表1　わが国における23価肺炎球菌ポリサッカライドワクチンの接種対象者

2歳以上で肺炎球菌による重篤疾患に罹患する危険が高い次のような個人および患者
(1) 脾摘患者における肺炎球菌による感染症の発症予防（脾臓摘出後の2歳以上の場合のみ保険適用）
(2) 肺炎球菌による感染症の予防
1) 鎌状赤血球疾患，あるいはその他の原因で脾機能不全である患者
2) 心・呼吸器の慢性疾患，腎不全，肝機能障害，糖尿病，慢性髄液漏などの基礎疾患のある患者
3) 高齢者
4) 免疫抑制作用を有する治療が予定されている者で治療開始まで少なくとも14日以上の余裕のある患者

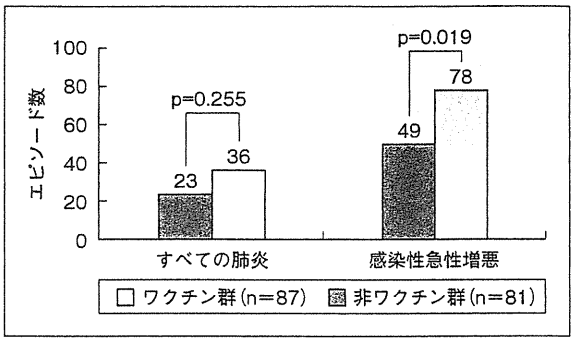

図2　慢性呼吸器疾患を対象としたPPVとインフルエンザワクチン併用接種の効果（文献[7]より引用）

肺炎球菌ワクチン

PPVは，現在93種類とされている肺炎球菌莢膜血清型のうち，23価を含む多価ワクチンである．肺炎球菌は呼吸器感染症の主な起炎菌であり，敗血症などの侵襲性感染症や肺炎などの表在性感染症を引き起こす．この23価の抗原は，わが国の市中肺炎の82.5%をカバーしていた（図1）[1]．したがって，23価ワクチンを用いれば大部分のわが国における肺炎球菌性肺炎の予防に有用と考えられる．

肺炎球菌ワクチンの適応

感染症のワクチンはインフルエンザワクチンなどウイルスに対するものが多数あるが，細菌感染症に対して実用化されているワクチンは未だ少ない．わが国でPPVが実用化されたのは1988年であり長い歴史があるが，保険適用は脾摘出後に限定されていることもあって，大規模な普及には至らなかった．近年，高齢化人口の増加とともに肺炎球菌感染症の予防対策としてPPVが注目されるようになり，現在では年間15万人以上が接種を受けるようになった．PPVの適応について表1に示す．主な接種対象者である65歳以上の高齢者は保険の適用がないため費用が自己負担となり，接種率向上の妨げとなっている．現在，全国の約30%の地方自治体で公費助成制度が実施されていて，65歳以上人口の推定接種率は11%に達している．しかし，この接種率には地方自治体の公費助成制度の有無などによる地域格差が生じており，今後は国による地域格差のない公費助成が望ましい状況にある．

副反応と再接種

PPVは比較的に副反応が少なく安全なワクチンである．副反応の多くは局所の発赤，腫脹であり，接種した腕全体が腫れることもあるが，現在までわが国において死亡につながる重篤な副反応は報告されていない．本ワクチンの接種後の有効期間は5年とされており，以前は一度接種した後の再接種が認められていなかったことが，ワクチン接種を躊躇させる原因の一つとなっていた．ようやく2009年10月に「65歳以上で最初のPPV接種から5年程度経過した場合に再接種が可能」と厚生労働省が認めた．このことにより，翌2010年くらいからは再接種も始まり，PPVの接種数はさらに増加傾向にある．

PPVの臨床効果について

PPVの侵襲性感染症にしては，古くからその有効性が報告されていた．2008年のWHOのstatement[2]では「免疫不全のない高齢者において，肺炎球菌ワクチン接種はワクチン血清型による侵襲性感染症を予防する」とされており，近年問題となったインフルエンザ感染との関わりでは「肺炎球菌ワクチンが使われている国々では，インフルエンザ流行時や新型インフルエンザパンデミック時の侵襲性感染症の頻度を減少する」とされている．しかし，表在性感染症である肺炎については「肺炎球菌ワクチン接種後の成人における

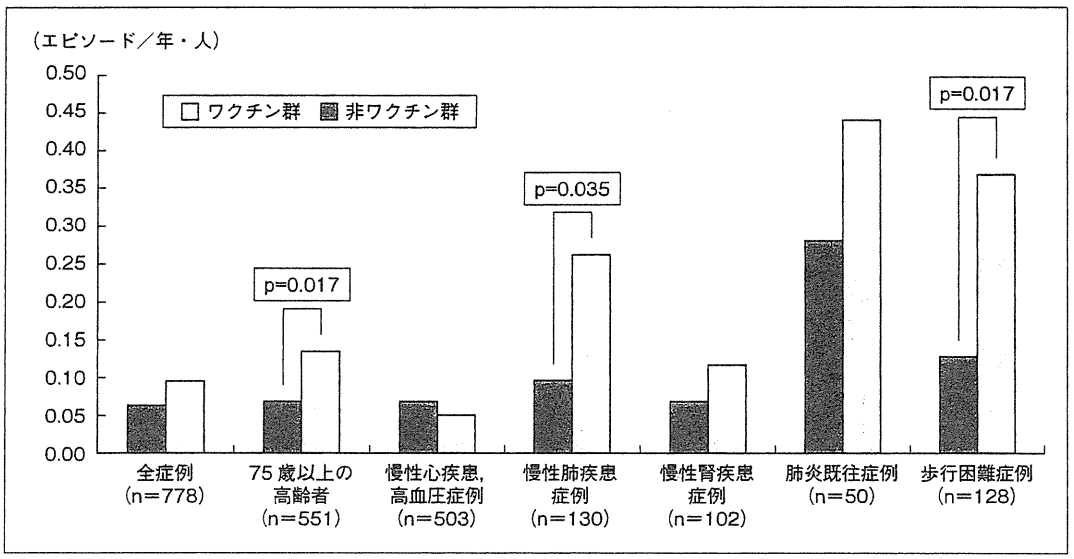

図3　すべての原因による肺炎のエピソード数—症例登録後1年間における比較(階層別)

市中肺炎はその生存率の改善，呼吸不全の頻度の低下，入院期間の短縮が認められ，重症化，死亡のリスクを軽減する」とされているものの，肺炎自体を減らすとは認められていなかった．この評価の元となる有効性に対するpositiveな報告としてFisman[3]やJhonstone[4]らの「市中肺炎患者の重症度と死亡のリスクを軽減する」という論文やAlfageme[5]らの「65歳未満のCOPD患者およびFEVが40%未満の重症COPD患者では肺炎を予防する」という論文がある．こういったことからPPVは2006年のGOLDではCOPDに対してBランクの推奨をされている．一方，肺炎のリスク低下に関するnegativeなエビデンスとしてJackson[6]らは「成人における全ての肺炎に対する効果が5つの無作為比較試験で検討されたが，いずれの試験でも有意な肺炎リスクの低下はみられていない」と報告している．

