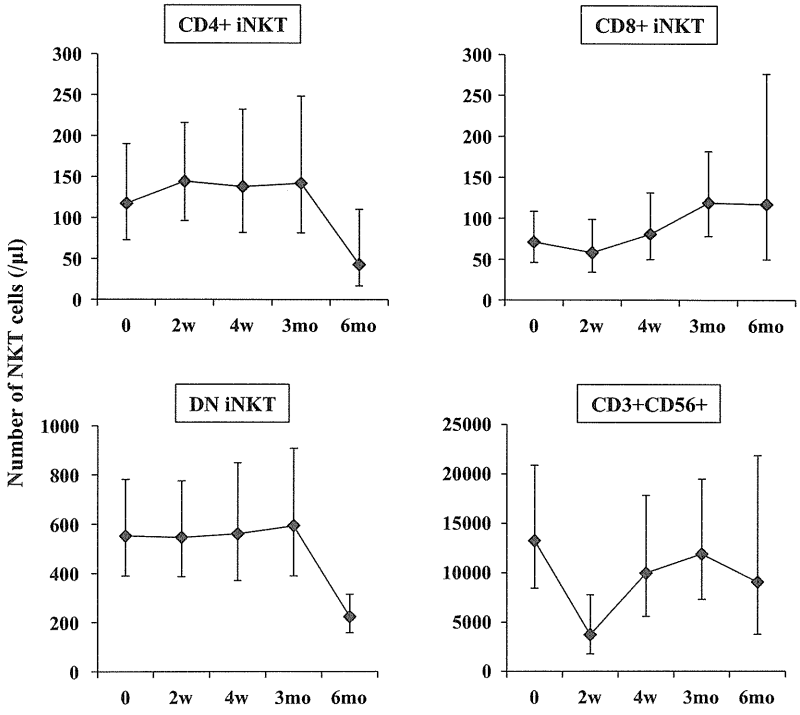

Fig. 2. NKT cells in the peripheral blood after PPV injection. Number of NKT cells in the peripheral blood was examined before PPV administration and 2 weeks, 4 weeks, 3 months and 6 months after PPV administration in 24 individuals. NKT cells were identified as the lymphocytes positively stained with α-GalCer-CD1d tetramer or expressing both CD3 and CD56, and α-GalCer-CD1d tetramer$^+$ lymphocytes were further divided into $CD4^+CD8^-$ ($CD4^+$ iNKT), $CD4^-CD8^+$ ($CD8^+$ iNKT) and $CD4^-CD8^-$ (double negative: DN iNKT) subsets. Data are shown as the geometric means and 95% confidence intervals in each NKT cell subset.

to be in accordance with those of previous investigations, which indicate that 16–31% of vaccinated subjects are low responders, whose anti-pneumococcal Ab levels increase less than two-fold for two among four to seven analyzed serotypes [27–29].

Previous studies have shown NKT cells to be involved in immune responses to TI-2 antigens, as a possible source of the secondary stimulatory signal for B cell activation [25] as well as in protection against pneumococcal infection [24]. These earlier observations suggest that NKT cells may play a certain role in the clinical effects of anti-pneumococcal vaccination. In agreement with this possibility, in the present study, a significant positive correlation was detected between changes in the number of DN iNKT cells, though not of $CD4^+$ iNKT cells, and increases in Ab levels against serotype 14 antigen. Moreover, the increase in DN iNKT cells was more marked in responders than in low responders, and this difference was statistically significant for serotype 19F. However, the positive correlation between DN iNKT cells and Ab levels and the difference in DN iNKT cells between responders and low responders were not significantly detected in other serotypes, although there were such tendencies with lower p values. The increase of study subjects would help in making these differences statistically significant. In addition, there is a possibility that the increase of DN iNKT cell number in responders may be due to overall immune activation of these individuals in response to vaccine, rather than selective effect on NKT cells. This may not apply to our case, because there was no tendency of difference between low responders and responders in other NKT cell subsets (data not shown).

$CD4^+$ and DN iNKT cells are major subsets in humans, both of which secrete large amounts of IFN-γ upon stimulation [21]. Yet these subsets differ in their secretion of such Th2 cytokines as IL-4, IL-5 and IL-13, and in their expression of chemokine receptors, integrins and NK receptors [21,30–32]. Galli and co-workers have demonstrated that iNKT cells promote immunoglobulin production by B cells, an activity that is more potent in $CD4^+$ iNKT cells than in DN iNKT cells [33]. The same group has also reported that activated human iNKT cells directly support the proliferation of and immunoglobulin production by naive and memory B cells. All these experiments were conducted *in vitro*, however, and frequent stimulation of iNKT cells during culture has been reported to cause a shift in their cytokine profile toward a Th2-dominant condition [34], raising the possibility that cultured NKT cells are not always equivalent to those in circulation *in vivo*. In the present clinical study of individuals receiving PPV, the relationship between iNKT cells and Ab production does not seem to be identical between $CD4^+$ and DN iNKT cells. Taken together, the data suggest that these subsets play distinct roles in Ab production by B cells after PPV administration. Further investigation is necessary to define the precise mechanism by which this occurs.

On the other hand, only a limited subset of NKT cells expressing NK cell markers, such as CD56 or CD161, is reactive to α-GalCer-loaded CD1d tetramer [31]. Therefore, $CD3^+CD56^+$ NKT cells, described as NKT-like cells, are distinguished from iNKT cells by certain characteristics, including the differences in their cytokine production profiles and their TCR αβ chains [18]. Our results suggest that iNKT cells rather than NKT-like cells may be particularly involved in IgG production caused by pneumococcal capsular polysaccharides, because no correlation was observed between $CD3^+CD56^+$ NKT cell count and Ab response.

To the best of our knowledge, the current study is the first report presenting clinical data that suggests a possible relationship between the activation of iNKT cells and Ab responses after PPV administration. The increase in DN iNKT cell count seems to be particularly correlated with serotype-specific IgG production, suggesting a higher contribution from DN iNKT cells than from other subsets. The population size in this study was limited, and the

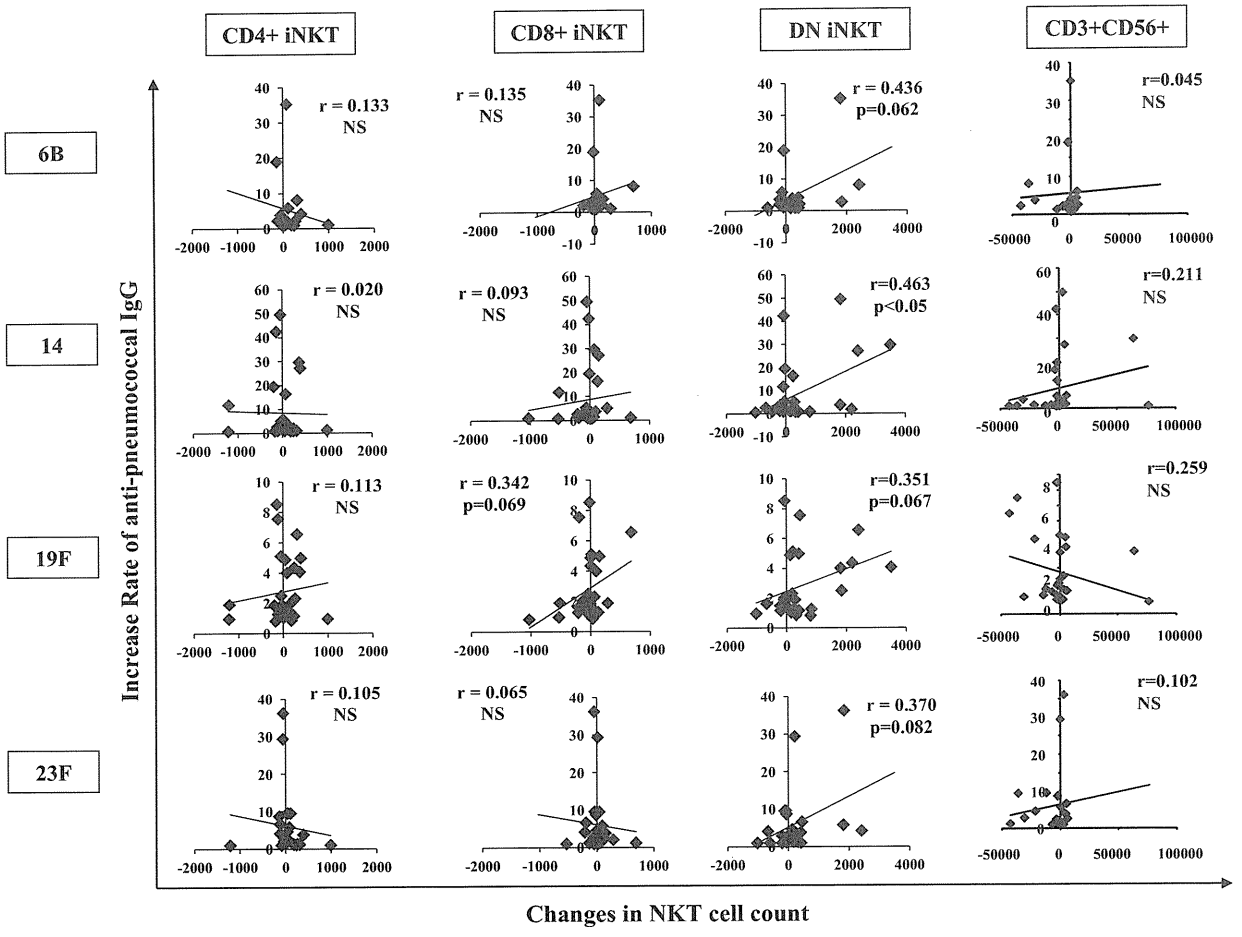

Fig. 3. Relationship between NKT cell counts and anti-pneumococcal IgG. Relationship between changes in NKT cell counts during the first 2 weeks post-vaccination and degree of change in serum anti-pneumococcal IgG levels from pre-vaccination to peak. Each symbol indicates the relationship for one subject. *R* and *P* values and number of subjects in each analysis are shown.

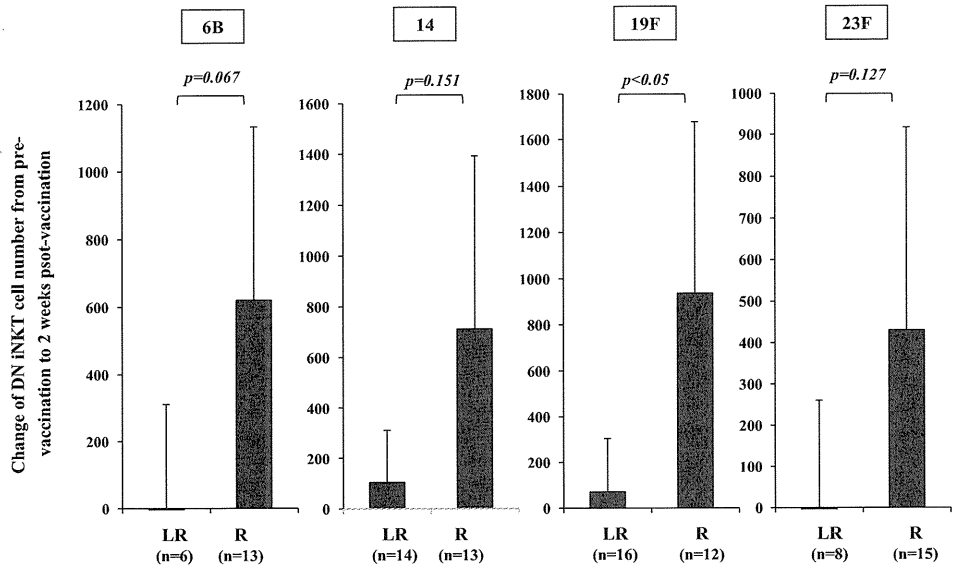

Fig. 4. Changes in DN iNKT cell counts in responders and low responders. Degree of change in DN iNKT cell count during the first 2 weeks after vaccination was compared between responders and low responders for each serotype. Data are expressed as the arithmetic means and 95% confidence intervals of indicated number of subjects. LR, low responders; R, responders.

enrolled subjects were aged (74.4 ± 6.6 years) and had underlying diseases that affected their immune condition. In these respects, there are some limitations in interpreting the results. At present, it remains to be elucidated how iNKT cells are involved in humoral immune responses to pneumococcal capsular polysaccharides in the clinical setting, but further investigations are already under way in our laboratory to define the precise mechanism underlying the relationship between iNKT cells and Ab responses.

