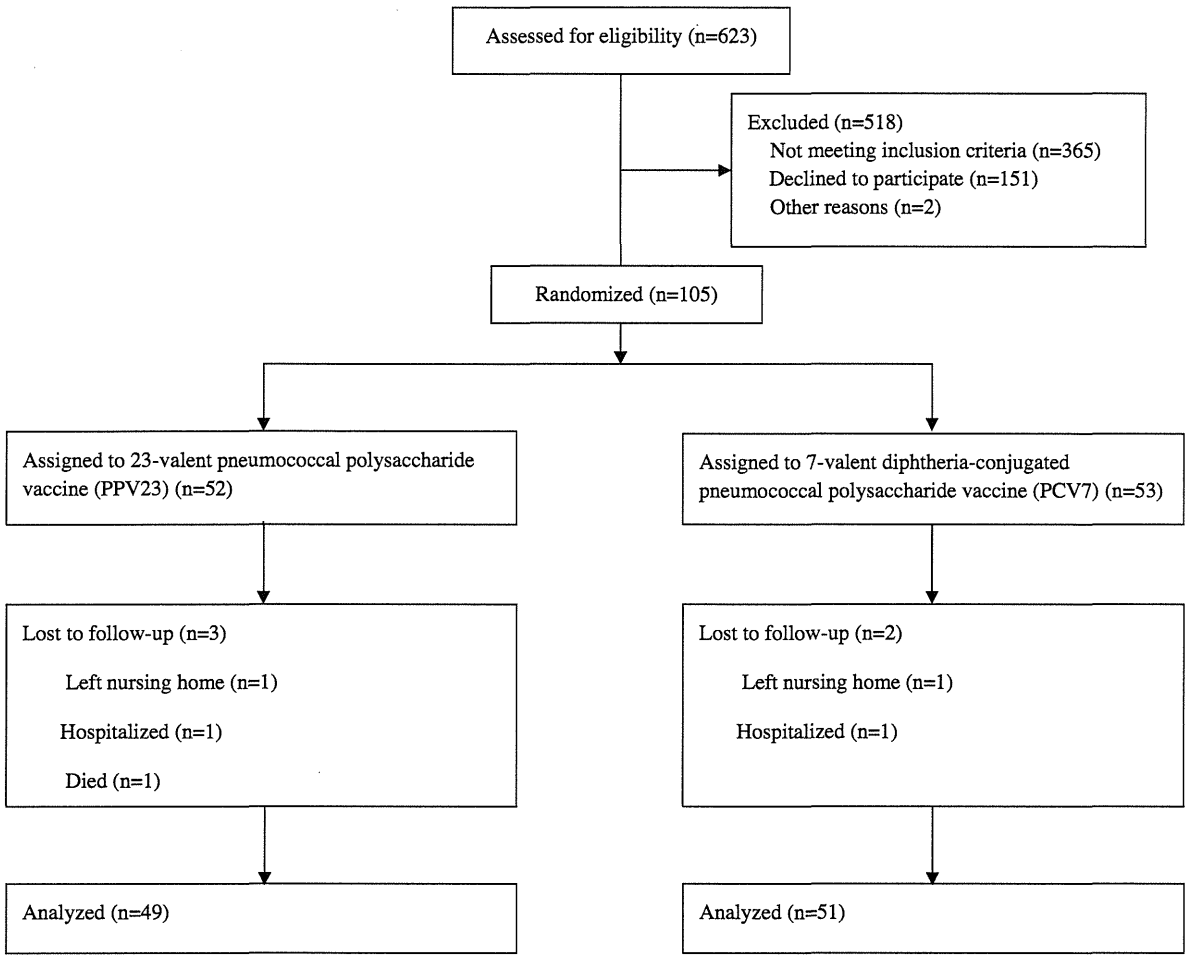

Fig. 1. Flow diagram of trial.

0.5-mL injections of PCV7 could have safely obtained more potent immunogenicity.

The mean number of co-morbidities of study participants staying at nursing homes was 3.34 in our study. According to the nationwide epidemiological study in Scotland by Barnett K et al., the mean number of co-morbidities is 2.60 in elderly people aged 65–84 years and 3.62 in elderly people aged 85 and over [15]. The cross sectional study of aged Medicare beneficiaries in the United States shows that the mean number of co-morbidities is 2.71 in elderly people aged 80 and over [16]. Considering these previous data, our study population of nursing homes could be regarded as not an unusual population of the elderly people in the developed countries.