わが国でのエビデンスとして，慢性呼吸器疾患を対象とした肺炎球菌ワクチンとインフルエンザワクチン併用接種の効果についてわれわれが検討[7]したところ，肺炎については減少効果を示したが有意差は証明できず，感染性急性増悪については有意に減少させるという結果であった(図2)．近年，Maruyama[8]らは国内高齢者施設に入所中の高齢者1,006人を対象に多施設二重盲検無作為化プラセボ対照試験を施行し報告した．対象者は平均84.8歳でperformance statusは平均2.2，長期臥床は10%前後，ほぼ全例がインフルエンザワクチンを併用していた．この報告では，PPV接種によってすべての肺炎の予防効果，肺炎球菌性肺炎の頻度および死亡頻度の減少効果を認めるという効果が証明された．また，われわれは国立病院機構長崎川棚医療センターおよび長崎県東彼杵郡医師会の10カ所の開業医をインフルエンザワクチン接種のために受診した65歳以上の高齢者778人を対象に多施設非盲検無作為比較試験を施行し報告した[9]．結果は図3に示すとおり，すべての肺炎の減少傾向は認めたものの有意差がなかった．しかし，階層別では，75歳以上の高齢者，慢性肺疾患，杖歩行や車椅子乗車を可能な歩行困難症例において有意に肺炎の発症を抑止していた．さらにこの検討では図4に示すように肺炎の医療費についても検討した．ここでは全症例，75歳以上の高齢者，慢性肺疾患，歩行困難症例においてPPVの接種が有意に医療費を抑制するという結果を得た．

PPVの医療費削減効果

予防接種部会ワクチン評価に関する小委員会(肺炎球菌ワクチン作業部会)は，2011年3月に

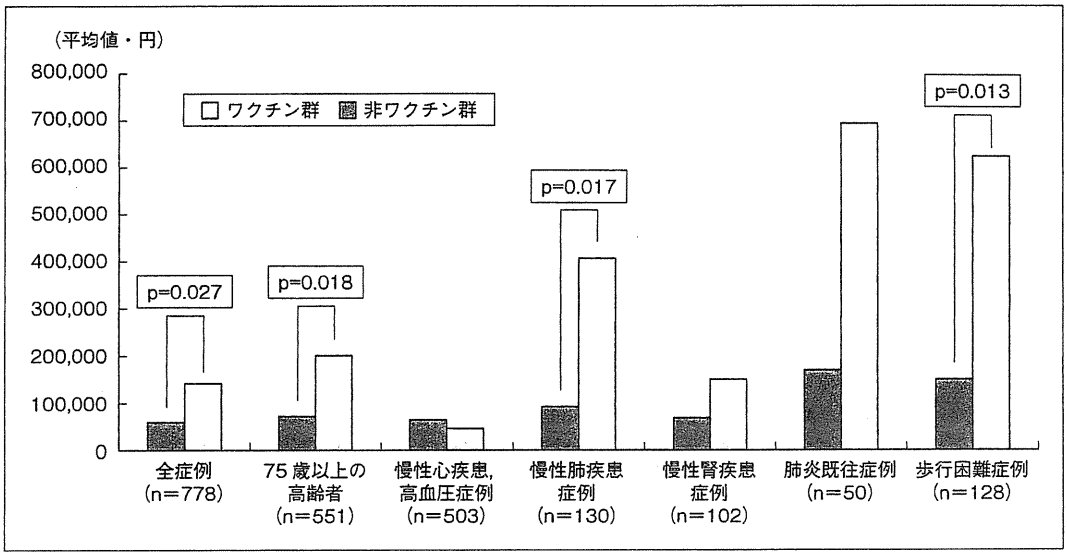

図4　すべての原因による肺炎の医療費総額―症例登録後1年間における比較(階層別)

表2　費用対効果分析結果(65歳コホート)

	一人当たりとして計算 (円, QALY)			コホート全体174.7万人 (億円, x10,000QALY)		
	投与	非投与	増分	投与	非投与	増分
ワクチン接種費	8,264	0	8,264	144	0	144
医療費	146,478	425,486	−279,008	2,559	7,433	−4,874
総費用	154,742	425,486	−270,744	2,703	7,433	−4,730
QALY	4.5470	4.5438	0.0032	794.3609	793.8019	0.5590

感度分析で割引率を0%から5%の間で変化させた場合，総費用は4,499億円～5,115億円の削減となる．効果に関しては65歳以上の集団データ[9]を使用して推定した．(文献[10]より引用)

PPVに関するファクトシートをまとめた．そのなかで医療経済評価を担当した厚生労働科学研究班は，毎年65歳の方全員にワクチン接種を行い，ワクチン接種の効果が5年間持続すると仮定すると保険医療費全体では毎年5,115億円が削減されるものと推定されると報告している[10]．ここで医療経済効果の指標としてQALY(quality adjusted life year)が用いられている．このQALYは単に生存年数の延長だけでなく生活の質を重み付けした指標で，日本語では質調整生存年と表記される．QALYが増えることは基本的に寿命が延びて生活の質が向上することを意味する．**表2**は医療コストとQALYの関係を示している．この表から，国の65歳コホート(65歳年齢の全人口)，75歳コホート，85歳コホートに対してPPVはその接種費用が問題にならないほどに医療費を削減する効果があり，しかも高齢者の健康アウトカム(QALY)を改善することが分かる(**図5**)．また，PPVの医療費削減効果は他の小児ワクチンと比較しても圧倒的に優れていることが示されている[10]．

おわりに

わが国のワクチン行政の節目の年にあたり，これまでわが国で蓄積されたPPVの安全性，臨床効果，医療費削減効果のエビデンスが，高齢者に対する地域格差のない公費助成，すなわち定期接種化の実現に繋がることが期待される．

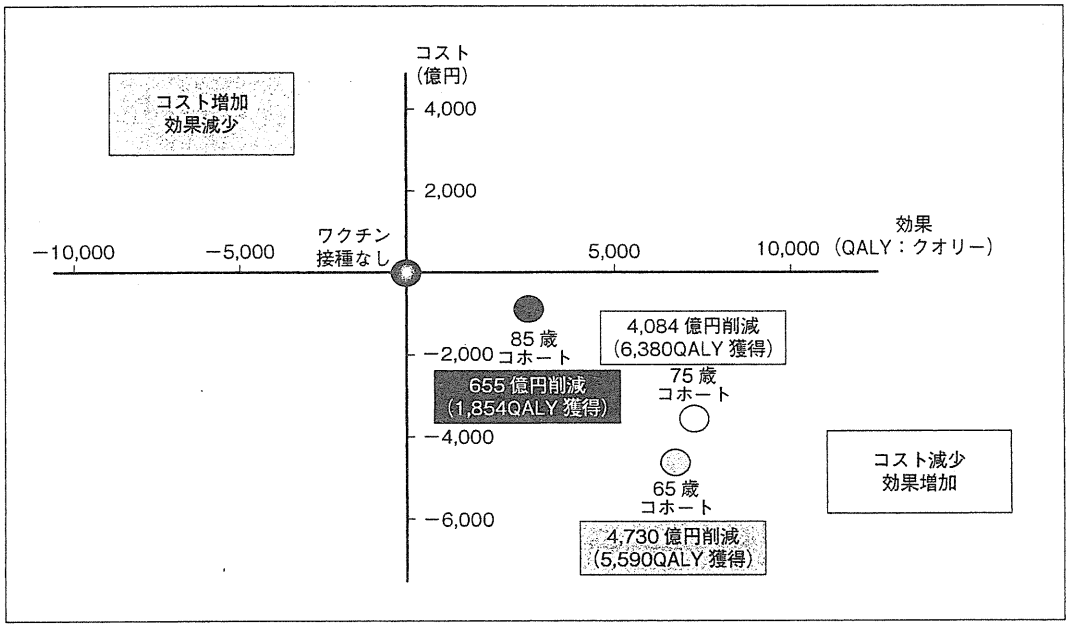

図5　費用対効果(文献[10]より引用)