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References

[1] Butler JC. Epidemiology of pneumococcal disease. In: Tuomanen EI, Mitchell TJ, Morrison DA, Spratt BG, editors. The pneumococcus. 1st ed Washington, DC: ASM Press; 2004. p. 148–68.
[2] Filice GA. Pneumococcal vaccines and public health policy. Consequences of missed opportunities. Arch Intern Med 1990;150(7):1373–5.
[3] Bennett NM, Buffington J, LaForce FM. Pneumococcal bacteremia in Monroe County, New York. Am J Public Health 1992;82(11):1513–6.
[4] Hofmann J, Cetron MS, Farley MM, Baughman WS, Facklam RR, Elliott JA, et al. The prevalence of drug-resistant *Streptococcus pneumoniae* in Atlanta. N Engl J Med 1995;333(8):481–6.
[5] Plouffe JF, Breiman RF, Facklam RR. Bacteremia with *Streptococcus pneumoniae*. Implications for therapy and prevention. Franklin County Pneumonia Study Group. JAMA 1996;275(3):194–8.
[6] Ishida T, Hashimoto T, Arita M, Ito I, Osawa M. Etiology of community-acquired pneumonia in hospitalized patients: a 3-year prospective study in Japan. Chest 1998;114(6):1588–93.
[7] Centers for Disease Control and Prevention (CDC). Bacterial coinfections in lung tissue specimens from fatal cases of 2009 pandemic influenza A (H1N1) – United States, May–August 2009. MMWR Morb Mortal Wkly Rep 2009;58(38):1071–4.
[8] Hussell T, Wissinger E, Goulding J. Bacterial complications during pandemic influenza infection. Future Microbiol 2009;4(3):269–72.
[9] O'Brien KL, Walters MI, Sellman J, Quinlisk P, Regnery H, Schwartz B, et al. Severe pneumococcal pneumonia in previously healthy children: the role of preceding influenza infection. Clin Infect Dis 2000;30(5):784–9.
[10] Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. J Infect Dis 2008;198(7):962–70.
[11] Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 1997;46(RR-8):1–24.
[12] Mond JJ, Lees A, Snapper CM. T cell-independent antigens type 2. Annu Rev Immunol 1995;13:655–92.
[13] Snapper CM, Mond JJ. A model for induction of T cell-independent humoral immunity in response to polysaccharide antigens. J Immunol 1996;157(6):2229–33.
[14] Vos Q, Lees A, Wu ZQ, Snapper CM, Mond JJ. B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. Immunol Rev 2000;176:154–70.
[15] Barrett DJ, Ayoub EM. IgG2 subclass restriction of antibody to pneumococcal polysaccharides. Clin Exp Immunol 1986;63(1):127–34.
[16] Snapper CM, McIntyre TM, Mandler R, Pecanha LM, Finkelman FD, Lees A, et al. Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. J Exp Med 1992;175(5):1367–71.
[17] Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1-specific NK1 T cells: development, specificity, and function. Annu Rev Immunol 1997;15:535–62.
[18] Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? Nat Rev Immunol 2004;4(3):231–7.
[19] Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science 1997;278(5343):1626–9.
[20] Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J Exp Med 2000;192(5):741–54.
[21] Liu TY, Uemura Y, Suzuki M, Narita Y, Hirata S, Ohyama H, et al. Distinct subsets of human invariant NKT cells differentially regulate T helper responses via dendritic cells. Eur J Immunol 2008;38(4):1012–23.
[22] Hammond KJ, Pellicci DG, Poulton LD, Naidenko OV, Scalzo AA, Baxter AG, et al. CD1d-restricted NKT cells: an interstrain comparison. J Immunol 2001;167(3):1164–73.
[23] Rogers PR, Matsumoto A, Naidenko O, Kronenberg M, Mikayama T, Kato S. Expansion of human Valpha24+ NKT cells by repeated stimulation with KRN7000. J Immunol Methods 2004;285(2):197–214.
[24] Kawakami K, Yamamoto N, Kinjo Y, Miyagi K, Nakasone C, Uezu K, et al. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against *Streptococcus pneumoniae* infection. Eur J Immunol 2003;33(12):3322–30.
[25] Kobrynski LJ, Sousa AO, Nahmias AJ, Lee FK. Cutting edge: antibody production to pneumococcal polysaccharides requires CD1 molecules and CD8+ T cells. J Immunol 2005;174(4):1787–90.
[26] World Health Organization Pneumococcal Serology Reference Laboratories. Training manual for enzyme linked immunosorbent assay for the quantitation of *Streptococcus pneumoniae* serotype specific IgG (Pn PS ELISA). Geneva, Switzerland: World Health Organization; 2000. http://www.vaccine.uab.edu/ELISA%20Protocol.pdf.
[27] Chen M, Hisatomi Y, Furumoto A, Kawakami K, Masaki H, Nagatake T, et al. Comparative immune responses of patients with chronic pulmonary diseases during the 2-year period after pneumococcal vaccination. Clin Vaccine Immunol 2007;14(2):139–45.
[28] Rubins JB, Puri AK, Loch J, Charboneau D, MacDonald R, Opstad N, et al. Magnitude, duration, quality, and function of pneumococcal vaccine responses in elderly adults. J Infect Dis 1998;178(2):431–40.
[29] Törling J, Hedlund J, Konradsen HB, Ortqvist A. Revaccination with the 23-valent pneumococcal polysaccharide vaccine in middle-aged and elderly persons previously treated for pneumonia. Vaccine 2003;22(1):96–103.
[30] Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. J Exp Med 2002;195(5):637–41.
[31] Kim CH, Johnston B, Butcher EC. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among Valpha 24(+)Vbeta 11(+) NKT cell subsets with distinct cytokine-producing capacity. Blood 2002;100(1):11–6.
[32] Thomas SY, Hou R, Boyson JE, Means TK, Hess C, Olson DP, et al. CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. J Immunol 2003;171(5):2571–80.
[33] Galli G, Nuti S, Tavarini S, Galli-Stampino L, De Lalla C, Casorati G, et al. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. J Exp Med 2003;197(8):1051–7.
[34] Burdin N, Brossay L, Kronenberg M. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. Eur J Immunol 1999;29(6):2014–25.

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PspA Family Distribution, Antimicrobial Resistance and Serotype of *Streptococcus pneumoniae* Isolated from Upper Respiratory Tract Infections in Japan

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Abstract

Background: The protection against pneumococcal infections provided by currently available pneumococcal polysaccharide conjugate vaccines are restricted to the limited number of the serotypes included in the vaccine. In the present study, we evaluated the distribution of the pneumococcal capsular type and surface protein A (PspA) family of pneumococcal isolates from upper respiratory tract infections in Japan.

Methods: A total of 251 *S. pneumoniae* isolates from patients seeking treatment for upper respiratory tract infections were characterized for PspA family, antibiotic resistance and capsular type.

Results: Among the 251 pneumococci studied, the majority (49.4%) was identified as belonging to PspA family 2, while most of the remaining isolates (44.6%) belonged to family 1. There were no significant differences between the distributions of PspA1 versus PspA2 isolates based on the age or gender of the patient, source of the isolates or the isolates' susceptibilities to penicillin G. In contrast, the frequency of the *mefA* gene presence and of serotypes 15B and 19F were statistically more common among PspA2 strains.

Conclusion: The vast majority of pneumococci isolated from the middle ear fluids, nasal discharges/sinus aspirates or pharyngeal secretions represented PspA families 1 and 2. Capsular serotypes were generally not exclusively associated with certain PspA families, although some capsular types showed a much higher proportion of either PspA1 or PspA2. A PspA-containing vaccine would potentially provide high coverage against pneumococcal infectious diseases because it would be cross-protective versus invasive disease with the majority of pneumococci infecting children and adults.

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Competing Interests: Some intellectual property relating to PspA that is held by the UAB Research foundation includes active patents on the alpha-helical and proline-rich domains of PspA, each of which covers the use of these domains of PspA in protection-eliciting vaccines. The patents for the alpha-helical domain only covers use in a vaccine in the USA. Patents for the proline-rich domain are being pursued worldwide. Drs. Briles and Hollingshead are among the inventors on these patents and could gain monetarily if a PspA-containing vaccine were licensed for human use. Drs. Briles and Hollingshead provided advice concerning PspA family determination and assisted with manuscript preparation, but all of the data was collected the authors' group in Japan, which has no conflicts of interest with the data obtained. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a major etiological agent causing various infectious diseases ranging from non-invasive diseases such as acute otitis media (AOM), rhinosinusitis and pneumonia to invasive disease such as sepsis and meningitis in young children and the elderly [1–4]. In recent decades, penicillin resistant *S. pneumoniae* (PRSP) have evolved at a rapid pace into a global problem [5–7]. The high prevalence of antimicrobial resistant pneumococci has further emphasized the importance of pneumococcal vaccines [8,9].

Currently available pneumococcal vaccines are based on capsular polysaccharides. Although the 23-valent polysaccharide vaccine (23 PPV) is immunogenic and protective in most adults, it has been shown to be poorly efficacious in children younger than 2 years of age [10]. In contrast, the 7-valent pneumococcal conjugate vaccine (7 PCV) is highly efficacious at preventing bacteremic disease in children under 5 years of age [11–14]. Promising results regarding the prevention of pneumonia and AOM, reducing nasopharyngeal carriage of vaccine serotypes, and elicitation of herd immunity against vaccine serotypes have also been reported for PCV [15]. However, the protection is restricted

to the limited number of the serotypes included in the vaccine. In recent years the protection afforded by the conjugate vaccine has begun to be eroded by an increasing frequency of infections with pneumococcal strains not covered by the vaccine [16,17]. An ideal pneumococcal vaccine would be immunogenic in all young children, the age group for whom pneumococcal infection and mortality is the highest in the developing world. An ideal vaccine would also protect against pneumococci regardless of their capsular types [18].

Pneumococcal surface protein A (PspA) is an important virulent factor expressed by all pneumococci that is essential for full virulence in invasive disease, and contributes to colonization [19–21]. It is highly immunogenic and protective against invasive disease as well as nasal colonization in mice. Protective antibody to PspA is elicited in the alpha helical and proline-rich domains of PspA. Of the ~300 amino acid alpha-helical domain protective, its 100 C-terminal amino acids, known as the clade/family-defining region are responsible for much of the elicited protection [22–25]. However, the N-terminal 100 amino acids are also protection-eliciting [24,25]. All three protection-eliciting regions exhibit variability in their mosque sequence but contain many shared sequences some of which are highly conserved [22,26]. These shared sequences outside the family-defining regions and shared sequence between families within the family-defining region explain why immunity to PspAs of one family can often elicit some or complete protection against strains expressing PspAs of other families. Since virtually all pneumococci have at least slightly different PspA sequences, we regard virtually all protection by antibody to PspA as cross-protection. Immunity to PspA is highly cross-protective against invasive disease [27,28]. Thus, a vaccine containing at least three different PspAs should be able to provide redundant protection against all pneumococci. Both intranasal and humoral immunization with PspA can also protect against colonization [29–31]. Consequently, PspA is an attractive candidate antigen for the development of new effective vaccines [32].

Since capsular type distribution is not uniform world wide, it is important to know the overall distribution of the PspA family expressed in pneumococcal strains at multiple sites around the world to make sure the PspA molecules represented in a vaccine will be effective world wide [33–35]. In the present study, we evaluated the distribution of PspA family types among pneumococcal isolates from upper respiratory tract infections in Japan.

Materials and Methods

S. pneumoniae Strains

Between January and May 2003, the Japanese Society of Infectious Disease in Otorhinolaryngology conducted the fourth nationwide surveillance of the bacterial pathogens responsible for otorhinolaryngological infections. A total of 251 *S. pneumoniae* isolates were collected from 251 patients treated for the upper respiratory tract infections including AOM, rhinosinusitis and pharyngotonsillitis during these periods. All pneumococcal strains were identified by alpha-hemolysis and colony morphology on 5% sheep blood agar, Gram's stained smear, optochin disk sensitivity, bile solubility, and the presence of *ply* gene by polymerase chain reaction (PCR). The patients ranged in age from 0 to 68 years old, with 125 females and 126 males. Among the isolates, 57 (22.7%) were from the middle ear fluids (MEFs), 88 (35.1%) were from nasal discharges or sinus aspirates and 106 (42.2%) were from the pharyngeal secretions (Table 1).

Susceptibility to penicillin G (PCG) was tested by a broth dilution standard method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The CLSI published revised susceptibility breakpoints for penicillin and *S. pneumoniae* in 2008. The revised susceptibility breakpoint is $\leq$2 μg/ml for non-meningeal infections treated with parental penicillin. In this study, categorization of penicillin susceptibility according to the former CLSI guidelines was applied because most of the cases were treated with oral penicillin. Strains with MICs of PCG $\geq$2 μg/ml were interpreted as penicillin resistant *S. pneumoniae* (PRSP), strains with MICs from 0.1 to 1 μg/ml were classified as penicillin intermediate resistant *S. pneumoniae* (PISP), and strains with MICs $\leq$0.06 μg/ml were interpreted as penicillin susceptible *S. pneumoniae* (PSSP). During the assay, *S. pneumoniae* strains ATCC 49619 and ATCC BAA-334 were used as susceptible controls for quality assurance [36].

Serotype

All isolates were serotyped or serogrouped by the capsular quellung reaction method with pneumococcal capsule specific antisera (Statens Serum Institute, Copenhagen, Denmark), as recommended by the manufacturer. Strains of serotypes 4 (ATCC BAA-334) and 19F (ATCC 49619) obtained from the American Type Culture Collection 169 (ATCC, Manassas, VA, USA) were used for quality control in every reaction.

PspA Family Classification

PspAs were classified into three families by PCR. Briefly, genomic DNA was extracted from pneumococcal isolates as described and stored at 4°C [37]. PCR were carried out in a standard PCR mixture (QIAGEN, Valencia, CA, USA) of 25 μl containing 2.5 mM $MgCl_2$, 200 μM dNTPs (each), 50 pmol of primers, and 2.5 U of *Taq* DNA polymerase. The oligonucleotide primers (LSM12, SKH63, SKH52, SKH41, SKH42, SKH02, ply1, and ply2) reported by Hollingshead et al were used in this study [37]. Primers for PspA family 1 (PspA1) and PspA family 2 (PspA2) were LSM12/SKH63 and LSM12/SKH52, respectively. Primers for PspA family 3 (PspA3) were SKH41 and SKH 42. Primers LSM12 and SKH02 were used for testing the presence of *pspA* gene. Primers ply1 and ply2 were used for testing the presence of the pneumolysin gene.

The PCR conditions were 95°C for 3 min; then 30 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 3 min, and finally 72°C for 10 min. The optimal annealing temperature was 62°C. The isolates that were not initially amplified were further processed with the same cycling pattern at an annealing temperature of 58°C, or, if that also failed, of 55°C. Isolate that were not typed after the lower annealing temperatures in the family 1, 2, and 3 tests were classified as nontypeable PspA (PspA NT). An additional two tests were used to verify that the PspA NT isolates were truly pneumococcal isolates. One test was for the presence of the pneumolysin gene and another test was for the presence of the *pspA* gene. A single isolate that was amplified by the ply primers and not amplified by any of the PspA primers was classified as PspA null.

Three microliters of the PCR products were loaded on 0.8% agarose gels, electrophoresed at 80 V for 1 h, and stained with 0.5 μg/ml ethidium bromide.

Statistical Analysis

All data were statistically analyzed by using Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA). A two tailed chi-square test or Fisher's exact test (for small group sizes) was used for categorical variables to test the significance of differences between groups. A p-value of <0.05 was considered statistically significant. The odds ratio (OR) and 95% confidential intervals (CIs) of individual

Table 1. Distribution of *S. pneumoniae* serotypes based on their susceptibilities to PCG.