This study has several limitations. Firstly, a vaccine type and an injection route have to be considered. In this study, we could not use PCV13 because of the lack of the license in Japan at the time of the current study, while PCV13 was launched in Japan in 2014. Therefore our study is out-of-date data at the present. Although PCV is usually administered intramuscularly, not subcutaneously, an intramuscular injection of PCV7 was not allowed in Japan at the time of our clinical study. In order to minimize injection dependent bias, we administered PCV7 subcutaneously by following that the most common route of PPV23 is the subcutaneous route in Japan. Even though, we should have administered both PPV23 and PCV7 intramuscularly.

Table 2
The geometric mean concentrations of serotype-specific IgG antibody before and one month after vaccination pneumococcal vaccines.

Serotype	Pre IgG μg/ml (95% CI)		Post IgG μg/ml (95% CI)		Bonferroni-adjusted *P* value
	PPV23[a] (*n* = 49)	PCV7[b] (*n* = 51)	PPV23 (*n* = 49)	PCV7 (*n* = 51)	
4*	0.44 (0.35–0.55)	0.52 (0.42–0.66)	1.02 (0.77–1.34)	3.38 (2.32–4.92)*	>0.001
6B	1.22 (1.00–1.64)	1.11 (0.84–1.39)	3.51 (2.66–5.30)	3.32 (2.08–4.84)	6.205
9V*	1.03 (0.81–1.38)	0.92 (0.70–1.18)	4.01 (3.12–5.66)	8.75 (5.80–12.14)*	0.003
14	1.88 (1.44–2.85)	2.26 (1.61–3.22)	7.66 (5.00–14.02)	11.41 (7.57–18.26)	3.723
18C*	1.12 (0.89–1.56)	1.08 (0.80–1.39)	4.93 (3.53–6.76)	10.02 (6.98–14.39)*	0.043
19F	1.69 (1.38–2.15)	2.24 (1.72–2.82)	5.26 (3.65–7.30)	6.10 (4.08–8.45)	2.467
23F*	1.28 (0.95–1.81)	1.31 (0.95–1.80)	5.39 (3.51–8.98)	14.68 (9.75–22.04)*	0.014

[a] 23-valent pneumococcal polysaccharide vaccine.
[b] 7-valent pneumococcal conjugate vaccine.
* A significant difference in absolute postvaccination IgG levels between vaccine groups.
Within each study group, postvaccination antibody levels were higher than baseline ($P < 0.01$) for all serotypes.

Table 3
The geometric mean titers of serotype-specific opsonization index before and one month after vaccination pneumococcal vaccines.

Serotype	Pre OI[a] (95% CI)		Post OI (95% CI)		Bonferroni-adjusted *P* value
	PPV23[b] (*n* = 49)	PCV7[c] (*n* = 51)	PPV23 (*n* = 49)	PCV7 (*n* = 51)	
4*	3.55 (2.55–5.22)	5.77 (3.53–9.44)	45.84 (25.55–104.83)	710.65* (307.45–1642.62)*	0.005
6B	17.97 (10.58–38.69)	23.34 (12.10–40.88)	271.51 (123.06–586.19)	700.27 (327.87–1188.63)	2.227
9V*	24.44 (13.77–49.77)	19.34 (9.97–34.30)	234.47 (138.37–478.25)	958.78* (559.49–1680.79)*	0.012
14	44.83 (24.67–101.30)	90.57 (38.43–183.84)	588.67 (262.75–1380.93)	1925.23 (1144.17–3430.09)	2.259
18C*	47.67 (25.48–89.65)	39.13 (19.53–69.75)	708.20 (329.96–1295.19)	2730.37* (1805.42–4118.33)*	0.016
19F	18.26 (10.23–32.94)	25.94 (13.43–45.32)	352.42 (163.69–628.10)	414.32 (196.85–707.47)	3.572
23F*	19.00 (10.50–35.76)	14.51 (7.30–26.69)	197.51 (80.78–466.58)	2076.51* (1129.01–3937.53)*	>0.001

[a] Opsonization index.
[b] 23-valent pneumococcal polysaccharide vaccine.
[c] 7-valent pneumococcal conjugate vaccine.
* Bolded items represent a significant difference in absolute postvaccination OI levels between vaccine groups.
Within each study group, postvaccination OI were higher than baseline ($P < 0.01$) for all serotypes.