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Q 肺炎球菌ワクチンの3回以降接種の可否

23価肺炎球菌莢膜ポリサッカライドワクチン（ニューモバックス®NP）を接種し，5年以降の再接種は国内でも認められているが，10年以降の再々接種，さらに15年以降の接種の可否，副反応についてはいかがか．海外の事情も含めて．

（静岡県　M）

A 米国，英国は23価肺炎球菌莢膜ポリサッカライドワクチン（PPV23）の3回以上の複数回接種を推奨するに至っていないが，我が国の平均寿命と肺炎球菌ワクチンの抗体維持期間を考慮すれば，複数回接種は必要と考えられる

現在，我が国では23価肺炎球菌莢膜ポリサッカライドワクチン（PPV23，ニューモバックス®NP）の製造販売が承認されている．1988年の薬事承認以来，本ワクチンを再接種・追加接種してはならない旨が添付文書に記載されていたが，2009年10月には初回接種から十分な間隔（5年以上）を確保することで再接種が承認されるに至っている．しかし，10年以降の再々接種，さらに15年以降の接種の可否については，異論のあるところである．

1994年に，米国アラスカ州は侵襲性感染症が高頻度な55歳以上のアラスカ住民に対して6年ごとのPPV23の接種を推奨した[1]．最近になって，Hammittらはこのアラスカ住民に対するPPV23の初回接種後（n=123），2回目接種後（n=121），3回目および4回目接種後（n=69）の3グループにおける主要な5血清型に対する血清型特異的血中抗体の推移，PPV23接種後の副反応について報告している[2]．

結果として，血清中血清型特異的IgG濃度は，血清型1を除けば，初回と複数回接種で同等であった．また，血清中血清型特異的オプソニン活性も，血清型6Bにおいて複数回接種で高い以外は，初回と複数回接種で同等であった．

一方，副反応については，複数回接種において関節痛，易疲労感，頭痛，局所の腫脹，中等度の腕の運動制限が，初回接種より多く認められた（表1）[2]．しかし，初回接種および複数回接種後30日間における死亡や重篤な副反応は認められていない．

これらの結果から，筆者らは6年以上ごとのPPV23の反復接種によって，これまで懸念されている複数回接種による低応答は認められず，さらにその安全性も容認できると考えている．

しかし，国内外を通じて未だPPV23の複数回接種による臨床効果のエビデンスはないのが現状であり，今後の検討が必要である．米国ACIP（Advisory Committee on Immunization Practices，予防接種勧奨委員会）は65歳以上の免疫能が正常で，肺炎球菌ワクチン接種後5年以上経過し，かつ前回接種が65歳未満であった場合には再接種を推奨しているものの，それ以後の複数回接種については言及していない[3]．また，2009年にはアラスカ州もACIPの推奨に従って，1回のみの再接種を推奨するとしている[4]．さらに，英国では無脾症，脾機能不全，慢性腎疾患などのハイリスク者では5年ごとの反復接種が

表1　接種回数による23価肺炎球菌ワクチン接種後4日以内の副反応頻度

副反応	初回接種 n=121	2回目接種 n=119	3回あるいは4回接種 n=69	P 初回接種との比較
発熱（37.5℃以上）	4 (3)	7 (6)	3 (4)	0.427
関節痛	24 (20)	45 (38)	3 (4)	0.004
悪寒	7 (6)	15 (13)	9 (13)	0.99
易疲労感	20 (17)	35 (29)	18 (26)	0.019
頭痛	11 (9)	30 (25)	9 (13)	0.014
発赤（すべての）	22 (18)	24 (20)	15 (22)	0.433
発赤（4cm以上）	1 (1)	3 (3)	4 (6)	0.133
腫脹（すべての）	21 (17)	36 (30)	23 (30)	0.006
腫脹（4cm以上）	2 (2)	3 (3)	2 (3)	0.701
強い腕の痛み	2 (2)	2 (2)	3 (4)	0.829
中等度の腕の運動制限	5 (4)	15 (13)	9 (13)	0.025

（　）内の数字は%.

（文献[2]より改変）

推奨されているものの，日常的な再接種は推奨されていない[5]．

すなわち，PPV 23の複数回接種の免疫原性，安全性からはその複数回接種は容認できるものの，複数回接種の臨床効果が明確になっていないことから，米国，英国は3回以上の複数回接種を推奨するに至っていないのが現状である．しかし，我が国における平均寿命（男性80歳，女性86歳）と肺炎球菌ワクチンの抗体維持期間を考慮すれば，PPV 23の複数回接種は必要と考えられる[6]．

一方，現在我が国においても成人用13価肺炎球菌コンジュゲートワクチン（PCV 13）の承認申請が準備中であるが，現時点では成人におけるPCV 13の役割は未だ明確ではない[7]．今後，PPV 23以外の選択肢としてPCV 13が加わることも予想されるが，我が国の高齢者に対する肺炎予防のための肺炎球菌ワクチンは1つであり，現時点で考えられる最善の肺炎予防対策を推進すべきである．

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▶回　答

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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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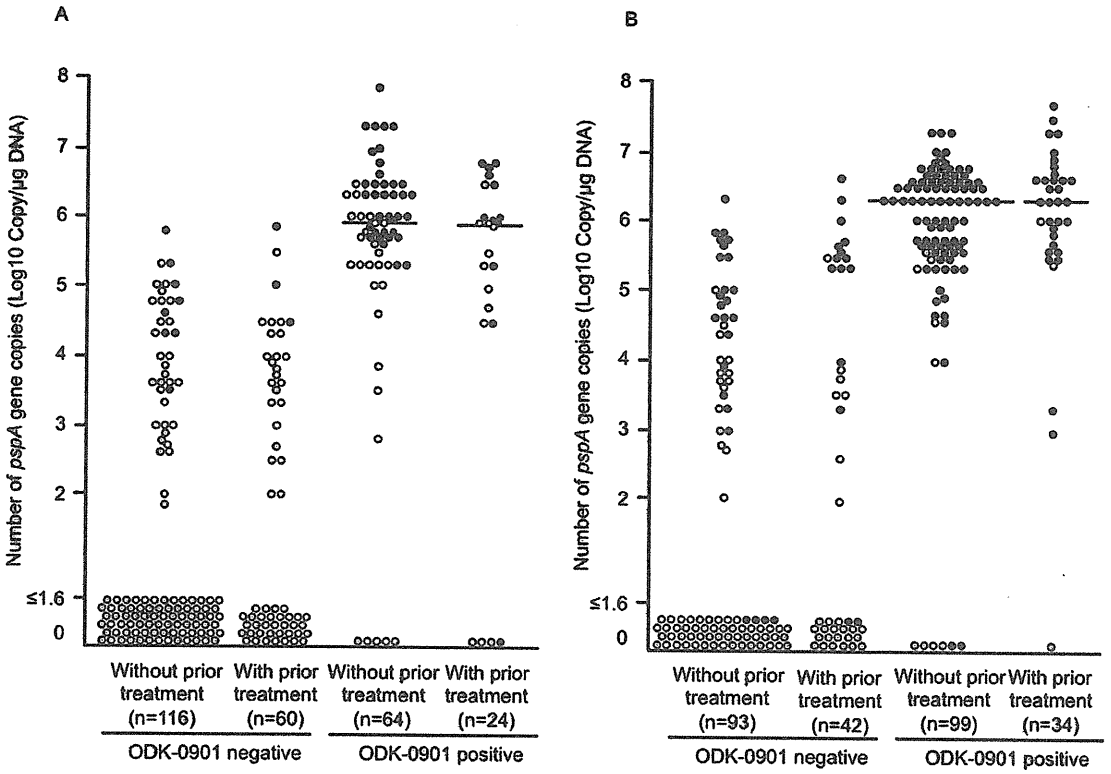