Category	Sub-category	Total	Susceptibility to PCG (μg/ml)			*p*-value
			PSSP	PISP	PRSP	DRSP v.s. PSSP
Gender	Female	125 (49.8%)	50 (19.9%)	48 (19.1%)	27 (10.8%)	$p=0.362$
	Male	126 (50.2%)	43 (17.1%)	56 (22.3%)	27 (10.8%)	
Age	0–2	92 (36.7%)	16 (6.4%)	50 (19.9%)	26 (10.4%)	$p<0.001$*
	3–5	37 (14.7%)	16 (6.4%)	17 (6.8%)	4 (1.6%)	
	6–12	25 (10%)	18 (7.2%)	7 (2.8%)	0 (0%)	
	13–20	7 (2.8%)	4 (1.6%)	2 (0.8%)	1 (0.4%)	
	21–50	72 (28.7%)	30 (12.0%)	22 (8.8%)	20 (8.0%)	
	≥51	18 (7.2%)	9 (3.6%)	6 (2.4%)	3 (1.2%)	
Origin	Middle ear fluids	57 (22.7%)	21 (8.4%)	27 (10.8%)	9 (3.6%)	$p=1.000$
	Nasal discharge/Sinus aspirates	88 (35.1%)	37 (14.7%)	34 (13.5%)	17 (6.8%)	$p=0.273$
	Pharyngeal secretions	106 (42.2%)	35 (13.9%)	43 (17.1%)	28 (11.2%)	$p=0.291$
Serotype	1	2 (0.8%)	1 (0.4%)	0 (0%)	1 (0.4%)	$p=1.000$
	3	14 (5.6%)	13 (5.2%)	1 (0.4%)	0 (0%)	$p<0.001$
	4	1 (0.4%)	0 (0%)	0 (0%)	1 (0.4%)	$p=1.000$
	6A	16 (6.4%)	6 (2.4%)	6 (2.4%)	4 (1.6%)	$p=1.000$
	6B	37 (14.7%)	14 (5.6%)	17 (6.8%)	6 (2.4%)	$p=1.000$
	9V	5 (2.0%)	3 (1.2%)	2 (0.8%)	0 (0%)	$p=0.667$
	14	20 (8.0%)	2 (0.8%)	13 (5.2%)	5 (2.0%)	$p=0.002$
	15B	7 (2.8%)	4 (1.6%)	3 (1.2%)	0 (0%)	$p=0.429$
	19A	5 (2.0%)	4 (1.6%)	1 (0.4%)	0 (0%)	$p=0.064$
	19F	52 (20.7%)	8 (3.2%)	25 (10.0%)	19 (7.6%)	$p<0.001$
	23F	41 (16.3%)	11 (4.4%)	18 (7.2%)	12 (4.8%)	$p=0.029$
	G23	6 (2.4%)	4 (1.6%)	2 (0.8%)	0 (0%)	$p=0.198$
	Others	45 (17.9%)	23 (9.2%)	16 (6.4%)	6 (2.4%)	$p=0.040$
Total		251 (100%)	93 (37.1%)	104 (41.4%)	54 (21.5%)	

G23: serogroup 23 strains except serotype 23F. PCG: penicillin G. PSSP: penicillin susceptible *S. pneumoniae*. PISP: penicillin intermediately resistant *S. pneumoniae*. PRSP: penicillin resistant *S. pneumoniae*. DRSP: PRSP+PISP. Others: serotypes not included in 23 PPV.
*comparison between ≤2 y.o. vs. ≥3 y.o.
doi:10.1371/journal.pone.0058124.t001

serotypes were calculated relative to all other serotypes in the samples.

Ethical Approval

The isolates used in this study are all clinical isolates obtained from patients with otorhinolaryngological infections as part of routine clinical diagnosis and management. The main ethical issue relates to specific consent for detailed characterization of an isolate from a clinical specimen taken from a patient on clinical ground. Because no information that would allow identification of the patients was collected in this study, this requirement was waived by the Institutional Review Board of the Ethical Committee of Wakayama Medical University. This study was therefore approved by the Institutional Review Board of the Ethical Committee of Wakayama Medical University.

Results

Distribution of Pneumococcal Serotypes Based on their Penicillin and Macrolide Susceptibilities

The distribution of *S. pneumoniae* serotypes based on their susceptibilities to PCG is listed in Table 1. Based on their susceptibility to PCG, the 251 pneumococcal isolates evaluated in this study were classified into three groups as follows: 93 (37.0%) PSSP, 104 (41.4%) PISP, and 54 (21.6%) PRSP. There were no significant differences in distributions of susceptibilities to PCG based on gender of the patients providing the strains, or based on the source of the isolates. Drug resistant *S. pneumoniae* (DRSP; PISP+PRSP) were frequently identified among children younger than 2 years old (OR 4.5, 95% CI 2.4–8.3, $p<0.001$).

The most common serotype was 19F (20.7%) followed by 23F (16.3%), 6B (14.7%), 14 (8.0%), 6A (6.4%) and 3 (5.6%). Among the serogroup 6 strains, we could not find the recently discovered serotype 6C and 6D strains. The distribution of *S. pneumoniae* serotypes based on their susceptibility to PCG was statistically significant ($p<0.001$). Serotype 3 (OR 25.5, 95% CI 3.3–198.6, $p<0.001$) was prevalent among the strains with MICs to PCG of ≤0.06 μg/ml. In contrast serotype 14 (OR 5.9, 95% CI 1.3–25.8, $p=0.002$) and serotype 19F (OR 4.1, 95% CI 1.8–9.2, $p<0.001$) were frequently identified among DRSP strains. The isolated strains identified as serotypes 6A and 6B showed a broad spectrum of antibiotic resistance regardless of their susceptibility to PCG. The most common five serotypes (19F, 23F, 6B, 6A and 14) represented about 79.1% of the DRSP strains.

The distribution of *S. pneumoniae* serotypes based on their susceptibilities to macrolide is listed in Table 2. Based on the

macrolide susceptibilities, the 251 isolates were classified into four groups as follows: 106 (42.2%) strains with the *ermB* gene, 75 (29.8%) strains with the *mefA* gene, 15 (6.0%) strains with both genes, and 55 (22.0%) strains without both genes. There were no significant differences in distributions of macrolide resistant traits based on the gender or based on the source of the isolates or age of the patients.

The distribution of *S. pneumoniae* serotypes based on their macrolide resistant traits is also statistically significant ($p = 0.001$). The *mefA* gene was most prevalent among isolates typed as serotype 19F (OR 4.7, 95% CI 2.5–8.9, $p<0.001$). Strains of the most predominant six serotypes (19F, 23F, 6B, 6A, 14, and 3) represented 60.6% of the total strains and about 77.6% of the strains with macrolide resistant genes.

Distribution of PspA Families Based on their Serotypes and Penicillin Susceptibilities

Among the 251 pneumococci isolates studied, the 49.4% were identified as belonging to family 2 (PspA2), and 44.6% to family 1 (PspA1). Thus, 94.0% of the isolates included in this study were PspA1- or PspA2-positive isolates. Eight isolates (3.2%) classified into PspA family 3 (PspA3). Four isolates (1.6%) were classified as PspA NT. Three isolates (1.2%) was identified as a PspA null strain.

Because the vast majority of PspA families were identified as PspA1 or PspA2, we further evaluated the distributions of PspA1 and PspA2 by the other parameters. There were no significant differences in the distributions of PspA1 and PspA2 based on the age and gender of the patients, the origin of the isolates (Fig. 1.). Although there were no significant differences in the distribution of PspA1 and PspA2 based on the isolates' susceptibilities to PCG, PspA2 were expressed at a higher frequency among the strains with the *mefA* gene (OR 2.4, 95% CI 1.4–4.1, $p = 0.003$) than the population of strains in general (Fig. 2.).

The distribution of PspA families based on their serotypes is shown in Fig. 3. The differences in distribution of PspA1 and PspA2 isolates based on their pneumococcal serotype were statistically significant ($p = 0.013$). Serotype 19F (OR 6.9, 95% CI 3.1–15.5, $p<0.001$), and serotype 15B (OR 12.3, 95% CI 0.7–221.8, $p = 0.031$) frequently expressed PspA2. Serotype 3 (OR 4.4, 95% CI 1.2–16.2, $p = 0.025$), serotype 6A (OR 19.0, 95% CI 1.7–146.6, $p<0.001$) and serotype 14 (OR 3.4, 95% CI 1.2–9.8, $p = 0.029$) tended to express PspA1. Serotypes 6B contained equal numbers of PspA1 and PspA2 isolates. In spite of these statistical differences in PspA family frequency among the different capsular

Table 2. Distribution of *S. pneumoniae* serotypes based on their macroride-resistant traits.

Category	Sub-category	Total	Macrolide resistance genes				*p*-value
			ermB	*mefA*	*ermB+mefA*	None	MLR v.s. MLS
Gender	Female	99 (39.4%)	51 (20.3%)	39 (15.5%)	9 (3.6%)	26 (10.4%)	$p=0.761$
	Male	97 (38.6%)	55 (21.9%)	36 (14.3%)	6 (2.4%)	29 (11.6%)	
Age	0–2	76 (30.3%)	39 (15.5%)	32 (12.7%)	5 (2.0%)	16 (6.4%)	$p=0.208$*
	3–5	28 (11.2%)	14 (5.6%)	12 (4.8%)	2 (0.8%)	9 (3.6%)	
	6–12	14 (5.6%)	11 (4.4%)	3 (1.2%)	0 (0%)	11 (4.4%)	
	13–20	4 (1.6%)	3 (1.2%)	0 (0%)	1 (0.4%)	3 (1.2%)	
	21–50	58 (23.1%)	29 (11.6%)	22 (8.8%)	7 (2.8%)	14 (5.6%)	
	≥51	16 (6.4%)	10 (4.0%)	6 (2.4%)	0 (0%)	2 (0.8%)	
Origin	Middle ear fluids	46 (18.3%)	34 (13.5%)	11 (4.4%)	1 (0.4%)	11 (4.4%)	$p=0.264$
	Nasal discharge/Sinus aspirates	65 (25.9%)	35 (13.9%)	25 (10.0%)	5 (2.0%)	23 (9.2%)	$p=0.716$
	Pharyngeal secretions	85 (33.9%)	37 (14.7%)	39 (15.5%)	9 (3.6%)	21 (8.4%)	$p=0.539$
Serotype	1	1 (0.4%)	0 (0%)	1 (0.4%)	0 (0%)	1 (0.4%)	$p=0.391$
	3	10 (4.0%)	9 (3.6%)	1 (0.4%)	0 (0%)	4 (1.6%)	$p=0.514$
	4	1 (0.4%)	0 (0%)	1 (0.4%)	0 (0%)	0 (0%)	$p=0.515$
	6A	13 (5.2%)	8 (3.2%)	4 (1.6%)	1 (0.4%)	3 (1.2%)	$p=1.000$
	6B	27 (10.8%)	17 (6.8%)	7 (2.8%)	3 (1.2%)	10 (4.0%)	$p=0.397$
	9V	4 (1.6%)	3 (1.2%)	0 (0%)	1 (0.4%)	1 (0.4%)	$p=1.000$
	14	17 (6.8%)	10 (4.0%)	6 (2.4%)	1 (0.4%)	3 (1.2%)	$p=0.579$
	15B	6 (2.4%)	5 (2.0%)	0 (0%)	1 (0.4%)	1 (0.4%)	$p=1.000$
	19A	1 (0.4%)	1 (0.4%)	0 (0%)	0 (0%)	4 (1.6%)	$p=0.009$
	19F	49 (19.5%)	15 (6.0%)	30 (12.0%)	4 (1.6%)	3 (1.2%)	$p=0.001$
	23F	36 (14.3%)	19 (7.6%)	14 (5.6%)	3 (1.2%)	5 (2.0%)	$p<0.001$
	G23	5 (2.0%)	4 (1.6%)	1 (0.4%)	0 (0%)	1 (0.4%)	$p=0.147$
	Others	26 (10.4%)	15 (6.0%)	10 (4.0%)	1 (0.4%)	19 (7.6%)	$p=1.000$
Total		196 (78.1%)	106 (42.2%)	75 (29.9%)	15 (6.0%)	55 (21.9%)	$p=0.001$

G23: serogroup 23 strains except serotype 23F. PCG: penicillin G. PSSP: penicillin susceptible *S. pneumoniae*. PISP: penicillin intermediately resistant *S. pneumoniae*. PRSP: penicillin resistant *S. pneumoniae*. DRSP: PRSP+PISP. Others: serotypes not included in 23 PPV.
*comparison between ≤2 y.o. vs. ≥3 y.o.
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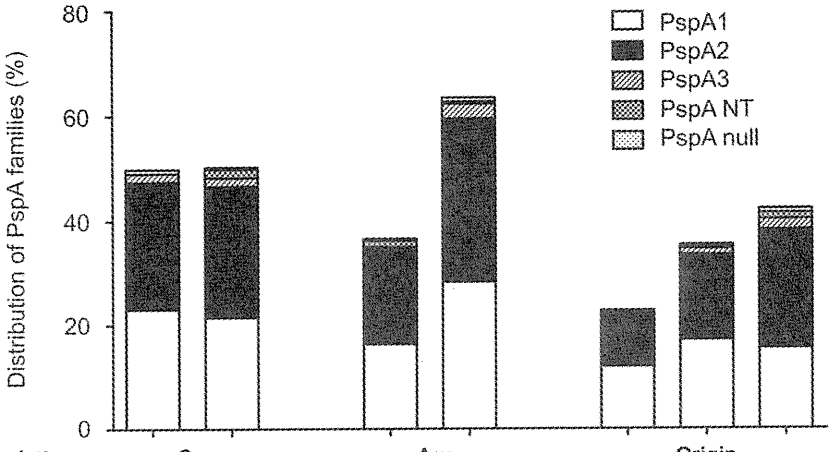

Characteristics	Sex		Age		Origin		
	Female	Male	≤2 y.o.	≥3 y.o.	MEF	ND/SA	PS
(n=)	125	126	92	159	57	88	106
(%)	(49.8)	(50.2)	(36.7)	(63.3)	(22.7)	(35.1)	(42,2)
PspA families (n=)							
PspA1	58	54	41	71	30	43	39
PspA2	61	63	46	78	26	41	57
PspA3	4	4	1	7	0	3	5
PspA NT	0	4	3	1	1	0	3
PspA null	2	1	1	2	0	1	2