Another limitation is that it was only one month after vaccination that the antibody levels were examined, thereby limiting our knowledge regarding long-term effects. Our recent study suggested sustained levels of serotype-specific IgG and OI after primary and secondary vaccination with PPV23 among elderly individuals with chronic lung diseases [17]. We therefore intend to compare the serotype-specific IgG and OI after primary vaccination between the study subjects immunized with PPV23 and PCV7 in this study.

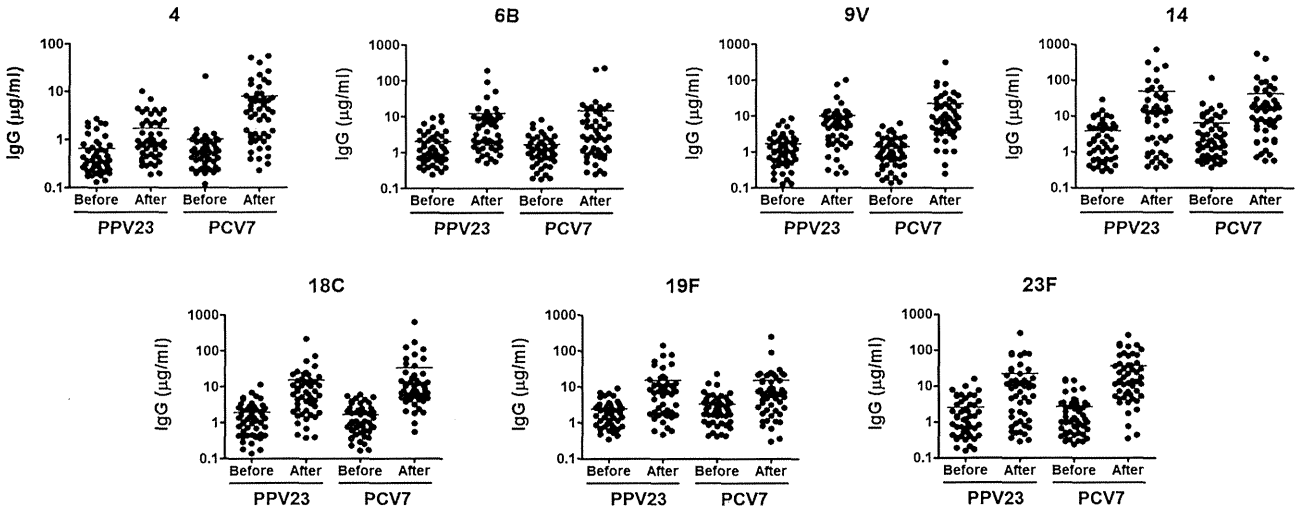

Fig. 2. The serotype-specific baseline and 1-month absolute IgG antibody levels are shown for each patient. The heptavalent diphtheria-conjugated pneumococcal polysaccharide vaccine (PCV7) resulted in statistically significantly higher antibody levels at one month to baseline for serotypes 4, 9V, 18C and 23F.