Figure 1. Distribution of the number of copies of *pspA* gene in MEF and NPS depending on prior antimicrobial treatment and the ODK-0901 test. The pneumococcal *pspA* gene was quantified by real-time PCR and the distribution was expressed. Open circles are culture-negative specimens. Closed circles are 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.

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Maternal Immunization with Pneumococcal Surface Protein A Protects against Pneumococcal Infections among Derived Offspring

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Abstract

Pathogen-specific antibody plays an important role in protection against pneumococcal carriage and infections. However, neonates and infants exhibit impaired innate and adaptive immune responses, which result in their high susceptibility to pneumococci. To protect neonates and infants against pneumococcal infection it is important to elicit specific protective immune responses at very young ages. In this study, we investigated the protective immunity against pneumococcal carriage, pneumonia, and sepsis induced by maternal immunization with pneumococcal surface protein A (PspA). Mother mice were intranasally immunized with recombinant PspA (rPspA) and cholera toxin B subunit (CTB) prior to being mated. Anti-PspA specific IgG, predominantly IgG1, was present at a high level in the serum and milk of immunized mothers and in the sera of their pups. The pneumococcal densities in washed nasal tissues and in lung homogenate were significantly reduced in pups delivered from and/or breast-fed by PspA-immunized mothers. Survival after fatal systemic infections with various types of pneumococci was significantly extended in the pups, which had received anti-PspA antibody via the placenta or through their milk. The current findings strongly suggest that maternal immunization with PspA is an attractive strategy against pneumococcal infections during early childhood. (191 words)

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Competing Interests: David E. Briles and Susan K. Hollingshead are faculty at the University of Alabama at Birmingham, which owns some intellectual property relating to PspA. 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 frequently colonize the nasopharynx asymptomatically. Especially following viral infections, *S. pneumoniae* are responsible for a significant proportion of bacterial infectious diseases such as meningitis, otitis media, bacteremia, and pneumonia [1]. The high incidence of pneumococcal disease starts in the neonatal period and peaks around the first birthday. Efforts have focused on the protection of children against pneumococcal infections by immunization with vaccines.

The current 23-valent pneumococcal polysaccharide vaccine (PPV) is efficacious in adults [2]. However, this polysaccharide-based vaccines evoke little or no immune response in infants younger than 2 years of age because of the weak immunogenicity of its T cell independent polysaccharides [3,4]. Protein-conjugated polysaccharide vaccines have been considered as an alternative means to induce protective immunity in infants and children [5,6]. Human trials of a 7-valent polysaccharide conjugate vaccine (PCV7) showed the capability to elicit solid protection against invasive pneumococcal infection in children [7–11]. However, PCV7 is not protective against strains with capsular types/groups not present in the vaccine [12,13]. Shortly after the vaccine was licensed, reports of serotype replacement began to appear [14–16]. Efforts to circumvent the problem of serotype replacement have included expanding the number of polysaccharides in the vaccine but this will not necessarily avoid the problem of subsequent serotype replacement [6,17,18].

Furthermore, children younger than 2-years old usually have low levels of IgG serum antibody to pathogen-specific antigens; a results of age-related immaturity of immune responses [19,20]. The recurrent bacterial infections are thought to be in part due to the subnormal levels of serum IgG antibody against causative pathogens due to age-related immaturity [20–23]. Virolainen et al showed that children who were infected most frequently with pneumococci had the lowest titer of antibody to PspA among children with invasive pneumococcal infections [24]. Simell et al have made a similar observation showing that higher salivary antibody levels to PspA are associated with a lower rate of pneumococcal otitis media [25]. The need for protein-based pneumococcal vaccines and their ability to protect against pneumococcal infections during infant period has been further emphasized by studies demonstrating a recent rapid increase in both the prevalence and levels of resistance of multiple antimicrobial resistant pneumococci [16]. Maternal immunization with PPV is reported to reduce acute lower respiratory infections in infants [26,27].

Pneumococcal surface protein A (PspA) is a promising candidate for inclusion in a cost-effective protein-based pneumococcal vaccine. PspA is an exposed virulence factor present in virtually all pneumococcal strains. It is a highly immunogenic antigen and affects host-pathogen interactions by inhibiting complement activation by the classical and alternative pathways [28–30]. PspA can elicit an antibody response that enhances complement deposition and protects against nasal carriage, pneumonia, and bacteremia in animal models [31–33]. Moreover, a human trial showed an increase in specific anti-PspA immunoglobulin G (IgG) levels after immunization with rPspA. Sera from the humans immunized with rPspA were able to passively protect mice against otherwise fatal challenge with various pneumococcal strains [34,35].

Our preliminary study evaluated the efficacy of maternal immunization with rPspA for protecting against lethal systemic pneumococcal infections [36]. In the current study, we further evaluated the relative roles of placental and milk/colostrum derived antibody in the protection against pneumococcal invasive disease and carriage in mouse pups following maternal immunization with rPspA. Prospective mother mice were intranasally immunized with rPspA. Antibody levels in the breast milk and serum of the mothers were measured and the transmission of antibody and protection to the pups was evaluated by challenging the offspring of mothers and the offspring of mice that were fostered on immunized and non-immunized mothers.

Results

Anti-PspA specific antibodies in sera and milk of mother mice

The levels of anti-PspA specific antibodies in sera of mother BALB/cByJ mice were evaluated on day 0, 7, and 14 after they gave birth (Fig. 1A). Anti-PspA specific IgG in sera of pups was present at the birth and maintained during nursing periods among PspA-immunized mother mice. Anti-PspA specific IgA and IgM were also identified among PspA-immunized mother mice although the levels of anti-PspA specific IgA and IgM in sera were relatively low rather than anti-PspA specific IgG. The levels of anti-PspA specific antibodies in sera among pre-immunized mother mice and sham-immunized mother mice were below the detections limit (data not shown).

The levels of anti-PspA specific antibodies in breast milk from mother mice were also evaluated on day 0, 7, and 14 after the birth (Fig. 1B). In breast milk higher levels of IgG PspA-specific antibody were detected relative to IgA or IgM specific antibody. The levels of anti-PspA specific antibodies in breast milk among pre-immunized mother mice and sham-immunized mother mice were below the detections limit (data not shown).