Figure 1. Distribution of PspA familes based on sex, age and origin of pneumococci. MEF: middle ear fluid, ND/SA: nasal discharge/sinus aspirate, PS: pharyngeal secretion. Each numbers shows numbers of isolates and percentage shows in parenthesis. There is no significant differences in PspA family distribution based on sex, age and origin of isolates.
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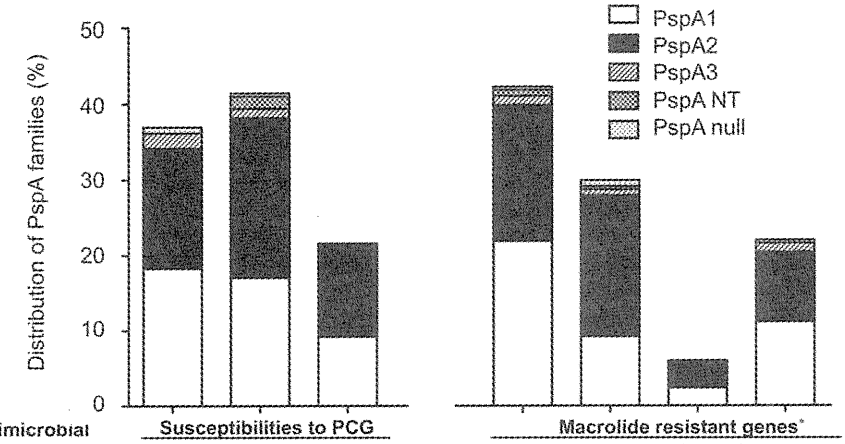

Antimicrobial Susceptibilities	Susceptibilities to PCG			Macrolide resistant genes*			
	PSSP	PISP	PRSP	*ermB*	*mefA*	*ermB+mefA*	none
(n=)	93	104	54	106	75	15	55
(%)	(37.1)	(41.4)	(21.5)	(42.2)	(29.9)	(6.0)	(21.9)
PspA families (n=)							
PspA1	46	43	23	55	23	6	28
PspA2	40	53	31	45	47	9	23
PspA3	5	3	0	3	2	0	3
PspA NT	0	4	0	2	1	0	1
PspA null	2	1	0	1	2	0	0

Figure 2. Distribution of PspA familes based on antimicoribila suscptibilities. PSSP: penicillin susceptible *S. pneumoniae*, PISP: Penicillin intermediately resistant *S. pneumoniae*, PRSP: penicillin resistant *S. pneumoniae*. Each numbers shows numbers of isolates and percentage shows in parenthesis. *$p<0.05$.
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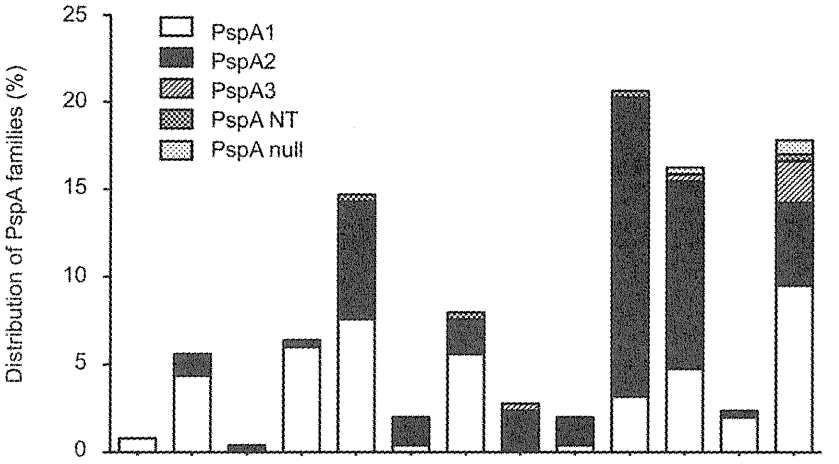

Serotypes	1	3*	4	6A**	6B	9V	14*	15B*	19A	19F*	23F	23G	others*
(n=)	2	14	1	16	37	5	20	7	5	52	41	6	45
(%)	(0.8)	(5.6)	(0.4)	(6.4)	(14.7)	(2.0)	(8.0)	(2.8)	(2.0)	(20.7)	(16.3)	(2.4)	(17.9)
PspA families (n=)													
PspA1	2	11	0	15	19	1	14	0	1	8	12	5	24
PspA2	0	3	1	1	17	4	5	6	4	43	27	1	12
PspA3	0	0	0	0	0	0	0	1	0	0	1	0	6
PspA NT	0	0	0	0	1	0	1	0	0	1	0	0	1
PspA null	0	0	0	0	0	0	0	0	0	0	1	0	2

Figure 3. Distribution of PspA familes based on pneumococcal serotypes. G23: serogroup 23 strains except serotype 23F. Others: serotypes not included in 23 PPV. Each numbers shows numbers of isolates and percentage shows in parenthesis. *$p<0.05$, **$p<0.01$.
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types, representative capsular types (except for serotype 15B) had isolates of both PspA families. Thus, in general the capsular types were found not to be restricted to particular PspA families and PspA families were not restricted to particular capsular types.

Coverage of Pneumococcal Vaccine Formulas

The coverage and 95% CI of pneumococcal vaccine formulas according to serotypes and PspA families are listed in Table 3. The total serotype coverage of the 7-valent, 10-valent (10 PCV), 13-valent (13 PCV), and 23-valent pneumococcal vaccines were 62.2%, 62.9%, 76.9% and 70.5%, respectively. The coverage of DRSP by the 7-valent, 10-valent, 13-valent, and 23-valent pneumococcal vaccines were 74.7%, 75.3%, 82.9% and 78.5%, respectively. The total coverage of *S. pneumoniae* with either the *mefA* gene or the *ermB* gene by the 7-valent, 10-valent, 13-valent, and 23-valent pneumococcal vaccines were 68.4%, 68.9%, 81.1%, and 74.5%, respectively. The percentages of total pneumococcal isolates, DRSP, and macrolide resistant *S. pneumoniae* (MRSP) having either *mefA* or *ermB* gene strains that would be covered by a PspA vaccine including the PspA1 and PspA2 families were 94.0%, 94.9%, and 94.4%, respectively. Consequently, the serotype coverage by a PspA vaccine was higher than the serotype coverage provided by the current 7 PCV, 10 PCV, 13 PCV, and 23 PPV vaccines ($p<0.001$).

Discussion

PspA consists of five domains including a signal peptide, alpha-helical charged region, a proline-rich domain, a choline-binding domain consisting of ten amino acids repeats, and a C-terminal amino acid tail [37–39]. Depending on the divergence of nucleotide sequences in the alpha-helical charged region, PspA is classified into three families, with no more than 50% sequence divergence within each family. The three PspA families are made up of six PspA clades that diverge from each other by no more than 20% sequence identity within each clade; family 1 (clades 1 and 2), family 2 (clades 3, 4, and 5), and family 3 (clade 6) [38,40,41].

Despite the great variation in the sequences of PspA, mouse and humans antibodies against PspA can be cross-reactive and cross protective against invasive disease in mice [27,28]. The serologic cross-reactivity of PspA has been found to be strongly associated with PspA, but not restricted to family [42,43]. Even so these antibodies can be more cross-protective than their level of cross-reactivity might suggest. Immunization of adult humans and mice with a PspA family 1 produced antibodies that could protect mice from infection with strains of PspA families 1 or 2 and from infections with strains of 3 different capsular types [42,44,45]. In addition successful fusion proteins have been made between family 1 and family 2 PspAs that can elicit antibody in mice protect against challenge strains of both PspA families [46]. In this study we focused on the distribution of PspA families among clinical isolates in Japan.

The Japanese strains were evenly distributed over family 1 and family 2. The proportions of the different PspA families can vary somewhat among countries. Hollingshead et al reported that the majority of PspAs in a collection of strains from Alabama fell into family 1 [37]. A study on invasive pneumococcal strains isolated from children less than 5 years of age in Colombia showed that 62.5% and 35.0% of strains belonged to families 1 and 2, respectively [43]. In Argentina 54.4% and 41.6% of the strains belonged to family 1 and family 2, respectively, with only 4.0% of the strains isolated from children being unclassifiable [47]. In Brazil, 50.5% of the isolates belonged to family 1, 43.2% were members of family 2, and 6.3% were not classified [48,49]. In contrast, the high prevalence of PspA family 2 among pneumo-

Table 3. Serotype coverage of pneumococcal vaccine formulas among *S. pneumoniae* isolates from upper respiratory tract infections in Japan.

Vaccine formulations	Number and percentable coverage of *S. pneumoniae*					
	DRSP (n = 158)		MRSP (n = 196)		Total (n = 251)	
	n (%)	95% CI	n (%)	95% CI	n (%)	95% CI
7-valent (4,6B,9V,14,18C,19F,23F)	118 (74.7%)	67.9%–81.5%	134 (68.4%)	61.9%–74.9%	156 (62.2%)	56.2%–68.2%
10-valent (1,4,5,6B,7F,9V,14,18C,19F,23F)	119 (75.3%)	68.6%–82.0%	135 (68.9%)	62.4%–75.4%	158 (62.9%)	57.0%–68.9%
13-valent (1,3,4,5,6A,6B,7F,9V,14,18C,19A,19F,23F)	131 (82.9%)	77.0%–88.8%	159 (81.1%)	75.6%–86.6%	193 (76.9%)	71.7%–82.1%
23-valent (1,2,3,4,5,6B,7F,8,9V,9N,10A, 11A,12F,14,15B,17F,18C,19A, 19F,20,22F,23F,33F)	124 (78.5%)	72.1%–84.9%	146 (74.5%)	68.4%–80.6%	177 (70.5%)	64.9%–76.2%
PspA (PspA1 and PspA2)	150 (94.9%)	91.5%–98.4%	185 (94.4%)	91.2%–97.6%	236 (94.0%)	91.1%–97.0%

DRSP: drug resistant *S. pneumoniae* (PISP+PRSP). MRSP: macrolide resistant *S. pneumoniae*.
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cocci isolated from invasive pneumococcal diseases has been reported from Spain, Poland, Canada, Sweden, Germany, the USA, and France [37,50–53]. A recent study of pneumococci isolates from nasopharyngeal carriage in Finnish children showed a prevalence of PspA family 1 and family 2 that was similar to our results [54]. The vast majority of pneumococci isolated from the middle ear fluid or nasopharyngeal secretion samples of the Finnish children less than 2 years old were from PspA families 1 and 2 [54]. Prior to our study, there had been a few reports of the PspA family distribution among pneumococci in Japan or any other countries in Asia [55,56].

In contrast to the similar frequencies of PspA1 and PspA2 in Japan the frequency of different capsular serotypes was highly variable with 19F, 23F, 14, 6A, 6B, and 3 being the predominant common capsular types we observed which together accounted for 71.7% of the pneumococci isolates in this study. However, the PspA family distribution varied somewhat among serotypes. Earlier studies found that both PspA families occurred within the most common capsular serotypes, but that some serotypes were associated more strongly with one PspA family than the other [57,58]. The capsular serotypes most strongly associated with a certain PspA family are 9N, 9V, 11A, 14, and 23F, whereas serotypes 6A, 6B, 19A, and 19F were equally associated with PspA families 1 and 2. This was most dramatic for the 24 different 23F isolates which were 25% PspA1 and 75% PspA2. In a study in France 37 different 23F strains were examined; 92% were PspA1 and 8% were PspA2 [54]. These findings indicate that there can be variations of distributions in PspAs in different geographic different regions although serotypes do not necessarily globally associate with certain PspAs. In some regions some capsular serotypes associated with a certain PspA family might be heavily clonal.

Based on the previously published information on PspA family distribution, there is still little information about the relationship between PspA families and antimicrobial-susceptibilities. In Japan, the rate of antimicrobial-resistant *S. pneumoniae* has increased continually since around 1990 and was about 49.0% between 1998 and 2000 [59,60]. As documented in previous reports, penicillin-resistant strains were frequently identified among children younger than 2 years old [61]. In our previous study most of the serotype 19F and 23F strains were classified as either PISP or PRSP, while all of serotype 3 stains were classified as PSSP in middle ear isolates [62]. In this study, PRSP strains consisted equally of family 1 and 2 PspA. This means that a PspA-based vaccine would show a higher coverage of PRSP compared to the polysaccharide-based vaccines that have been available in the market.

Previous studies showed that PspA clades were independent of capsular serotypes [49,50,51]. Pneumococci of the same serotype were associated with different PspA clades from the same or a different family. This means that PspA-containing vaccines may be able to improve the protective efficacy of pneumococcal vaccines compared with the currently available serotype-based vaccines and may be able to avoid the serotype replacement that has been observed with conjugate vaccines [16]. The coverage of serotypes and PRSP by the 7 PCV was reported to be 62.8% and 88.0% for middle ear isolates, respectively. A PspA-based vaccine that contained representatives of PspA families 1 and 2 would potentially provide a high coverage rate because it would be cross-protective against invasive disease caused by the bulk of pneumococci infecting children and adults. It will be important however that data relating to both serotype and antibiotic resistance, similar to those reported here for Japan, should be collected in other geographical areas. Such a study would help to determine if a vaccine covering PspA families 1 and 2 would be appropriate for the geographic region in question.

In conclusion, even conjugate vaccine formulations with 13 pneumococcal capsular polysaccharides will not reach the coverage of 90% or more achieved by a vaccine containing family 1 and 2 PspA. The addition of PspA to the existing conjugate vaccine formulations may be a possible alternative for future development of pneumococcal vaccine.

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Author Contributions

Conceived and designed the experiments: MH NY. Performed the experiments: MH AT MK YI ST. Analyzed the data: MH NY. Contributed reagents/materials/analysis tools: MH MK. Wrote the paper: MH SKH DEB KS NY.