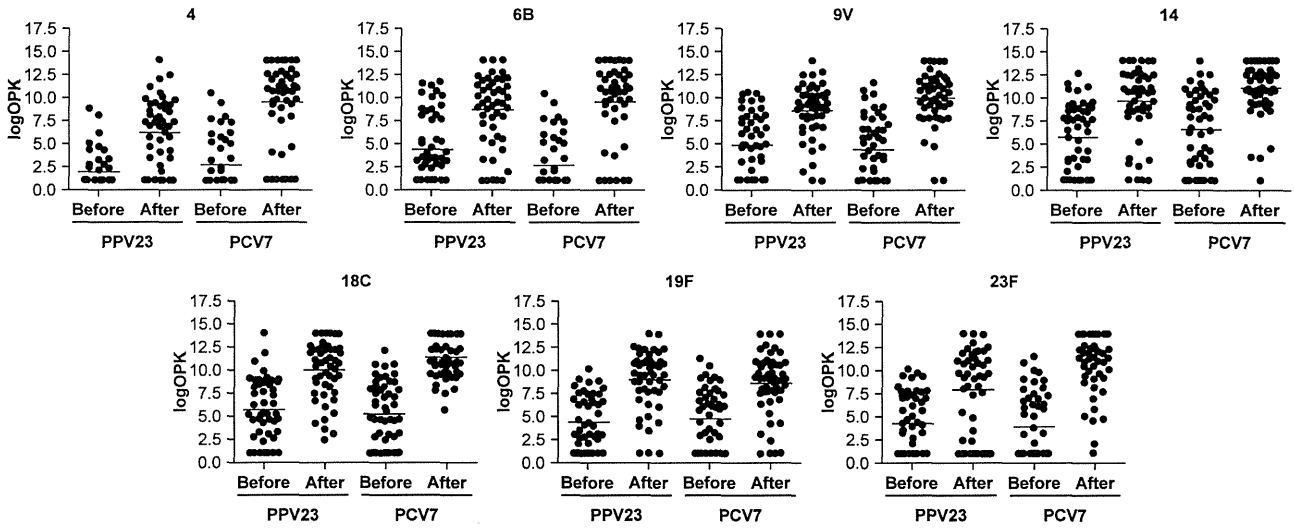

Fig. 3. The serotype-specific baseline and one-month geometric mean opsonophagocytic killing index are shown for each patient. The heptavalent diphtheria-conjugated pneumococcal polysaccharide vaccine (PCV7) resulted in statistically significantly higher geometric mean opsonophagocytic killing index at one month to baseline for serotypes 4, 9V, 18C and 23F.

Table 4
Comparison of adverse reactions among elderly individuals after vaccination with pneumococcal vaccines.

Characteristics	PPV23[a] (n=49)	PCV7[b] (n=51)	P value
Fatigue			
Grade I	3	3	0.96
Grade II	0	0	-
Muscle aches			
Grade I	0	0	-
Grade II	0	0	-
Headache			
Grade I	0	0	-
Grade II	0	0	-
Itching of vaccinated arm			
Grade I	2	4	0.43
Grade II	0	0	-
Pain of vaccinated arm			
Grade I	0	0	-
Grade II	0	0	-
Fever			
Grade I	4	3	0.65
Grade II	0	0	-
Redness			
Grade I (<8 cm)	9	16	0.13
Grade II (>8 cm and <15 cm)	3	5	0.50
Grade III (>15 cm)	0	0	-
Localized swelling			
Grade I (<8 cm)	11	19	0.11
Grade II (>8 cm and <15 cm)	0	0	-

[a] 23-valent pneumococcal polysaccharide vaccine.
[b] 7-valent pneumococcal conjugate vaccine.

There are several unsolved issues for pneumococcal vaccination. The titer of correlate of protection for adults who received pneumococcal vaccines has not yet been established, while a titer of 0.35 μg/mL has been defined as a correlate of protection against invasive diseases among infants who received the pneumococcal conjugate vaccine. In this respect, as for adults, the advantage of the higher immunogenicity in the PCV7 group is not clear in protection against pneumococcal diseases. Moreover, the difference of the serotypes covering range by each pneumococcal vaccine has to be taken into consideration. Based on the newest domestic reports on the serotype distribution of community-acquired pneumonia (CAP) [18] and invasive pneumococcal disease (IPD) [19], the ratio of serotypes of CAP covered by PPV23, PCV7 and PCV13 are 82.5%, 61.4% and 83.3% while the ratio of serotypes of IPD covered by PPV23, PCV7 and PCV13 are 85.4%, 39.8% and 61.9%, respectively. Taken together, to make best of our current study, the nationwide surveillance of *S. pneumoniae* infections is essential in Japan. Beyond the scope of this current study, the most important aspect is to establish the vaccine policy which produce clinical efficacy for preventing *S. pneumoniae* infections.

In conclusion, we demonstrated higher increases in the GMCs of serotype-specific IgG levels and the GMTs of OIs in the PCV7 group compared to the PPV23 group, and confirmed the safety of vaccinations with PCV7 and PPV23 for subjects aged 80 years and older.

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Conflict of interest: Dr. Hasegawa has received grants from MSD and Pfizer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.11.023.