IgG subclasses of antibody to PspA in sera and milk of mother mice

On the day of birth (day 0) the predominant IgG subclass of IgG antibody to PspA in sera from PspA-immunized mother mice was IgG1, followed by IgG2a and IgG2b (Fig. 2A). The levels of anti-PspA specific IgG2a gradually increased from day 0 to day 14 ($p<0.05$), while the levels of anti-PspA specific IgG1 and IgG2b did not change significantly. The mean IgG1/IgG2a ratio in the sera of individual mice gradually decreased from 3.1 on day 0 to 1.1 on day 14 ($p<0.05$). The levels of IgG3 were below the detection limit.

In the breast milk, IgG1 was also the predominant anti-PspA specific IgG subclass followed by IgG2a and IgG2b from PspA-immunized mother mice (Fig. 2B). The levels of anti-PspA specific IgG2a and IgG2b increased from day 0 to day 14 ($p<0.05$), while IgG1 did not change. The mean IgG1/IgG2a ratio in breast milk from individual mice gradually decreased from 5.7 on day 0 to 1.7 on day 14 ($p<0.05$). The levels of IgG3 were below the detection limit.

Anti-PspA specific antibodies in sera of offspring

The changes of anti-PspA specific IgG in the sera of offspring were evaluated on day 0, 7, and 14 after the birth (Fig. 3). Offspring delivered from PspA-immunized mothers (Group A and Group C) had high levels of anti-PspA specific IgG in sera at birth. The levels of anti-PspA specific IgG in sera from offspring of immune mothers that were breast-fed by PspA-immunized mother mice (Group A) were maintained at the high levels on day 7 and day 14. In contrast the levels of anti-PspA specific IgG in sera from offspring of immune mothers who were breast-fed by sham-immunized mother mice (Group C) rapidly declined after the birth. On the other hand, offspring delivered from sham-immunized mother mice (Group B and Group D) did not have anti-PspA specific IgG in sera at the birth. The PspA-specific IgG in sera from offspring of sham-immune mothers that were breast-fed by PspA-immunized mothers (Group B) gradually increased and reached levels similar to those of Group A on day 7 to day 14. The control offspring (Group D) from sham-immunized mothers who were nursed on sham-immune mothers did not have anti-PspA specific IgG in their sera. Anti-PspA specific IgA and IgM were not detected in sera of all offspring.

Anti-PspA specific IgG subclasses in sera of offspring

The levels of anti-PspA specific IgG subclass in sera of offspring were also evaluated on day 0, 7, and 14 after the birth (Fig. 4). The predominant IgG subclass in sera of offspring in all groups was IgG1 followed by IgG2a and IgG2b at the birth. In all offspring, the levels of IgG3 were below the detection limit.

In Group A, the levels of IgG1 and IgG2a were not changed during the period from day 0 to day 14. In contrast to the results of group A mothers' sera, the mean IgG1/IgG2a ratio calculated from the individual pup sera also did not change during the period from day 0 to day 14. This indicates that IgG2 antibody was less efficiently transported to the progeny by nursing than the IgG1 antibody. In Group B, offspring did not have anti-PspA specific IgG in sera at the birth. The levels of anti-PspA specific IgG1 in sera gradually increased and reached the similar levels to those of the offspring in Group A on day 7 to day 14 ($p<0.05$ and $p<0.01$, respectively). The mean IgG1/IgG2a ratio for group B was higher on day 7 than that of Group A ($p<0.05$). On day 14 the mean IgG1/IgG2a ratio for Group B was higher than that of Group A, but the difference was not statistically significant. In Group C, anti-PspA specific serum IgG1 was predominant on day 0 and the levels did not change during the period from day 0 to day 14. The IgG2b levels in the Group C mice gradually decreased from day 0 to day 14. The mean IgG1/IgG2a ratios for Group C mice were higher than those of Group A mice on day 0 and increased from day 7 to day 14 ($p<0.01$).

Protection against nasal carriage of pneumococci by maternal immunization with PspA among offspring

The carriage density of pneumococci in nasal washes and homogenized washed nasal tissue were evaluated at day 2 after intranasal challenge with 5×10^5 CFUs of TIGR4 pneumococci. The numbers of CFUs in nasal washes were not different among groups. The median Log_{10} CFUs of pneumococci in nasal washes of Group A, B, C, and D was 4.55, 4.45, 4.24, and 4.49,

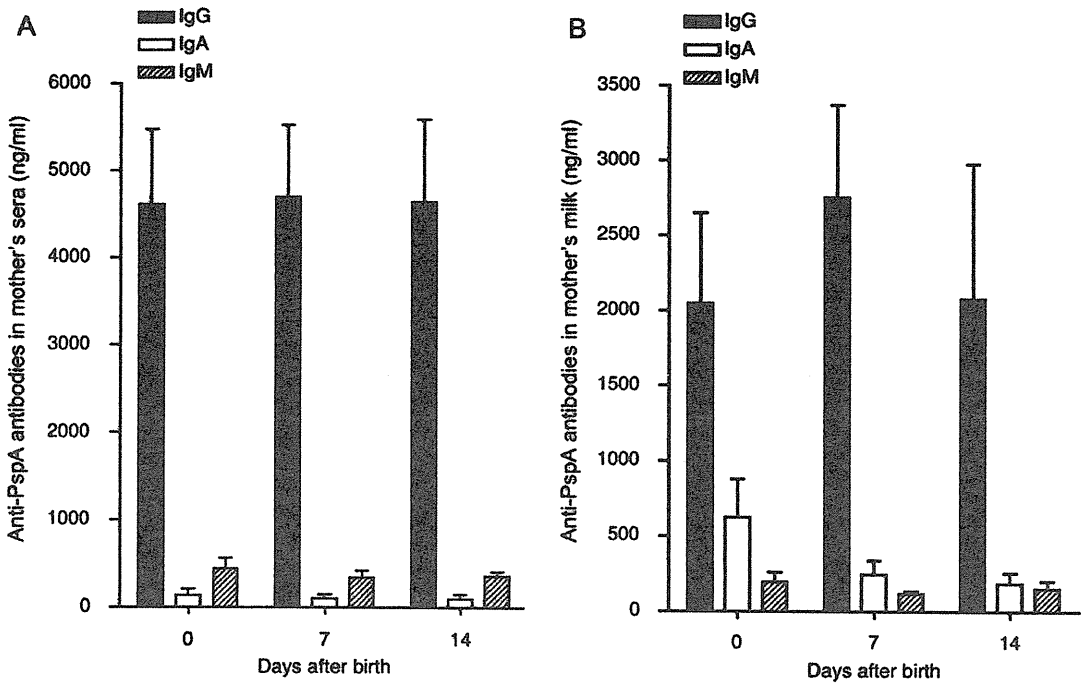

Figure 1. Anti-PspA specific antibodies in sera and milk of mother mice. Female mice were intranasally immunized twice each week with 1 μg of rPspA and 4 μg CTB for first 2 weeks and with 1 μg rPspA alone for the last week. The levels of anti-PspA specific IgG, IgA and IgM antibodies in sera (A) and breast milk (B) were determined by PspA-specific ELISA on day 0, 7 and 14 after the birth. The values shown are the mean ± S.E. concentrations (ng/ml) taken from PspA-immunized mother (n = 16) and sham-immunized mother (n = 14). The levels of anti-PspA specific antibodies in sera and breast milk from sham-immunized mice were below the limit of detection.
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respectively. On the other hand, the carriage density of the homogenized washed nasal tissue was significantly different among groups (Fig. 5). The median Log_{10} CFUs of pneumococci in nasal tissue of Group A, B, C, and D was 4.56, 4.77, 4.91, and 5.01, respectively. The carriage density of washed nasal tissues of Group A was statistically lower than those of Group D (control) ($p<0.05$). The Log_{10} CFUs of washed nasal tissue of Group B tended to be lower than that of Group D ($p<0.1$). There was no difference in CFUs in nasal tissue of Groups C and D. Thus, maternal immunization with PspA appeared to result in only a modest reduction of nasal colonization of nasal tissue among offspring nursed on immunized dams.