References

1. Greenwood B (1999) The epidemiology of pneumococcal infection in children in the developing world. Philos Trans R Soc Lond B Biol Sci 354: 777–785.
2. Hollingshead SK, Briles DE (2001) *Streptococcus pneumoniae*: new tools for an old pathogen. Curr Opin Microbiol 4: 71–77.
3. Musher DM, Breiman RF, Tomasz A (2000) *Streptococcus pneumoniae*: at the threshold of the 21st century. In: Tomaz A editor. *Streptococcus pneumoniae*: molecular biology and mechanisms of disease: Mary Ann Liebert, Inc., 485–491.
4. Yamanaka N, Hotomi M, Billal DS (2008) Clinical bacteriology and immunology in acute otitis media in children. J Infect Chemother 14: 180–187.
5. Appelbaum PC (1992) Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. Clin Infect Dis 15: 77–83.
6. Breiman R, Butler JC, Tenover FC, Elliot J, Facklam RR (1994) Emergence of drug-resistant pneumococcal infections in the United States. JAMA 271: 1831–1835.
7. Klugman KP (1990) Pneumococcal resistance to antibiotics. Clin Microbiol Rev 3: 171–196.
8. Dagan R, Klugman KP (2008) Impact of conjugate pneumococcal vaccines on antibiotic resistance. Lancet Infect Dis 8: 785–795.
9. Whitney CG, Farley MM, Hadler J, Harrison LH, Lexau C, et al. (2000) Increasing prevalence of multidrug-resistant Streptococcus pneumoniae in the United States. N Engl J Med 343: 1917–1924.
10. Ortqvist A, Hedlund J, Burman LA, Elbel E, Höfer M, et al. (1998) Randomised trial of 23-valent pneumococcal capsular polysaccharide vaccine in prevention of pneumonia in middle-aged and elderly people. Swedish Pneumococcal Vaccination Study Group. Lancet 351: 399–403.
11. Cutts FT, Zaman SM, Enwere G, Jaffar S, Levine OS, et al. (2005) Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. Lancet 365: 1139–1146.
12. Hsu HE, Shutt KA, Moore MR, Beall BW, Bennett NM, et al. (2009) Effect of pneumococcal conjugate vaccine on pneumococcal meningitis. N Engl J Med 360: 244–256.
13. Poehling KA, Talbot TR, Griffin MR, Craig AS, Whitney CG, et al. (2006) Invasive pneumococcal disease among infants before and after introduction of pneumococcal conjugate vaccine. JAMA 295: 1668–1674.
14. Whitney CG, Pilishvili T, Farley MM, Schaffner W, Craig AS, et al. (2006) Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. Lancet 368: 1495–1502.
15. Eskola J, Kilpi T, Palmu A, Jokinen J, Haapakoski J, et al. (2001) Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 344: 403–409.
16. Klugman KP (2009) The significance of serotype replacement for pneumococcal disease and antibiotic resistance. Adv Exp Med Biol 634: 121–128.
17. Muñoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia J, et al. (2008) Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. Clin Infect Dis 46: 174–182.
18. Razzaque R, Shah P, Watt JM, Mirza S, Coats MT, et al. (2012) Intranasal immunization of mice with neuraminidase A elicits virtually complete protection against nasopharyngeal colonization and subsequent invasion by *Streptococcus pneumoniae*. Infect Immun In submission.
19. Briles DE, Yother J, McDaniel LS (1988) Role of pneumococcal surface protein A in the virulence of *Streptococcus pneumoniae*. Rev Infect Dis 10: S372–S374.
20. Ren B, Li J, Genschmer K, Hollingshead SK, Briles DE (2012) The absence of PspA or presence of antibody to PspA facilitates the complement-dependent phagocytosis of pneumococci in vitro. Clin Vaccine Immunol 19: 1574–1582.
21. Ogunniyi AD, Lemessurier KS, Graham RM, Watt JM, Briles DE, et al. (2007) Contribution of pneumolysin, PspA, and PspC (CbpA) to pathogenicity of *Streptococcus pneumoniae* D39 in a mouse model. Infect Immun 75: 1843–1851.
22. Hollingshead SK, Becker RS, Briles DE (2000) Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun 68: 5889–5900.
23. Coral MCV, Fonseca N, Castaneda E, Di Fabio JL, Hollingshead SK, et al. (2001) Families of pneumococcal surface protein A (PspA) of *Streptococcus pneumoniae* invasive isolates recovered from Colombian children. Emerging Infectious Diseases 7: 832–836.
24. McDaniel LS, Sheffield JS, Swiatlo E, Yother J, Crain MJ, et al. (1992) Molecular localization of variable and conserved regions of *pspA*, and identification of additional *pspA* homologous sequences in *Streptococcus pneumoniae*. Microb Pathog 13: 261–269.
25. Roche H, Hakansson A, Hollingshead SK, Briles DE (2003) Regions of PspA/EF3296 best able to elicit protection against *Streptococcus pneumoniae* in a murine infection model. Infect Immun. 71: 1033–1041.
26. Brooks-Walter A, McDaniel LS, Hollingshead SK, Crain MJ, Briles DE (1995) RFLP of the *psp*A gene from different isolates of *Streptococcus pneumoniae* reveal families of *pspA*. 35th ICAAC, San Franscisco, CA.: ASM Press.
27. Briles DE, Hollingshead SK, Nabors GS, Paton JC, Brooks-Walter A (2000) The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*. Vaccine 19: S87–S95.
28. Briles DE, Hollingshead SK, King J, Swift A, Braun PA, et al. (2000) Immunization of humans with rPspA elicits antibodies, which passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 182: 1694–1701.
29. Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, et al. (2000) Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect Immun 68: 796–800.
30. Fukuyama Y, King JD, Kataoka K, Kobayashi R, Gilbert RS, et al. (2010) Secretory-IgA antibodies play an important role in the immunity to Streptococcus pneumoniae. J Immunol 185: 1755–1762.
31. Ferreira DM, Oliveira ML, Moreno AT, Ho PL, Briles DE, et al. (2010) Protection against nasal colonization with *Streptococcus pneumoniae* by parenteral immunization with a DNA vaccine encoding PspA (Pneumococcal surface protein A). Microb Pathog 48: 205–213.
32. Briles DE, Paton JC, Swiatlo E, Crain MJ (2006) Pneumococcal Vaccines. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, editors. Gram Positive Pathogens. 2nd ed: ASM Press. 289–298.
33. Franco CM, Andrade AL, Andrade JG, Almeida e Silva S, Oliveira CR, et al. (2010) Survey of nonsusceptible nasopharyngeal *Streptococcus pneumoniae* isolates in children attending day-care centers in Brazil. Pediatr Infect Dis J 29: 77–79.
34. Shouval DS, Greenberg D, Givon-Lavi N, Porat N, Dagan R (2009) Serotype coverage of invasive and mucosal pneumococcal disease in Israeli children younger than 3 years by various pneumococcal conjugate vaccines. Pediatr Infect Dis J 28: 277–282.
35. Chiba N, Morozumi M, Sunaoshi K, Takahashi S, Takano M, et al. (2010) Serotype and antibiotic resistance of isolates from patients with invasive pneumococcal disease in Japan. Epidemiol Infect 138: 61–68.
36. Weinstein MP, Klugman KP, Jones RN (2009) Rationale for revised penicillin susceptibility breakpoints versus *Streptococcus pneumoniae*: coping with antimicrobial susceptibility in an era of resistance. Clin Infect Dis 48: 1596–1600.
37. Hollingshead SK, Baril L, Ferro S, King J, Coan P, et al. (2006) Pneumococcal surface protein A (PspA) family distribution among clinical isolates from adults over 50 years of age collected in seven countries. J Med Microbiol 55: 215–221.
38. Hollingshead SK, Becker R, Briles DE (2000) Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun 68: 5889–5900.
39. Briles DE, Tart RC, Swiatlo E, Dillard JP, Smith P, et al. (1998) Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). Clin Microbiol Rev 11: 645–657.
40. Jedrzejas MJ, Hollingshead SK, Lebowitz J, Chantalet L, Briles DE, et al. (2000) Production and characterization of the functional fragment of pneumococcal surface protein A. Arch Biochem Biophys 373: 116–125.
41. Jedrzejas MJ, Lamani E, Becker RS (2001) Characterization of selected strains of pneumococcal surface protein A. J Biol Chemi 276: 33121–33128.
42. Nabors GS, Braun PA, Herrmann DJ, Heise ML, Pyle DJ, et al. (2000) Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine 18: 1743–1754.
43. Vala Coral MC, Fonseca N, Castaneda E, Di Fabio JL, Hollingshead SK, et al. (2001) Pneumococcal surface protein A of invasive *Streptococcus pneumoniae* isolates form Colombian children. Emerg Infect Dis 7: 832–836.
44. Tart RC, McDaniel LS, Ralph BA, Briles DE (1996) Truncated *Streptococcus pneumoniae* PspA molecules elicit cross-protective immunity against pneumococcal challenge in mice. J Infect Dis 173: 380–386.
45. Briles DE, Hollingshead SK, King J, Swift A, Braun PA, et al. (2000) Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 182: 1694–1701.
46. Darrieux M, Miyaji EN, Ferreira DM, Lopes LM, Lopes AP, et al. (2007) Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody-mediated enhancement of complement deposition. Infect Immun 75: 5930–5938.
47. Mollerach M, Regueira M, Bonofiglio L, Callejo R, Pace L, et al. (2004) *Streptococcus pneumoniae* Working Group: Invasive *Streptococcus pneumoniae* isolates from Argentinean children: serotypes, families of pneumococcal surface protein A (PspA) and genetic diversity. Epidemiol Infect 132: 177–184.
48. Brandileone MC, Andrade AL, Teles EM, Zanella RC, Yara TI, et al. (2004) Typing of pneumococcal surface protein A (PspA) in *Streptococcus pneumoniae* isolated during epidemiological surveillance in Brazil: towards novel pneumococcal protein vaccines. Vaccine 28: 3890–3896.
49. Pimenta FC, Ribeiro-Dias F, Brandileone MCC, Miyaji EN, Leite LCC, et al. (2006) Genetic diversity of PspA types among nasopharyngeal isolates collecting during an ongoing surveillance study of children in Brazil. J Clin Microbiol 44: 2838–2843.
50. Beall B, Gherardi G, Fracklam RR, Hollingshead SK (2000) Pneumococcal pspA sequence types of prevalent multiresistant pneumococcal strains in the United States and of internationally disseminated clones. J Clin Microbiol 38: 3663–3669.
51. Heeg C, Franken C, van der Linden M, Ai-Lahlam A, Reiner RR (2007) Genetic diversity of pneumococcal surface protein A of *Streptococcus pneumoniae* meningitis in German children. Vaccine 25: 1030–1035.

52. Rolo D, Ardanuy C, Fleites A, Martín R, Liñares J (2009) Diversity of pneumococcal surface protein A (PspA) among prevalent clones in Spain. BMC Microbiol 9: 80–86.
53. Sadowy E, Skoczynska A, Fiett J, Gniadkowski M, Hryniewicz W (2006) Multilocus sequence types, serotypes, and variants of the surface antigen PspA in *Streptococcus pneumoniae* isolates from meningitis patients in Poland. Clin Vaccine Immunol 13: 139–144.
54. Melin MM, Hollingshead SK, Briles DE, Hanage WP, Lahdenkari M, et al. (2008) Distribution of pneumococcal surface protein A families 1 and 2 among *Streptococcus pneumoniae* isolates from children in finland who had acute otitis media or were nasopharyngeal carriers. Clin Vaccine Immunol 15: 1555–1563.
55. Imai S, Ito Y, Ishida T, Hirai T, Ito I, et al. (2011) Distribution and clonal relationship of cell surface virulence genes among *Streptococcus pneumoniae* isolates in Japan. Clin Microbiol Infect 17: 1409–1414.
56. Ito Y, Osawa M, Isozumi R, Imai S, Ito I, et al. (2007) Pneumococcal surface protein A family types of *Streptococcus pneumoniae* from community-acquired pneumonia patients in Japan. Eur J Clin Microbiol Infect Dis 26: 739–742.
57. Crain MJ, Turner JS, Robinson DA, Coffey TJ, Brooks-Walter A, et al. (1996) Evidence for the simultaneous expression of two PspAs by a clone of capsular serotype 6B *Streptococcus pneumoniae*. Microb Pathog 21: 265–275.
58. Robinson DA, Turner JS, Facklam RR, Parkinson AJ, Breiman RF, et al. (1999) Molecular characterization of a globally distributed lineage of serotype 12F *Streptococcus pneumoniae* causing invasive disease. J Infect Dis 179: 414–422.
59. Niki Y, Hanaki H, Matsumoto T, Yagisawa M, Kohno S, et al. (2009) Nationwide surveillance of bacterial respiratory pathogens conducted by the Japanese Society of Chemotherapy in 2007: general view of the pathogens' antibacterial susceptibility. J Infect Chemother 15: 156–167.
60. Niki Y, Hanaki H, Yagisawa M, Kohno S, Aoki N, et al. (2008) The first nationwide surveillance of bacterial respiratory pathogens conducted by the Japanese Society of Chemotherapy. Part 1: a general view of antibacterial susceptibility. J Infect Chemother 14: 279–290.
61. Hotomi M, Billal DS, Shimada J, Suzumoto M, Yamauchi K, et al. (2006) High prevalence of *Streptococcus pneumoniae* with mutations in *pbp1a*, *pbp2x*, and *pbp2b* genes of penicillin-binding proteins in the nasopharynx in children in Japan. ORL J Otorhinolaryngol Relat Spec 68: 139–145.
62. Hotomi M, Billal DS, Kamide Y, Kanesada K, Uno Y, et al. (2008) Serotype distribution and penicillin resistance of *Streptococcus pneumoniae* isolates from middle ear fluids of pediatric patients with acute otitis media in Japan. J Clin Microbiol 46: 3808–3810.

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Evaluation of a Rapid Immunochromatographic ODK-0901 Test for Detection of Pneumococcal Antigen in Middle Ear Fluids and Nasopharyngeal Secretions

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Abstract

Since the incidence of penicillin-resistant *Streptococcus pneumoniae* has been increasing at an astonishing rate throughout the world, the need for accurate and rapid identification of pneumococci has become increasingly important to determine the appropriate antimicrobial treatment. We have evaluated an immunochromatographic test (ODK-0901) that detects pneumococcal antigens using 264 middle ear fluids (MEFs) and 268 nasopharyngeal secretions (NPSs). A sample was defined to contain *S. pneumoniae* when optochin and bile sensitive alpha hemolytic streptococcal colonies were isolated by culture. The sensitivity and specificity of the ODK-0901 test were 81.4% and 80.5%, respectively, for MEFs from patients with acute otitis media (AOM). In addition, the sensitivity and specificity were 75.2% and 88.8%, respectively, for NPSs from patients with acute rhinosinusitis. The ODK-0901 test may provide a rapid and highly sensitive evaluation of the presence of *S. pneumoniae* and thus may be a promising method of identifying pneumococci in MEFs and NPSs.