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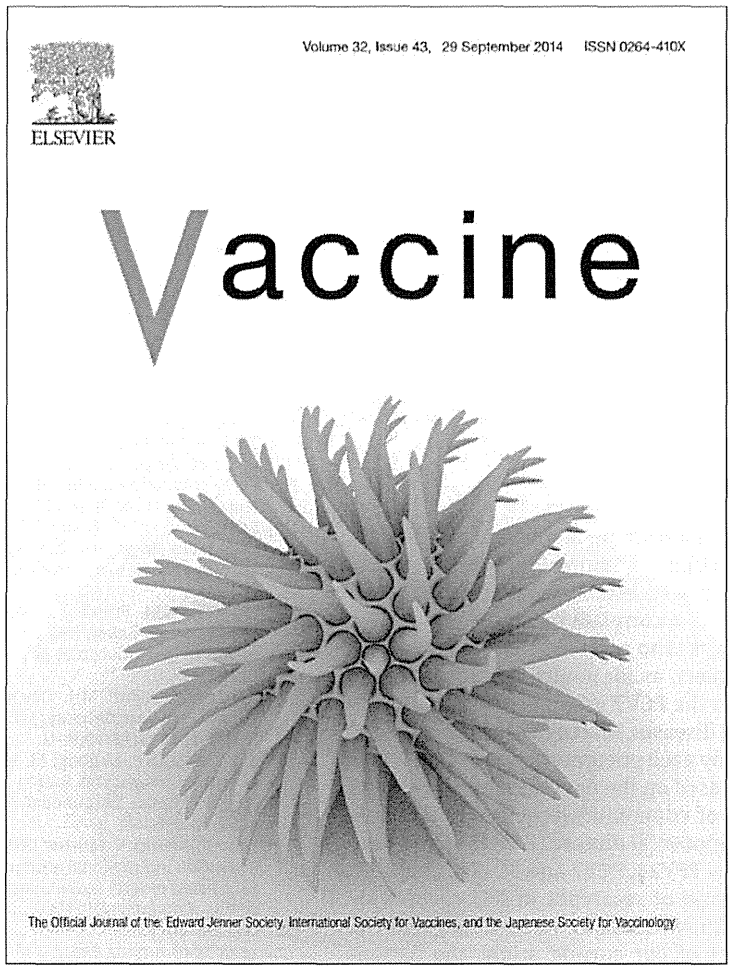

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Protective properties of a fusion pneumococcal surface protein A (PspA) vaccine against pneumococcal challenge by five different PspA clades in mice

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ABSTRACT

An increase in the appearance of nonvaccine serotypes in both children and adults with invasive pneumococcal disease (IPD) after introduction of pneumococcal conjugate vaccine represents a limitation of this vaccine. In this study, we generated three recombinant pneumococcal surface protein A (PspA) proteins comprising PspA families 1 and 2, and we examined the reactivity of antisera raised in mice immunized with a PspA fusion protein in combination with CpG oligonucleotides plus aluminum hydroxide gel. The protective effects of immunization with PspA fusion proteins against pneumococcal challenge by strains with five different PspA clades were also examined in mice. Flow cytometry demonstrated that PspA3+2-induced antiserum showed the greatest binding of PspA-specific IgG to all five challenge strains with different clades. PspA2+4- or PspA2+5-induced antiserum showed the lowest binding of PspA-specific IgG to clade 3. Immunization with PspA3+2 afforded significant protection against pneumococcal challenge by five strains with different clades in mice, but immunization with PspA2+4 or PspA2+5 failed to protect mice from pneumococcal challenge by strains with clades 1 and 3. The binding of PspA-specific IgG in antisera raised by three PspA fusion proteins was examined in 68 clinical isolates from adult patients with IPD. Immunization of mice with PspA3+2-induced antiserum with a high binding capacity for clinical isolates expressing clades 1–4, but not clade 5. Our results suggest that the PspA3+2 vaccine has an advantage over the PspA2+4 or PspA2+5 vaccine in terms of a broad range of cross-reactivity with clinical isolates and cross-protection against pneumococcal challenge in mice.

1. Introduction

Streptococcus pneumoniae is a major cause of morbidity and mortality caused by pneumonia, bacteremia, and meningitis worldwide [1]. After introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) in children, significant declines in the incidence of invasive pneumococcal disease (IPD) caused by vaccine serotypes were reported in children and adults [2,3]. However, an increase in the incidence of IPD caused by non-PCV7 serotypes has been also observed in children and adults [3–5]. In addition, after introduction of a 13-valent pneumococcal conjugate vaccine (PCV13) in children, serotypes not included in PCV13 have been isolated with increasing frequency in pediatric and adult patients with IPD [6,7]. Because there are >90 different pneumococcal capsular serotypes, continuous supplementation of pneumococcal conjugate vaccines with new serotypes for serotype replacement may not be a practical strategy.