Protection against lung infection by maternal immunization with PspA among offspring

Inoculation of 7-day old offspring with a relatively large volume of inoculum intranasally under anesthesia caused enough pneumococci to be aspirated to cause infection of the lung. The 5×10^5 CFUs of TIGR4 pneumococci in 10 μl sterile Ringer's solution were inoculated intranasally into anesthetized offspring. At 3 days after inoculation, all mice were alive and were euthanized so that the numbers of CFU in their lungs and blood could be determined (Fig. 6). The median Log_{10} CFUs of pneumococci in Group A, B, C, and D was 1.91, 1.58, 1.90, and 2.73, respectively. The mean number of Log_{10} CFUs in lung homogenate in Group A and Group B were each significantly reduced in comparison to Group D ($p<0.05$ and $p<0.01$, respectively). Mice from immunized mothers who were not nursed by immune mothers (Group C) had fewer median CFU than the non-immune Group D mice, this difference was not statistically significant. In all cases there were no CFU or only a few in the blood. As a result, we can be confident that the protection seen was the result of events in the lung and not protection against sepsis. Based on these results immunity achieved through nursing appeared to be especially important for protection in this model.

Protection against fatal systemic pneumococcal infections among offspring through maternal immunization with rPspA

Survival of offspring after intraperitoneal infection with 1×10^4 CFUs of TIGR4 strain was evaluated. The survival after the otherwise fatal systemic pneumococcal infection was significantly extended in Groups A, B, and C as compared to the sham-immunized Group D controls ($p<0.01$ for each group) (Fig. 7). With this model, transfers of antibody by the placenta and/or nursing were all exhibited significant protection.

In all of the above studies we immunized with TIGR4 derived PspA and challenged with TIGR4 capsular type 4 strain. However, there is some variability in PspA and it is found in two broad serologically cross-reactive families; PspA serologic/sequence family 1 (PspA1) and PspA serologic/sequence family 2 (PspA2) [37]. It is generally recommended that in the development of a human vaccine that one PspA1 protein and one to two PspA2 proteins be used [38]. However, there have also been findings that in some studies that strong cross-protection could be observed between PspA1 and PspA2 families [39–43].

TIGR4 strain is PspA2 and we evaluated its ability to elicit immunity to four different PspA1 strains. In each case the highest

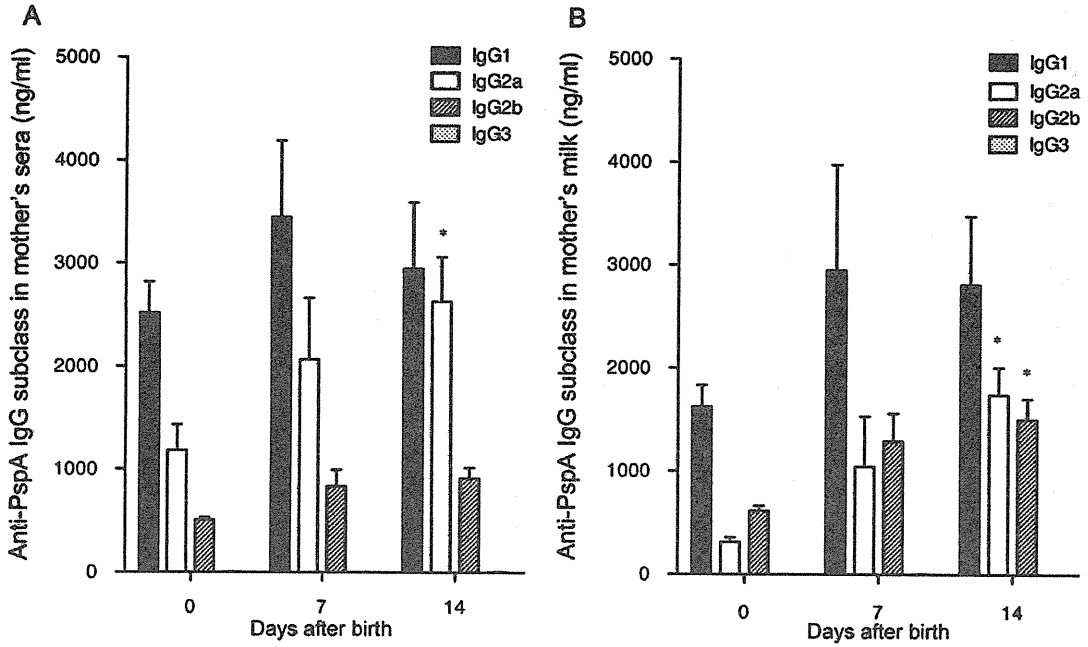

Figure 2. Anti-PspA specific IgG subclasses in sera and milk of mother mice. Female mice were intranasally immunized twice each week with 1 μg of rPspA and 4 μg CTB for first 2 weeks and with 1 μg rPspA alone for the last week. The levels of anti-PspA specific IgG1, IgG2a, IgG2b and IgG3 antibodies in sera (A) and breast milk (B) were determined by PspA-specific ELISA on day 0, 7 and 14 after the birth. The values shown are the mean ± S.E. concentrations (ng/ml) taken from PspA-immunized mother (n = 16) and sham-immunized mother (n = 14). The mean values of IgG1/IgG2a antibody to PspA in the sera of the individual mother's sera were 3.1, 2.1 and 1.1 for day 0, 7, and 14, respectively. The mean IgG1/IgG2a anti-PspA values for the individual mother's milk samples were 5.7, 6.5, and 1.7 for day 0, 7, and 14, respectively. The levels of anti-PspA specific IgG subclasses in sera and breast milk from sham-immunized mice were below the detections limit. * $p<0.05$ when compared with mice at day 0 by ANOVA test or Kruskal-Wallis test with Dunn's multiple comparison test. n.d. not determined.
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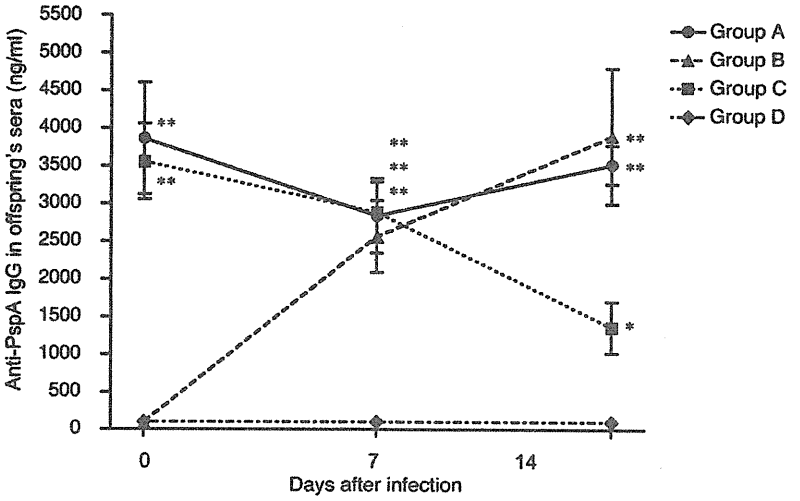