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Introduction

Acute otitis media (AOM) is a common bacterial infection during childhood and frequently accompanies acute rhinosinusitis. By 3 years of ages, 50–70% of children have experienced at least one episodes of AOM, with 15–20% of children suffering from recurrent episodes of AOM [1,2]. *Streptococcus pneumoniae* is the major bacterial cause of both AOM and acute rhinosinusitis, followed by *Haemophilus influenzae* and *Moraxella catarrhalis* [3]. Simultaneous cultures from middle ear fluids (MEFs) and nasopharyngeal secretions (NPSs) demonstrate the presence of identical pathogens [4].

Antibiotics are prescribed frequently for initial treatment of these infectious diseases. However, the choice of appropriate antibiotics is difficult since there is no rapid and accurate diagnostic test to identify the pathogen in the middle ear cavity. As a result, selective pressure by the frequent use of empirical antimicrobial treatment has increased the incidence of antibiotic-resistant pathogens in childhood [5–8]. One alternative to antibacterial therapy for physicians would be to observe the affected children without administering antibiotics [9,10]. However, this alternative is undoubtedly accompanied by the risk of worsening infections [11,12]. A safer approach to the treatment of AOM and acute rhinosinusitis would be the accurate and rapid determination of the causative pathogen such as *S. pneumoniae* followed by prompt antibacterial therapy using antibiotics appropriate for the detected pathogen. Identification of the causative pathogen as early as possible and selection of a suitable antibacterial drug are thus desirable to prevent persistent and recurrent infections. Unfortunately, an effective tool for the rapid diagnosis of middle ear infections had not yet been developed.

While bacterial culture with Gram stain is the gold standard for identifying *S. pneumoniae*, bacterial culture requires several days to complete [13–15]. In addition, the prior administration of antimicrobial agents reduces the ability of conventional bacterial cultures to accurately identify the pathogen. An antigen detection test can demonstrate the presence of non-viable bacteria. The latex agglutination test and countercurrent immunoelectrophoresis have both been applied to *S. pneumoniae* identification, but both are insufficient as routine diagnostic methods in general practice because of their lack of sensitivity and specificity [16–19]. At present, polymerase chain reaction (PCR) is considered the most sensitive and specific test, but it is both expensive and requires a complicated process [20].

The rapid immunochromatograpy test for the urinary pneumococcal antigen, the Binax NOW® *Streptococcus pneumoniae* test (Binax, Inc., Portland, MA), shows high specificity (>90%) and high sensitivity (50–80%) in adult pneumonia and is thus a useful tool for identifying severe pneumococcal pneumonia in adults [21–23].

However, the use of a urine sample as a diagnostic tool for AOM and acute rhinosinusitis has limited value, especially for children. Most children with pneumococcal AOM have antigen-negative urine samples [24]. In addition, the majority of healthy children carrying *S. pneumoniae* in the nasopharynx may have antigen-positive urine samples [25–28]. In children who recently received pneumococcal vaccination, the urinary test may be positive [29]. Sampling of urine of infants with AOM or rhinosinusitis may be inconvenient. The Binax NOW® urinary antigen test and the RAPIRUN® *S.pneumoniae* antigen detection test for sputum have been adapted for respiratory infections [23,30–33].

The ODK-0901 test (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) is a diagnostic kit that uses polyclonal antibodies to detect pneumococcal polysaccharides directly from MEFs and NPSs. The present study was designed to evaluate the ability of ODK-0901 test to detect *S. pneumoniae* among patients with AOM and acute rhinosinusitis in a clinical setting.

Materials and Methods

Study populations

This case-control study was conducted between December 2009 to March 2010 in seven hospitals and clinics in Japan. Patients with AOM and/or acute rhinosinusitis were eligible to be enrolled into this study without regard to age and gender, previous or current antimicrobial treatments, and inpatient or outpatient status. Diagnostic criteria for AOM included an acute onset of symptoms including ear pain, fever, and, for young children, crying combined with abnormal tympanic membrane findings with redness, bulging, and obliteration of landmarks. Specimens of MEFs were collected with the ATOMS® tap middle ear aspirator (LUMENIS Co., Ltd., Tokyo, Japan) or sterile swabs after myringotomy under anesthesia of the tympanic membrane. The diagnostic criteria for acute rhinosinusitis included acute onset of symptoms including nasal discharge, headache/irritability, and moist cough combined with postnasal discharge. Specimens of NPSs were obtained with the ATOMS® tap or sterile swabs. Immediately after testing via the ODK-0901 test and plating the specimens for cultures, the samples were stored at −80°C until real-time PCR was performed.

This study was approved by the Institutional Review Board of the Ethical Committee of Nishinomiya Kyoritsu Neurosurgical Hospital and Tohoku Rosai Hospital. Before collecting samples, informed consent was obtained from the patients or from the parents or guardians for pediatric patients.

Bacterial cultures

Bacterial culture was used as the gold standard for the presence of *S. pneumoniae* in samples. Approximately 10 µl of sample collected by sterile swabs was plated on blood and chocolate agar plates and then incubated for 24 to 48 h at 37°C under a 5% CO_2 environment according to standard laboratory procedures. *S. pneumoniae* were identified by alpha-hemolysis and colony morphology on 5% sheep blood agar, Gram stain characteristics, optochin sensitivity, and bile solubility. *H. influenzae* were identified by growth on chocolate agar, colony morphology, Gram stain characteristics, and a growth requirement for X and V factors. *M. catarrhalis* were identified by colony morphology, Gram stain characteristics, and the biochemical reaction of butyrate esterase.

Detection of pneumococcal antigen by the ODK-0901 test

The ODK-0901 test uses rapid immunochromatography to detect pneumococcal C-polysaccharides (teichoic acid) (C-ps (TA)) and capsular polysaccharides by rabbit anti-pneumococcal polysaccharide polyclonal antibody immobilized on a nitrocellulose membrane. After a 5-min extraction of the sample using the extraction reagent, approximately 0.2 ml (4–5 drops) of the extracted sample was applied to an additional reservoir cup and incubated for 15 min with the ODK-0901 test at room temperature.

If there are any pneumococcal C-ps (TA) and capsular polysaccharides in the sample, it forms an immune complex with gold-colloid-binding anti-pneumococcal polysaccharide polyclonal antibody during the development of the sample and, subsequently, produces a red line, which is complemented by anti-pneumococcal polysaccharide polyclonal antibody on the test line. The gold-colloid-labeled antibody not complemented on the test line presents a red line complemented by anti-rabbit IgG goat polyclonal antibody on the control line after it passed the test line. Therefore, the sample is determined to be positive for pneumococcal polysaccharide when two red lines appear and is determined to be negative when there is only a control line. If the control line does not appear, the sample should be retested. Although the test requires approximately 15 minutes, if the two test lines are observed within 15 minutes, the sample can be determined to be positive.

Extractions of the 13 pneumococcal strains including D39 strain serotype 2, TIGR4 strain serotype 4, EF3030 strain serotype 19F and 10 clinical isolates of serotype 3, 6A (2 strains), 6B (2 strains), 4, 19A, 19F, 23F (2 strains) from the nasopharynx of children with AOM were used as positive controls. As negative controls extractions of 10 non-pneumococcal bacteria including nontypeable *H. influenzae* (3 strains), *Moraxella catarrhalis* (3 strains), *Streptococcus pyogenes* (4 strains) were used in this study.

The ODK-0901 test showed cross-reactivity only *Streptococcus mitis* (1 strain) but not for other streptococcus species including *S. anginosus* (1 strain), *S. agalactiae* (1 strain), *S. constellatus* (2 strains), *S. equi* (1 strain), *S. intermedius* (2 strains), *S. oralis* (1 strain), and *S. sanguinis* (1 strain). The ODK-0901 test also recognized the purified LTA/TA, C-ps (the Statens Serum Institute, Copenhagen, Denmark) and typed purified pneumococcal capsular antigen, type 1, 2, 3, 4, 5, 8, 9N, 12F, 14, 17F, 19F, 20, 22F, 23F, 25, 6B, 10A, 11A, 7F, and 15B (the American Type Culture Collection, Manassas, VA) (manufacture data).

Real-time PCR

The detection of pneumococci by real-time PCR was done in accordance with a previously reported assay procedure in Kitasato Otsuka Biomedical Assay Laboratories, Kanagawa, Japan [32].

Briefly, total genomic DNA was extracted by the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA). The relative amount of pneumococcal DNA genome was quantified by real-time PCR using primers and the TaqMan probe established for the region of the *pspA* gene of *S. pneumoniae*. Real-time PCR was then proceeded on a thermal cycler ABI7700 or ABI7900 (Applied Biosystems, Foster City, CA). The nucleotide sequences of the primers were as follows: forward primer: 5′-CAAGTCTAGCCAGCGTCGCTAT-3′; reverse primer: 5′-GGGAGATTCTTCTGCTCTTACAAAAG-3′, 5′-GGGAGATTCTTCTGCTCTTACCAAAG-3′, and 5′-GGGAGATTCTTCTGCTCTTACAACAG-3′; and carboxyfluorescein-labeled probe: 5′-(FAM)-CTGAGACGCAACAAAACCAGCCCC-(TAMRA)-3′, 5′-(FAM)-CTGAGACGTAACAAAACCAGCCCC-(TAMRA)-3′ and 5′-(FAM)-CGAAGACGCAACAAAACCAGCCCC-(TAMRA)-3′. RNase free water and DNA extracted from the *S. pneumoniae* ATCC6303 strain were used for negative and positive controls, respectively. After an initial denaturation at 95°C for 15 min, the PCR reaction

was followed by 50 cycles of amplification at 94°C for 15 sec and at 60°C for 1 min. Positive and negative controls were included for every PCR run. The standard curves depended on the cycle threshold (Ct) values of the positive controls. The number of copies of the *pspA* gene in the samples was calculated based on the standard curve. The sample with less than 40 copies was defined as negative for real-time PCR.

Statistics

Statistical analysis was done by Prism 5 (GraphPad Software, Inc., CA). A two tailed chi-square test or Fisher's exact test was used for categorical variables to test the significance of differences between groups. The pneumococcal DNA densities were compared by Mann-Whitney U test. A p-value of ≤ 0.05 was considered statistically significant. A 95% confidential interval (CI) was calculated.

Results

Populations

The 264 patients with AOM (250 with simple AOM and 14 with intractable OM) were enrolled in this study ranged in age from 0 to 56 years with a median age of 1 year and included 117 females and 147 males. There were 257 children (0 to 14 years old) and 7 adults (18 to 56 years old). The population of 268 patients with acute rhinosinusitis enrolled in this study included 264 patients with acute rhinosinusitis and 4 patients with acute exacerbation of chronic sinusitis and ranged in age from 0 to 75 years with a median age of 1 year. They were 249 children (0 to 14 years old) and 19 adults (18 to 75 year old). There were 124 females and 144 males. Two hundred and four patients had AOM and acute rhinosinusitis concurrently. Because of the small numbers of adult samples, we analyzed both MEF and NPS samples without regarding ages of patients. Finally, we obtained 264 MEFs including 14 otorrhea samples from patients with AOM and 268 NPSs from patients with acute rhinosinusitis.

Bacterial cultures

When the samples were tested via conventional bacterial cultures, *S. pneumoniae* was identified in 59 samples out of 264 MEFs (22.3%). Of these 59 samples, 51 *S. pneumoniae* strains were a single pathogen and 8 *S. pneumoniae* strains were combined with other pathogens. Seven MEFs contained *S. pneumoniae* combined with either or both *H. influenzae* and *M. catarrhalis* (6 MEFs contained *H. influenzae* and oneMEF contained both *H. influenzae* and *M. catarrhalis*). *H. influenzae* and *M. catarrhalis* were identified in 84 (31.8%) and 9 (3.4%) MEFs, respectively. In 96 (36.4%) MEFs, no pathogenic bacteria were identified.

Out of 268 NPSs, *S. pneumoniae* was detected in 161 (60.1%) samples. Twenty-six NPSs contained *S. pneumoniae* as a single pathogen. In contrast to the MEF samples, for the NPS samples 134 strains were combined with either or both *H. influenzae* or *M. catarrhalis* (41 NPSs contained *H. influenzae*, 36 NPSs contained *M. catarrhalis* and 57 contained both pathogens). Only one strain was combined with other pathogenic bacteria. *H. influenzae* and *M. catarrhalis* were identified in 159 (59.3%) and 139 (51.9%) NPSs, respectively. No pathogenic bacteria were identified in 16 NPSs.

Sensitivity and specificity of the ODK-0901 test for MEFs and NPSs

When the samples were evaluated with the ODK-0901 test, the pneumococcal antigen was detected in 88 (33.3%) MEFs. Compared with results obtained by conventional bacterial culture, the sensitivity, specificity, positive predicting value, and negative predicting value of the ODK-0901 test for MEFs were 81.4% (95% CI: 71.4%–91.2%), 80.5% (95% CI: 75.0%–85.9%), 54.5% (95% CI: 44.1%–64.9%), and 93.8% (95% CI: 90.1%–97.3%), respectively (Table 1).

On the other hand, the pneumococcal antigen was detected in 133 (49.6%) NPSs. The sensitivity was 75.2% (95% CI: 68.4%–81.8%), the specificity was 88.8% (95% CI: 82.8%–94.7%), and the positive and negative predicting values were 91.0% (95% CI: 86.1%–95.8%) and 70.4% (95% CI: 62.6%–78.0%) for NPSs, respectively, when compared with the results of conventional bacterial cultures (Table 1).