Previous studies have demonstrated that several pneumococcal proteins are potential vaccine candidates [8–11]. One candidate protein antigen is pneumococcal surface protein A (PspA), which is

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an exposed virulence factor found in virtually all pneumococcal strains [12,13]. Anti-PspA antibodies overcome the anticomplement effect of PspA, allowing for increased complement activation and C3 deposition on PspA-bearing bacteria [14,15]. Serum from humans immunized with PspA can passively protect mice against challenge with various pneumococcal strains [16]. Importantly, a recent study confirmed that the rabbit antibodies to PspA could mediate killing in the modified opsonophagocytosis killing assay [17].

PspA is composed of five domains: (i) a signal peptide, (ii) an α-helical highly charged (N-terminal) domain, (iii) a proline-rich region domain, (iv) a choline-binding domain, and (v) a short hydrophobic tail [18,19]. The α-helical domain of PspA has an antiparallel coiled-coil motif and is considered to be the most exposed part of the molecule [20]. The α-helical domain binds to protective monoclonal antibodies and inhibits killing of pneumococci by at least two host cationic peptides [21,22]. The proline-rich domain is composed of many repetitive sequences shared by other proline-rich domains making its inclusion important for achieving broad protection [23].

PspA proteins have been grouped into three families encompassing six different clades based on the C-terminal 100 amino acids of the α-helical region [24]. Family 1 comprises clades 1 and 2; family 2 comprises clades 3, 4 and 5; and family 3 comprises clade 6 [22,24]. Pneumococcal strains expressing family 1 or 2 PspA proteins constitute >96% of clinical isolates from patients with IPD [6,13,25]. Although different PspA proteins induce antibodies with different degrees of cross-reactivity in vitro and cross-protection of mice [26,27], our previous studies demonstrated that no single PspA construct can elicit complete protection against challenge by strains with all PspA clades and families [28]. To accommodate this variability, it was proposed that a combination of two PspA antigens, one from PspA family 1 and one from PspA family 2, should elicit protection against the vast majority of pneumococcal strains [29–31]. Thus, it is important to determine which PspA fragments show the broadest cross-reactivity. In this study, we prepared fusion proteins of three pairs of PspA molecules, and determined which provided the broadest cross-reaction with clinical isolates of *S. pneumoniae*.

2. Materials and methods

2.1. Pneumococcal strains

Six laboratory strains (all originally from patients), including BG9739 (serotype 4, PspA clade 1), D39 (serotype 2, PspA clade 2), WU2 (serotype 3, PspA clade 2), TIGR4 (serotype 3, PspA clade 3), EF5668 (serotype 4, PspA clade 4), and ATCC 6303 (serotype 3, PspA clade 5) were used to construct the fusion PspA proteins. These laboratory strains and a recent clinical isolate, KK1162 (serotype 3, PspA clade 4), were used for bacterial challenge. Sixty-eight clinical isolates, including KK1162 strain, from Japanese adult patients with IPD were also used [32]. These isolates were serotyped using agglutination assay, and their PspA clades were determined using a method published previously [32,33].

2.2. Construction of fusion PspA fragments

Our previous study demonstrated a significant protection against sepsis caused by WU2 strain (PspA clade 1) by immunization with full-length BG9739 derived PspA (clade 1) but only a weak protection against homologous challenge with BG9739 [28]. Therefore, we excluded PspA clade 1 derived from BG9739 strain from the fusion PspA proteins. In this study, we prepared the fusion proteins from three pairs of PspA clade 2 from family 1 and PspA clades 3, 4 and 5 from family 2. All cloning procedures were performed with *Escherichia coli* DH5α grown in Luria–Bertani medium (Sigma-Aldrich, St. Louis, MO) supplemented with kanamycin (30 μg/ml). DNA fragments encoding portions of the N-terminal regions (containing the α-helix domain and proline-rich region) of PspA clades 2 and 3 were amplified by PCR using strains D39 and TIGR4. The primers used in this procedure are available in Appendix 1. The resulting PCR products were digested with *NdeI* and *EcoRI*, and were ligated to the pET28a (+) vector (Novagen, Madison, WI), and the sequences were confirmed by DNA sequencing. The pET28a–PspA constructs digested with *EcoRI* and *XhoI*, and the resulting fragments, which encoded portions of the N-terminal regions of PspA clades 4, 5, or 2 were amplified by PCR using strains EF5668 (Accession no. U89711), ATCC6303 (Accession no. AF071820), or WU2 (Accession no. AF071814), respectively, and were ligated to the linearized vector. The fusion PspA proteins were obtained with primers that allowed the removal of the signal sequence. The fusion PspA2+4 was constructed by fusing the 3′ terminus of PspA clade 2 of D39 strain (Accession no. AF071814) with the 5′ terminus of PspA clade 4 of EF5668 strain, through the *Eco*RI ligated to pET28a–6 × His. The fusion PspA2+5 was constructed by fusing the 3′ terminus of PspA clade 2 of D39 strain with the 5′ terminus of PspA clade 5 of ATCC6303 strain, through the *Eco*RI ligated to pET28a–6 × His. The fusion PspA3+2 was constructed by fusing the 3′ terminus of PspA clade 3 of TIGR4 strain (Accession no. AE005672.3) with the 5′ terminus of PspA clade 2 of WU2 strain, through the *Eco*RI ligated to pET28a–6 × His.