Figure 3. Anti-PspA specific antibodies in sera of offspring. The levels of anti-PspA specific IgG in sera of offspring were determined by PspA-specific ELISA at days 0, 7, and 14 after the birth. Group A mice were the offspring delivered from PspA-immunized mothers and breast-fed by the same mothers (n = 26). Group B mice were offspring from sham-immunized mothers and breast-fed by PspA-immunized mothers (n = 22). Group C mice were offspring from PspA-immunized mothers and breast-fed by sham-immunized mothers (n = 27). Group D mice were offspring from sham-immunized mother and breast-fed by the same mother (n = 18). The values shown are the mean ± S.E. concentrations (ng/ml). * $p<0.05$ and ** $p<0.01$ when compared with offspring in Group D by ANOVA test with Dunn's multiple comparison test.
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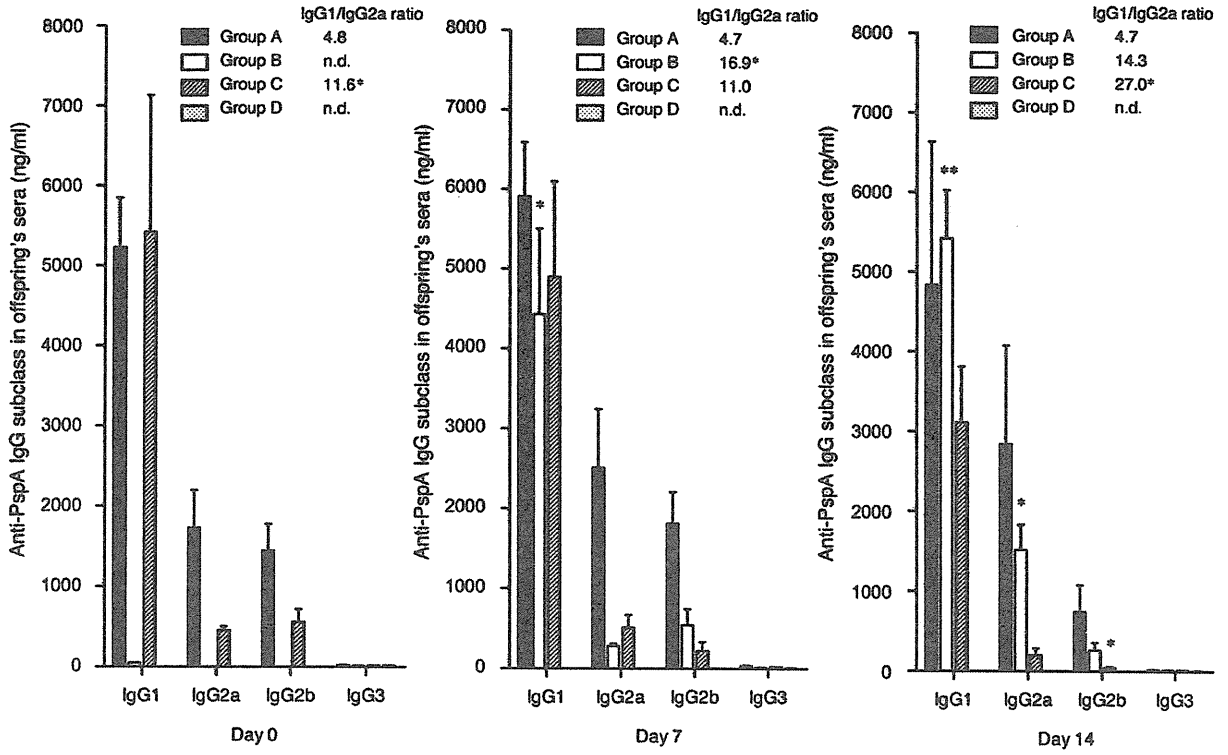

Figure 4. Anti-PspA specific IgG subclasses in sera of offspring. The levels of anti-PspA specific IgG subclasses in offspring's sera were determined by PspA-specific ELISA on day 0, 7, and 14 after birth. Group A (n = 26), B (n = 22), C (n = 27), and D (n = 18) mice were the same mice as described in figure 3. The values shown are the mean ± S.E. concentrations (ng/ml). The mean values of IgG1/IgG2a ratio were also shown. * $p<0.05$ and ** $p<0.01$ are for comparisons with offspring in Group D for PspA-specific IgG subclasses or with offspring on day 0 for the IgG1/IgG2a ratio by ANOVA test with Dunn's multiple comparison test.
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challenge dose was used to reproducibly kill 100% of non-immune control mice. Survival of offspring infected intraperitoneally with PspA1 strain D39 (capsular serotype 2, 50 CFUs/mouse) was significantly extended ($p<0.01$) compare to that of controls. Survival of offspring infected with two other PspA1 strains of EF3030 (capsular serotype 19F, 5×10^6 CFUs/mouse) and BG7322 (capsular serotype 6B, 20 CFUs/mouse) were weakly extended compared to that of controls (Fig. 8). However, there were no significant differences in survival times among offspring infected the PspA1 strain with L82016 (capsular serotype 6B, 5×10^6 CFUs/mouse). These finding makes it clear that even when immunization is not with the homologous PspA family it is still sometimes possible to see a protective response in the pups. However, these data are consistent with the expectation that a PspA vaccine should include PspAs of both major PspA families.

Discussion

During the first few months of life, when the human immune system is still immature, infants depend largely on passively acquired maternal IgG antibodies to protect themselves against invasive pathogens [20]. As in young humans, neonatal and infant mice are more susceptible to pneumococcal colonization and subsequent infection than are adults. Neonatal mouse macrophages show impaired innate and adaptive immune responses to pneumococci, which might explain the increased susceptibility to pneumococcal colonization in vivo [44]. The vaccines used to induce protective antibody early in childhood serve to minimize this window of natural susceptibility, but all such vaccines leave a window of susceptibility during the time that the initial immune response is induced. Immunization of women before pregnancy is a strategy that has been proven to reduce infection risks in mothers and infants for more than one pathogen [45–50].

Many recent pre-clinical studies in animals have focused on developing effective mucosal vaccines to combat the susceptibility of children to respiratory bacteria [51–53]. Our previous immunization studies using the outer membrane protein P6 of *Haemophilus influenzae* showed that maternal intranasal immunization could induce anti-P6 specific IgG antibody responses in mother's sera and breast milk at birth and that the immune response was maintained for 14 days during the nursing period [54]. Similar to those previous results, our present studies showed that anti-PspA specific antibody predominant in IgG was observed in PspA-immunized mother mice and was transported to their offspring via placenta and breast milk.