Influence of prior antimicrobial treatment on the ODK-0901 test

The sensitivity and specificity of the ODK-0901 test for MEFs of patients who had undergone prior antimicrobial treatment were further evaluated. We defined "prior antimicrobial treatment" as antimicrobial treatment within 4 weeks before the MEFs and NPSs were obtained. The sensitivity, specificity, positive predicting value, and negative predicting value of the ODK-0901 test on MEFs from patients with prior antimicrobial treatment compared with from patients without prior antimicrobial treatment were 80.0% (95% CI: 59.7%–100%), 82.6% (95% CI: 73.6%–91.5%), 50.0% (95% CI: 30.0%–70.0%), and 95.0% (95% CI: 89.4%–100%) compared with 81.8% (95% CI: 70.4%–93.2%), 79.4% (95% CI: 72.6%–86.2%), 56.3% (95% CI: 44.1%–68.4%), and 93.1% (95% CI: 88.4%–97.7%), respectively (Table 2). The sensitivity, specificity, positive predicting value, and negative predicting value of the ODK-0901 test on NPSs from patients with prior antimicrobial treatment compared with patients without prior antimicrobial treatment were 67.4% (95% CI: 53.8%–80.9%), 90.0% (95% CI: 79.2%–100%), 91.2% (95% CI: 81.6%–100%), and 64.3% (95% CI: 49.7%–78.7%) compared with 78.3% (95% CI: 70.7%–85.8%), 88.3% (95% CI: 81.1%–95.4%), 90.9% (95% CI: 85.2%–96.5%), and 73.1% (95% CI: 64.1%–82.1%), respectively (Table 3). There were no statistically significant differences between the sensitivity, specificity, positive predicting value, and negative predicting value of the MEFs and NPSs from patients with prior antimicrobial treatment and those from patients without prior antimicrobial treatment. However, a tendency was observed for the sensitivity of the ODK-0901 test to decrease for NPSs from patients with prior antimicrobial treatment.

Quantification of the *pspA* gene in MEFs and NPSs by real-time PCR

The number of *pspA* gene copies in MEFs was significantly higher among samples positive for the ODK-0901 test than among those of samples negative for the ODK-0901 test ($p<0.001$) (Fig. 1A). The median number of *pspA* gene copies in ODK-0901-

Table 1. Sensitivity and specificity of the ODK-0901 test for MEFs and NPSs.

		Culture for NPSs			Culture for MEFs		
		Positive	Negative	Total	Positive	Negative	Total
ODK-0901	Positive	48	40	88	121	12	133
	Negative	11	165	176	40	95	135
	Total	59	205	264	161	107	268

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Table 2. Sensitivity and specificity of the ODK-0901 test for MEFs depending on prior antimicrobial treatment.

		With prior treatment			Without prior treatment		
		Culture positive	Culture negative	total	Culture positive	Culture negative	Total
ODK-0901	Positive	12	12	24	36	28	64
	Negative	3	57	60	8	108	116
	Total	15	69	84	44	136	180

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positive and -negative MEFs were 8.0×10^5 copies/μg DNA and 40 copies/μg DNA, respectively. Sixty-four (36.4%) out of 176 MEFs negative for the ODK-0901 test contained the *pspA* gene, but 11 (6.3%) of them showed the growth of *S. pneumoniae* by the conventional culture. Nine (10.2%) out of 88 MEFs positive for the ODK-0901 test did not present the *pspA* gene. Only 1 (1.1%) MEF among the samples was positive by culture but not by the ODK-0901 test. There were no significant differences between the numbers of *pspA* gene copies in MEFs with or without prior antimicrobial treatment. The median number of *pspA* gene copies in ODK-0901-positive MEFs from patient having prior antimicrobial treatment compared with the median number from patients without prior antimicrobial treatment was 7.5×10^5 copies/μg DNA versus 8.0×10^5 copies/μg DNA.

The number of *pspA* gene copies in the ODK-0901-positive NPSs was also significantly higher than the number of copies in the ODK-0901-negative NPSs ($p<0,001$) (Fig. 1B). The median numbers of *pspA* gene copies in ODK-0901-positive and -negative NPSs were 2.0×10^6 copies/μg DNA and 40 copies/μg DNA, respectively. Similar to the results from MEFs, the number of *pspA* gene copies in NPSs exhibiting growth of *S. pneumoniae* was significantly higher than the number of copies in NPSs negative for *S. pneumoniae* by culture ($p<0.001$). Fifty-six (41.5%) out of 135 ODK-0901-negative NPSs contained the *pspA* gene, and 34 (25.2%) of them were culture positive. Seven (5.3%) out of 133 NPSs positive for the ODK-0901 test did not have the *pspA* gene, but only 2 (1.5%) of them were culture positive. The median numbers of *pspA* gene copies in ODK-0901-positive NPSs from both patients having prior antimicrobial treatment and patients without prior antimicrobial treatment were 4.0×10^6 copies/μg DNA.

Predictive value for middle ear pneumococci by evaluating nasopharyngeal secretions

For 204 cases of AOM, we evaluated the ability of the ODK-0901 test on NPSs to accurately make a bacteriologic assessment of AOM when compared with the bacteriologic assessment resulting from conventional bacterial cultures. The positive and negative predictive values of nasopharyngeal conventional bacterial cultures to detect the presence of pneumococci in MEFs were 31.6% (95% CI: 23.6%–39.4%) and 100%, respectively. In contrast, the positive and negative predictive values of the ODK-0901 test to detect the presence of pneumococci in MEFs were 32.7% (95% CI: 23.8%–41.6%) and 92.8% (95% CI: 87.6%–97.9%), respectively (Table 4). There were no statistically significant differences between the abilities of conventional bacterial cultures and the ODK-0901 test to negatively predict middle ear pathogens.

Discussion

Some attempts have been made to develop an immunochromatographic test suitable for the rapid detection of pneumococci in MEFs and NPSs in clinical situations. The advantage of such a test would be its ability to allow physicians to make earlier and more accurate decisions concerning the appropriate antimicrobial treatment for patients with AOM [24,34–37]. In the present study, we evaluated the clinical significance of a novel immunochromatographic ODK-0901 test that would allow the rapid and accurate detection of pneumococci in MEFs and NPSs.

The ODK-0901 test works better than bacterial culture in detecting the presence of *S. pneumoniae* because it recognizes C-ps (TA) and capsular polysaccharides from *S. pneumoniae*, even though *S. pneumoniae* have died out by prior antimicrobial treatment or inappropriate culture conditions. Further, no cross-reactivity was seen with type b *H. influenzae* contained phosphorylcholine, suggesting that the antibody did not react with phosphorylcholine carried in C-ps [38–40].

In this study, conventional bacterial cultures showed that 22.3% of MEFs and 60.1% of NPSs contained viable *S. pneumoniae*. In contrast, the ODK-0901 test used for the study detected *S. pneumoniae* antigen in 33.3% of MEFs and 49.6% of NPS. The ODK-0901 test yielded 81.4% sensitivity and 80.5% specificity for MEFs and 75.2% sensitivity and 88.8% specificity for NPSs. Faden et al. first reported the application of the Binax NOW test for detecting *S. pneumoniae* in MEFs from otitis media with effusion (OME) with a sensitivity of 80.0% and a specificity of 83.0% [35]. On the other hands, Gisselesson-Solen et al. reported that the Binax NOW test had the relatively high sensitivity of 90.5% and specificity of 82.4% for severe AOM and associated complications

Table 3. Sensitivity and specificity of the ODK-0901 test for NPSs depending on prior antimicrobial treatment.

		With prior treatment			Without prior treatment		
		Culture positive	Culture negative	total	Culture positive	Culture negative	Total
ODK-0901	Positive	31	3	34	90	9	99
	Negative	15	27	42	25	68	93
	Total	46	30	76	115	77	192

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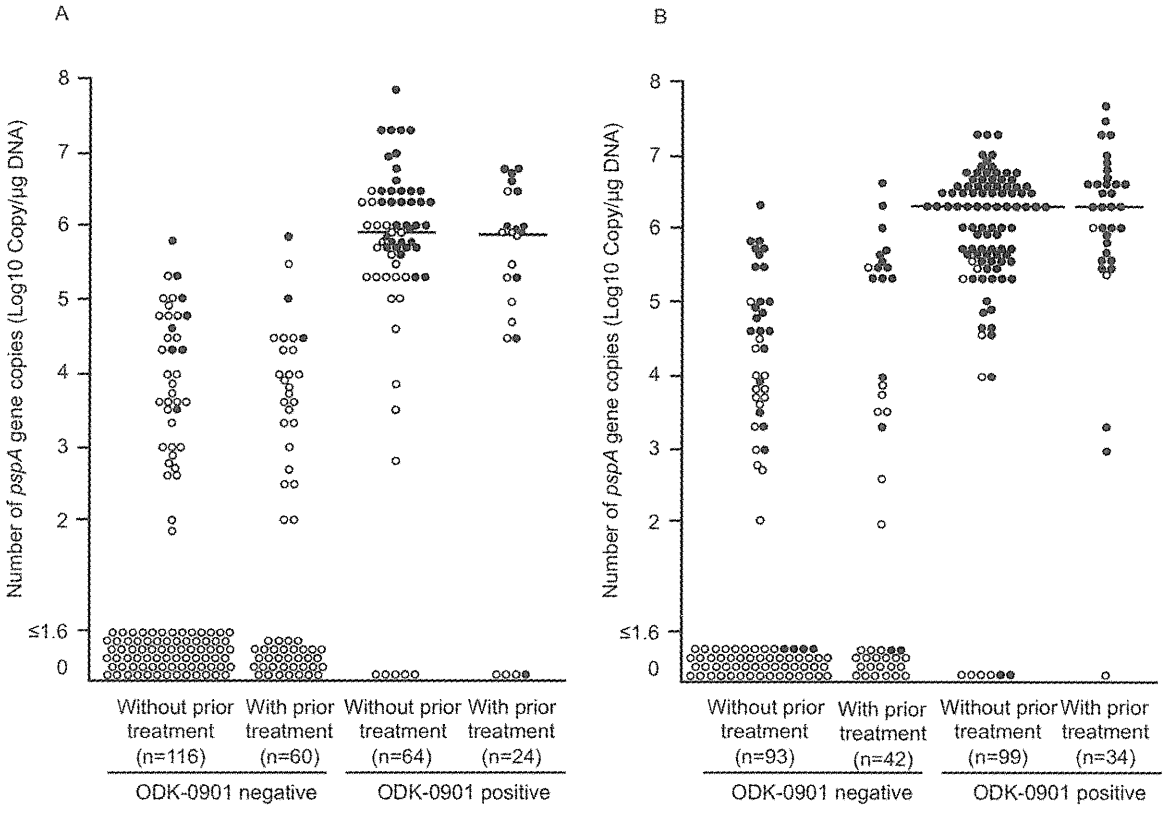

Figure 1. Distribution of the number of copies of *pspA* gene in MEF and NPS depending on prior antimicrobial treatment and the ODK-0901 test. The pneumococcal *pspA* gene was quantified by real-time PCR and the distribution was expressed. Open circles are culture-negative specimens. Closed circles are culture-positive specimens. A) Middle ear fluid; B) nasopharyngeal secretion.
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[24]. While this study focused on simple AOM, it did compare the sensitivity and specificity of the ODK-0901 test with the previous results by the Binax NOW test. The ODK-0901 test can directly apply to MEFs and may prove useful in selecting the most appropriate therapy for AOM. In contrast, the sensitivity of ODK-0901 test for NPSs was low at 75.2%. The sensitivity and specificity of the Binax NOW test in previous reports varied from 92.2% to 95.0% and from 78.0% to 97.7%, respectively [34,41].

Eleven samples out of 264 MEFs and 40 samples out of 268 NPSs exhibited false negatives to the ODK-0901 test due to the lower quantity of pneumococci in those MEFs and NPSs. Relatively small amounts of sample collected by swabs may lead to the false negative results for the ODK-0901 test. Some patients in this study with inconsistent ODK-0901 test results were concurrently undergoing or had undergone treatment with antibiotics. However, the study found that whether the patients had or had not undergone prior antimicrobial treatment made no differences in the pneumococcal density in MEFs and NPSs. As results, there were no differences in sensitivity and specificity of the ODK-0901 test based on the presence or absence of prior antimicrobial treatment. However, in particular, the ability of the ODK-0901 test to detect pneumococcal antigens in NPSs tended to be affected but not statistically significantly by the presence or absence of previous antibiotic treatment. Another possibility will be degeneration of polysaccharides of non-viable *S. pneumoniae* cells because real-time PCR indicated the presence of a relatively small amount of pneumococcal DNA.

Table 4. Prediction of middle ear pathogen by nasopharyngeal test.

			Middle ear fluid culture		
			Positive	**Negative**	**Total**
Nasopharyngeal secretion	Culture	Positive	42	91	133
		Negative	0	71	71
	ODK-0901	Positive	35	72	107
		Negative	7	90	97
	Total		42	162	204

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In contrast, false positives were observed in 40 MEFs and 12 NPSs. Like the Binax NOW test, the ODK-0901 test has already been confirmed to exhibit cross-reactivity with *S. mitis*, part of the bacterial flora of pharynx (manufacture data) [42–44]. However, alpha-streptococcus species including *S. mitis* was not identified in the MEFs from the 40 false positive patients. Another possibility is that *S. pneumoniae* in MEFs are affected by various products of inflammation. Thus, the pathogen sometimes does not grow well in conventional culture tests and is thus very difficult to identify. The samples for the 75% of the MEFs and NPSs that were false positive for the ODK-0901 test but were found to contain pneumococcal DNA via real-time PCR may have been of such degraded quality that bacterial culture was not able to detect *S.*

pneumoniae. The polysaccharide detection ability of ODK-0901 test as well as real-time PCR may be effective in *S. pneumoniae* with low biological activity. Furthermore, it is possible that some of the false positive samples contained a PspA null strain, which would result in the sample being negative for *pspA* gene via real-time PCR. Samples of 9 MEF and 7 NPSs were found to be negative by real-time PCR but were found to be positive by culture.