2.3. PspA expression and purification

Competent *E. coli* BL21 (DE3) cells were transformed with pET28a (+) vectors containing the fusion PspA or the single PspA constructs. The recombinant proteins were purified and stored as described elsewhere [34].

2.4. Immunization of mice

Female C57/BL6j mice (6–8 weeks old) were purchased from CLA Japan. Mice were immunized subcutaneously three times at 7-days intervals with 0.1 μg of recombinant fusion PspA derivatives in lipopolysaccharide-free phosphate-buffered saline (PBS) (Sigma) in combination with 2.5 μg of TLR9 ligand adjuvants K3 CpG oligonucleotides (CpG ODNs) and 5 μg of aluminum hydroxide gel (AHG) (A gift from The Research Foundation for Microbial Diseases of Osaka University) or CpG ODNs alone (final volume of 200 μl per mouse). A subcutaneous route of immunization was chosen because our preliminary study demonstrated the levels of PspA-specific IgG in mice subcutaneously immunized with 0.1 μg of PspA plus 2.5 μg of CpG ODNs were significantly higher than those in mice nasally immunized with 0.1 μg of PspA plus 2.5 μg of CpG ODNs (data not shown). CpG ODNs were prepared as described previously [35]. Because the PspA clade-specific IgG levels tended to be higher in mice immunized with each PspA fusion protein with CpG ODNs plus AHG than in those immunized with PspA fusion protein with CpG ODNs alone (see Appendix 2), we used the CpG ODNs plus AHG (define as the double adjuvants), for the immunization of mice with PspA fusion proteins in this study. These double adjuvants were safe in nonhuman primate models, and were applicable to humans [36]. Serum was collected from mice by retro-orbital bleeding 1 week after the third immunization. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, Japan (Permit Number: Biken-AP-H23-05-0).

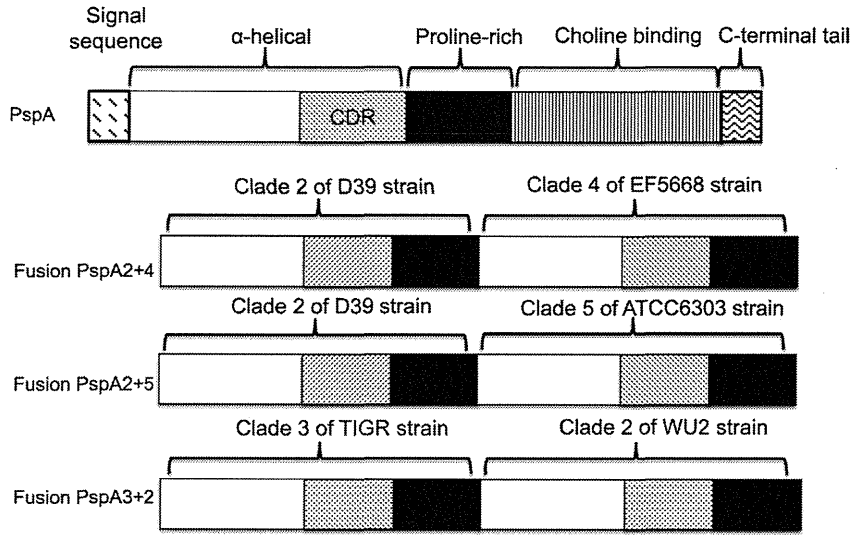

Fig. 1. Schematic diagram of PspA and three fusion PspA proteins. The entire PspA molecule containing the N-terminal α-helical domain, which contains the clade-defining region (CDR), the proline-rich region, the choline-binding domain, and the C-terminal tail (upper column). Each recombinant fusion protein is shown with its different composition (three lower rows).

2.5. Binding of PspA-specific IgG to pneumococcal strains by flow cytometry

Five pneumococcal strains for bacterial challenge and 68 clinical isolates were grown in blood agar plates overnight and then subcultured again on blood agar plates for 4–5 h. The bacteria were collected in PBS, harvested by centrifugation, and washed once with PBS. Ninety microliters of the bacterial suspension at a concentration of 1×10^8 colony-forming units (cfu)/ml in PBS was incubated with 10 μl of mouse antisera for 30 min at 37 °C. After incubation, the suspension was washed once with PBS, resuspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100), and incubated for 30 min on ice. After the incubation, the bacterial suspension was washed twice with PBS and suspended in 500 μl of 1% formaldehyde. The samples were kept on ice in the dark until analyzed by flow cytometry using a BD FACSCalibur™ with CellQuest software (BD Sciences, San Jose, CA), and the percentage of fluorescent bacteria (>1 fluorescence intensity unit) in each group was determined. Sera from mice immunized with double adjuvants only were used as the negative controls.

2.6. Protection against pneumococcal challenge

The mice immunized with the PspA fusion protein plus double adjuvants were challenged intranasally with 2×10^7 cfu of strain BG9739 (clade 1), 2×10^7 cfu of strain WU2 (clade 2), 5×10^6 cfu of strain TIGR4, 2×10^7 cfu of strain KK1162 (clade 4), or 5×10^5 cfu of strain ATCC6303 (clade 5). Bacterial challenges were performed 2 weeks after the final immunization. Mortality was monitored for 2 weeks following pneumococcal challenge. The mice immunized with double adjuvants alone were used as a control.

2.7. Statistical analysis

Analysis of variance followed by an unpaired Mann–Whitney *U* test was used to evaluate differences in antibody titer. The percent binding by immune sera to each pneumococcal strain was compared by paired *t*-test. Survival rates were analyzed by the Kaplan–Meier log-rank test. All analyses were performed using GraphPad Prism Software (GraphPad software, La Jolla, CA). *p* values <0.05 were considered significant.

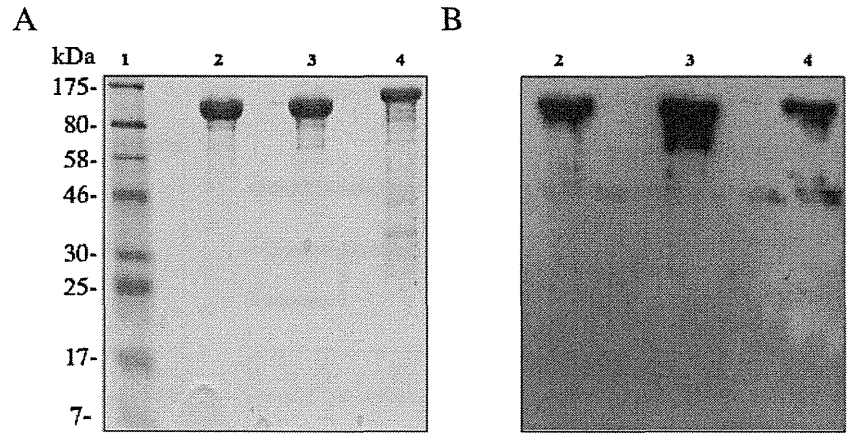

Fig. 2. Characterization of three purified fusion PspA proteins by SDS–PAGE (A) and Western blot analysis (B). The proteins were subjected to SDS–PAGE and detected by direct staining with Coomassie brilliant blue. Lane 1, standard molecular weight markers; lane 2, PspA2+4; lane 3, PspA2+5; 4, lane PspA3+2. The values on the left are molecular sizes in kilodaltons. Mouse antiserum against PspA recombinant protein (clade 2) was used for Western blot analysis. Lane 2, PspA2+4; lane 3, PspA2+5; lane 4, PspA3+2.