Since *S. pneumoniae* is one of the normal inhabitants of the nasopharynxes of children, its presence may create positives for antigen detection. Thus, the ODK-0901 test's detection of indigenous *S. pnuemoniae* in the nasopharynx will lead to overdiagnosis. In the current study, we further evaluated the pneumococcal DNA density in both MEFs and NPSs. The volume of MEFs obtained from AOM children is usually small and contains only a small number of organisms. It is important to evaluate the pneumococcal density in both types of specimens. Our study's use of real-time PCR proved that the ODK-0901 test yielded a positive result when the pneumococcal bacteria load was high at the affected site. Our previous study of nasopharyngeal carriage used real-time PCR to show that about 65% of children with upper respiratory infection had *S. pneumoniae* in the nasopharynx while conventional bacterial cultures of the same samples indicated that only 61% of the children were positive [45]. In practice, MEFs is not always available, and so nasopharyngeal secretions are sometimes used for bacteriological documentation. With the goal of using samples from nasopharyngeal colonization to predict the organism causing AOM, we evaluated the sensitivity, specificity, positive predictive value, and negative predictive value of culture test results from samples of middle ear fluid and nasopharyngeal secretions. We found essentially the same results as previous reports [24,34,35,41]. It was reported that nasopharyngeal cultures has meaningful negative predicting value for determining middle ear pathogens [46,47]. Based on the results of middle ear cultures as the gold standard, negative predicting value for *S. pnuemoniae* in MEFs were 100% by cultures and 92.8% by the ODK-0901 test, respectively.

The ODK-0901 test can thus be expected to be useful in infants from whom middle ear fluid cannot be collected. Because AOM may become persistent in young children, administration of appropriate antibiotics at an early stage of the treatment becomes especially important in both disease treatment and in the prevention of the development of drug-resistant bacteria. The current immunochromatography ODK-0901 test can become an important tool to help in the more rapid diagnosis of *S. pneumoniae* infections and in the subsequent administration of appropriate antibiotics earlier in the treatment cycle than was previously possible.

Author Contributions

Conceived and designed the experiments: MH NY. Performed the experiments: MH AT ST GS RS MK YF YK AU KK SS NO. Analyzed the data: MH NY. Contributed reagents/materials/analysis tools: MH YT YS NY. Wrote the paper: MH NY.

References

1. Faden H, Duffy L, Boeve M (1998) Otitis media: back to basics. Pediatr Infect Dis J 17: 1105–1113.
2. Teele DW, Klein JO, Rosner B (1989) Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. J Infect Dis 160: 83–94.
3. Bluestone CD, Stephenson JS, Martin LM (1992) Ten-year review of otitis media pathogens. Pediatr Infect Dis J 11: S7–11.
4. Hotomi M, Yamanaka N, Billal DS, Sakai A, Yamauchi K, et al. (2004) Genotyping of *Streptococcus pneumoniae* and *Haemophilus influenzae* isolated from paired middle ear fluid and nasopharynx by pulsed-field gel electrophoresis. ORL 66: 233–240.
5. Tähtinen PA, Laine MK, Huovinen P, Jalava J, Ruuskanen O, et al. (2011) A placebo-controlled trial of antimicrobial treatment for acute otitis media. N Engl J Med 364: 116–126.
6. Dagan R, Leibovitz E, Leiberman A, Yagupsky P (2000) Clinical significance of antibiotic resistance in acute otitis media and implication of antibiotic treatment on carriage and spread of resistant organisms. Pediatr Infect Dis J 19: S57–65.
7. Jacobs MR (2000) Increasing antibiotic resistance among otitis media pathogens and their susceptibility to oral agents based on pharmacodynamic parameters. Pediatr Infect Dis J 19: S47–56.
8. Hotomi M, Billal DS, Kamide Y, Kanesada K, Uno Y, et al. (2008) Serotype distribution and penicillin resistance of *Streptococcus pneumoniae* isolates from middle ear fluids of pediatric patients with acute otitis media in Japan. J Clin Microbiol 46: 3808–3810.
9. Damoiseaux RA, van Balen FA, Hoes AW, Verheij TJ, de Melker RA (2000) Primary care based randomized, double blind trial of amoxicillin versus placebo for acute otitis media in children aged under 2 years. BMJ 320: 350–354.
10. Van Buchem FL, Peeters MF, Van't Hof MA (1985) Acute otitis media: a new treatment strategy. Br Med J 290: 1033–1037.
11. Rovers MM, Glasziou P, Appelman CL, Burke P, McCormick DP, et al. (2007) Predictors of pain and/or fever at 3 to 7 days for children with acute otitis media not treated initially with antibiotics: a meta-analysis of individual patient data. Pediatrics 119: 579–585.
12. Rovers MM, Schilder AG, Zielhuis GA, Rosenfeld RM (2004) Otitis media. Lancet 363: 465–473.
13. Garcia-Vazquez E, Marcos MA, Mensa J, de Roux A, Puig J, et al. (2004) Assessment of the usefulness of sputum culture for diagnosis of community-acquired pneumonia using the PORT predictive scoring system. Arch Intern Med 164: 1807–1811.
14. Gleckman R, DeVita J, Hibert J, Pelletier C, Martin R (1988) Sputum gram stain assessment in community-acquired bacteremic pneumonia. J Clin Microbiol 26: 846–849.
15. Reed WW, Byrd GS, Gates RH, Jr., Howard RS, Weaver MJ (1996) Sputum gram's stain in community-acquired pneumococcal pneumonia. A meta-analysis. West J Med 165: 197–204.
16. Ballard TL, Roe MH, Wheeler RC, Todd JK, Glode MP (1987) Comparison of three latex agglutination kits and counterimmunoelectrophoresis for the detection of bacterial antigens in a pediatric population. Pediatr Infect Dis J 6: 630–634.
17. Slotved HC, Kaltoft M, Skovsted IC, Kerrn MB, Espersen F (2004) Simple, rapid latex agglutination test for serotyping of pneumococci (Pneumotest-Latex). J Clin Microbiol 42: 2518–2522.
18. Feigin RD, Wong M, Shackelford PG, Stechenberg BW, Dunkle LM, et al. (1976) Countercurrent immunoelectrophoresis of urine as well as of CSF and blood for diagnosis of bacterial meningitis. J Pediatr 89: 773–775.
19. Naiman HL, Albritton WL (1980) Counterimmunoelectrophoresis in the diagnosis of acute infection. J Infect Dis 142: 524–531.
20. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, et al. (2001) Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using Real-time PCR. J Clin Microbiol 39: 1553–1558.
21. Domínguez J, Galí N, Blanco S, Pedroso P, Prat C, et al. (2001) Detection of *Streptococcus pneumoniae* antigen by a rapid immunochromatographic assay in urine samples. Chest 119: 243–249.
22. Gutierrez F, Masia M, Rodriguez JC, Ayelo A, Soldan B, et al. (2003) Evaluation of the immunochromatographic Binax NOW assay for detection of *Streptococcus pneumoniae* urinary antigen in a prospective study of community-acquired pneumonia in Spain. Clin Infect Dis 36: 286–292.
23. Murdoch DR, Laing RT, Mills GD, Karalus NC, Town GI, et al. (2001) Evaluation of a rapid immunochromatographic test for detection of *Streptococcus pneumoniae* antigen in urine samples from adults with community-acquired pneumonia. J Clin Microbiol 39: 3495–3498.
24. Gisselsson-Solen M, Bylander A, Wilhelmsson C, Hermansson A, Melhus A (2007) The Binax NOW test as a tool for diagnosis of severe acute otitis media and associated complications. J Clin Microbiol 45: 3003–3007.
25. Dowell SF, Garman RL, Liu G, Levine OS, Yang YH (2001) Evaluation of Binax NOW, an assay for the detection of pneumococcal antigen in urine samples, performed among pediatric patients. Clin Infect Dis 32: 824–825.
26. Hamer DH, Egas J, Estrella B, MacLeod WB, Griffiths JK, et al. (2002) Assessment of the Binax NOW *Streptococcus pneumoniae* urinary antigen test in children with nasopharyngeal pneumococcal carriage. Clin Infect Dis 34: 1025–1028.
27. Navarro D, Garcia-Maset L, Gimeno C, Escribano A, Garcia-de-Lomas J, et al. (2004) Performance of the Binax NOW *Streptococcus pneumonieae* urinary antigen assay for diagnosis of pneumonia in children with underlying pulmonary diseases in the absence of acute pneumococcal infection. J Clin Microbiol 42: 4853–4855.
28. Murdoch DR, Reller LB (2003) Immunochromatographic test for rapid detection of *Streptococcus pneumoniae* in the nasopharynx. J Clin Microbiol 41: 2271.

29. Priner M, Cornillon C, Forestier D, Valero S, Paccalin M (2008) Might *Streptococcus pneumoniae* urinary antigen test be positive because of pneumococcal vaccine? J Am Geriatr Soc 56: 170–171.
30. Marcos MA, Jimenez de Anta MT, de la Bellacasa JP, Gonzalez J, Martinez E, et al. (2003) Rapid urinary antigen test for diagnosis of pneumococcal community-acquired pneumonia in adults. Eur Respir J 21: 209–214.
31. Ehara N, Fukushima K, Kakeya H, Mukae H, Akamatsu S, et al. (2008) A novel method for rapid detection of *Streptococcus pneumoniae* antigen in sputum and its application in adult respiratory tract infections. J Med Microbiol 57: 820–826.
32. Izumikawa K, Akamatsu S, Kageyama A, Okada K, Kazuyama Y, et al. (2009) Evaluation of a rapid immunochromatographic ODK0501 assay for detecting *Streptococcus pneumoniae* antigen in sputum samples from patients with lower respiratory tract infection. Clin Vaccine Immunol 16: 672–678.
33. Murdoch DR, Laing RT, Cook JM (2003) The NOW *S. pneumoniae* urinary antigen test positivity rate 6 weeks after pneumonia onset and among patients with COPD. Clin Infect Dis 37: 153–154.
34. Faden H, Heimerl M, Goodman G, Winkelstein P, Varma C (2002) New technique (the NOW test) for rapid detection of *Streptococcus pneumoniae* in the Nasopharynx. J Clin Microbiol 40: 4748–4749.
35. Faden H, Poje C, Pizzuto M, Nagy M, Brodsky L (2002) A new technique (the NOW test) for the detection of *Streptococcus pneumoniae* in the effusions of otitis media. J Laryngol Otol 116: 499–501.
36. Faden H, Heimerl M, Varma C, Goodman G, Winkelstein P (2002) Urinary excretion of pneumococcal cell wall polysaccharide in children. Pediatr Infect Dis J 21: 791–793.
37. Okitsu N, Yano H, Ohshima H, Sagai S, Irimada M, et al. (2011) Binax NOW® *Streptococcus pneumoniae* test of middle ear fluid for detecting causative pathogens in children with acute otitis media. J Microbiol Methods 84: 341–342.
38. Kolberg J, Hoiby EA, Jantzen E (1997) Detection of the phosphorylcholine epitope in streptococci, Haemophilus and pathogenic Neisseriae by immunoblotting. Microbial Pathogenesis 22: 321–329.
39. Sorensen UBS (1995) Pneumococcal polysaccharide antigens: capsules and C-polysaccharide. An immunochemical study. Danish Medical Bulletin 42: 47–53.
40. Poxton IR, Tarelli E, Baddiley J (1978) The structure of C-polysaccharide from the walls of streptococcus pneumoniae. Biochem J 175: 1033–42.
41. Vuorenoja K, Jalava J, Lindholm L, Tähtinen PA, Laine MK, et al. (2011) Detection of *Streptococcus pneumoniae* carriage by the Binax NOW test with nasal and nasopharyngeal swabs in young children. Eur J Clin Microbiol Infect DisIn press.
42. Alonso-Tarres C, Cortes-Lletget C, Casanova C, Domenech A (2001) False-positive pneumococcal antigen test in meningitis diagnosis. Lancet 358: 1273–1274.
43. Gillespie SH, McWhinney PHM, Patel S, Raynes JG, McAdam KPWJ, et al. (1993) Species of alpha-hemolytic streptococci possessing a C-polysaccharide phosphorylcholine-containing antigen. Infect Immun 61: 3076–3077.
44. Sjogren AM, Holmberg H, Krook A (1987) Etiologic diagnosis of pneumonia by antigen detection: crossreactions between pneumococcal C-polysaccharide and oral microorganisms. Diagn Microbiol Infect Dis 6: 239–248.
45. Ogami M, Hotomi M, Togawa A, Yamanaka N (2010) A comparison of conventional and molecular microbiology in detecting differences in pneumococcal colonization in healthy children and children with upper respiratory illness. Eur J Pediatr 169: 1221–1225.
46. Eldan M, Leibovitz E, Piglansky L, Raiz S, Press J, et al. (2000) Predictive value of pneumococcal nasopharyngeal cultures for the assessment of nonresponsive acute otitis media in children. Pediatr Infect Dis J 19: 298–303.
47. Gehanno P, Lenoir G, Barry B, Bons J, Boucot I, et al. (1996) Evaluation of nasopharyngeal cultures for bacteriologic assessment of acute otitis media in children. Pediatr Infect Dis J 15: 329–332.

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Review article

A review of the burden of disease due to otitis media in the Asia-Pacific

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ABSTRACT

Objective: The burden of disease due to otitis media (OM) in Asia Pacific countries was reviewed to increase awareness and raise understanding within the region.
Methods: Published literature and unpublished studies were reviewed.
Results: In school-age children, OM prevalence varied between 3.25% (Thailand) and 12.23% (Philippines) being highest (42%) in Aboriginal Australian children. OME prevalence at school age varied between 1.14% (Thailand) and 13.8% (Malaysia). Higher prevalence was reported in children with hearing impairment, HIV, pneumonia and rhinitis. CSOM prevalence was 5.4% in Indonesia (all ages), 15% in Aboriginal Australian children and 2–4% in Thailand, Philippines, Malaysia and Vietnam (WHO estimate). OM prevalence/incidence and service utilisation were highest in children 2–5 years of age. The disease burden was substantially higher in Pacific Island children living in New Zealand (25.4% with OME), and was highest in indigenous Australians (>90% with any OM). *Streptococcus pneumoniae* and *Haemophilus influenzae* dominated as primary causes of AOM in all studies. Few studies examined pneumococcal serotype distribution. Health-related cost estimates for OM, when available, were substantial. In developing countries, significant investment is needed to provide facilities for detection and treatment of ear disease in children, if long term hearing deficits and other sequelae are to be prevented.
Conclusion: The available evidence suggests an important burden of disease and economic cost associated with OM in most Asia Pacific countries and a potential benefit of prevention through vaccination. Large, prospective community-based studies are needed to better define the prevalence of ear disease in children, and to predict and track pneumococcal conjugate vaccine impacts. AOM prevention through vaccination may also provide a means of reducing antibiotic use and controlling antibiotic-resistant disease in children. This review highlights the need for additional research, and provides a basis on which to build and develop regional guidelines for OM management.

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