Mouse colostrums or breast milk have been reported to contain higher amounts of IgG antibody compared to IgA and IgM antibodies [55]. In mice, IgG antibody in mother's sera is transferred from mother to fetus through placenta by neonatal Fc receptor, FcRn. This antibody is initially collected in the yolk sacs of prenatal mice and rats [56,57]. Moreover, IgG antibody in breast milk is also transferred from intestine lumen to systemic circulation in neonate mice [56,57]. This transport of IgG antibody is mediated by FcRn expressed in the intestine of mice

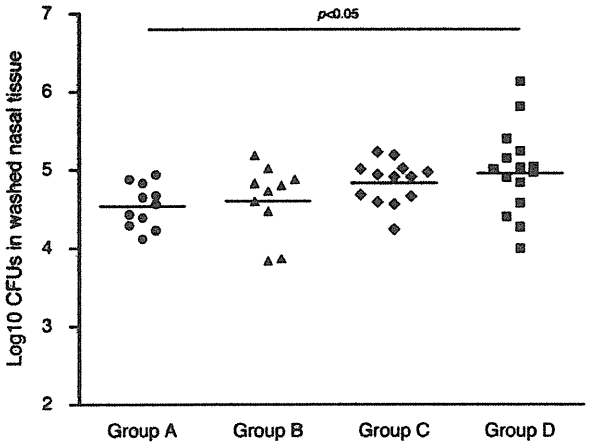

Figure 5. Protection against nasal carriage of pneumococci by maternal immunization with PspA among offspring. Offspring at 7-day-old were intranasally challenged with 1×10^5 CFU TIGR4 strain (5 µl/mouse) without anesthesia. Two days after challenge, nasal washes and homogenized washed nasal tissues were collected and the numbers of pneumococci colonies were determined. No evidence of protection was observed in CFUs in nasal washes (not shown). Results are shown for CFU in homogenized washed nasal tissue. Each dot represents the Log_{10} CFU/mouse. Each horizontal line depicts the median Log_{10} CFU/mouse. Group A (n = 11), B (n = 10), C (n = 13), and D (n = 15) mice were produced in the same manner as the corresponding groups in figure 3. Group A differed from Group D at $p<0.05$ by Kruskal-Wallis test with Dunn's multiple comparison test.
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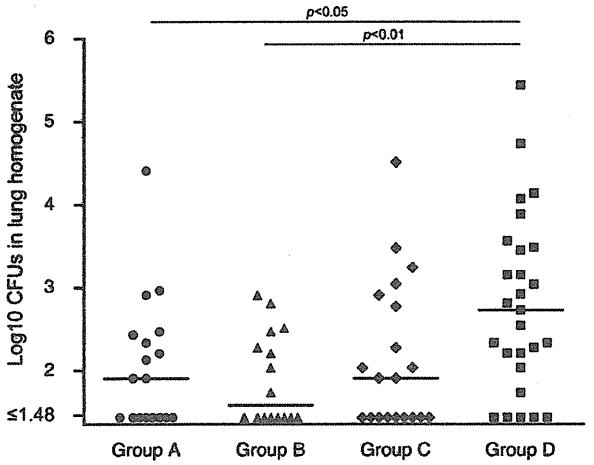

Figure 6. Protection against lung infection by maternal immunization with PspA among offspring. Seven-day-old mice were intranasally challenged with 5×10^5 CFU TIGR4 strain (10 µl/mouse) with anesthesia. Three days after challenge, lungs were collected and the numbers of pneumococci colonies in the lung homogenate were determined. Each dot shows the Log_{10} CFU/mouse. Each horizontal line shows the median Log_{10} CFU/mouse. Group A (n = 18), B (n = 16), C (n = 20), and D (n = 27) mice were produced in the same manner as the corresponding groups in figure 3. $p<0.05$ and $p<0.01$ are p-values for differences between the indicated group and the non-immune mice in Group D by Kruskal-Wallis test with Dunn's multiple comparison test.
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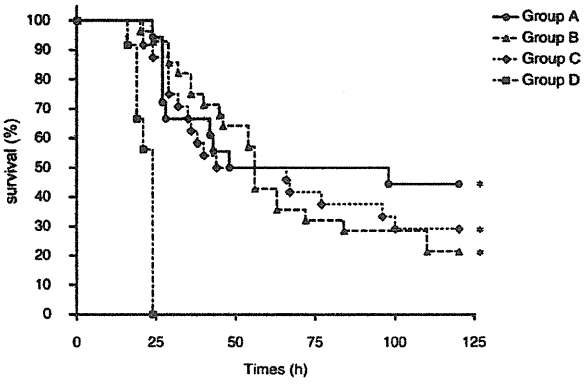

Figure 7. Protection against fatal systemic pneumococcal infections by maternal immunization with PspA among offspring. Offspring at 10-days of age were intraperitonally challenged with 1×10^4 CFU TIGR4 strain (100 µl/mouse) with anesthesia. After challenge, offspring were monitored for 5 days to determine survival. Group A (n = 24), B (n = 28), C (n = 24), and D (n = 38) mice were produced in the same manner as the corresponding groups in figure 3. Group A mice are offspring delivered from PspA-immunized mothers and breast-fed by the same mothers (n = 24). Group B mice are offspring from sham-immunized mothers and breast-fed by PspA-immunized mothers (n = 28). Group C mice are offspring from PspA-immunized mothers and breast-fed by sham-immunized mothers (n = 24). Group D mice are offspring from sham-immunized mothers and breast-fed by the same mothers (n = 38). * $p<0.01$ when compared with control offspring in Group D by Kaplan-Meier test with Log rank test.
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and rats [58–61]. The idea that IgG may cross epithelial barriers by receptor-mediated transcytosis in humans and other animals represents a novel concept in mucosal immunology. Recent studies have also demonstrated receptor-mediated IgG transport demonstrated across the lung of mice [62]. The FcRn expressing bronchia epithelial cells transports IgG across the mucosal surface of lung from lumen to serosa. The neonatal FcRn mediates the transport of IgG across polarized epithelial cells lining mucosal surface [63,64]. Furthermore, not only is IgG transmitted to progeny, but functional maternal immunoglobulin secreting cell or B cells can also be transferred to the neonate in both mouse and human [65,66].

In the present study of antibody to PspA, we further evaluated the protection of offspring against nasal carriage, lung infection, and fatal sepsis caused by pneumococci. Nasopharyngeal colonization is the initial step in the pathogenesis of infection caused by *S. pneumoniae*. Since carriage is considered to precede the development of subsequent fatal pneumococcal invasive diseases, the protection against carriage can also protect against subsequent disease [67].

The numbers of pneumococci colonizing closely associated with the nasal tissue were reduced by maternal immunization with PspA. On the other hand, the numbers of pneumococci washed from nasal surfaces were not different among groups (data not shown). Our earlier study demonstrated that during nasal colonization of mice with pneumococci, the majority of the colonizing pneumococci are tissue-associated and of the opaque phenotype [68]. This observation suggests that the opaque pneumococci may have either invaded the nasal tissue or may be sequestered in deep crypts and are not removed by the nasal wash. While secretory-IgA (SIgA) antibodies play an important role in the protection against nasopharyngeal colonization of

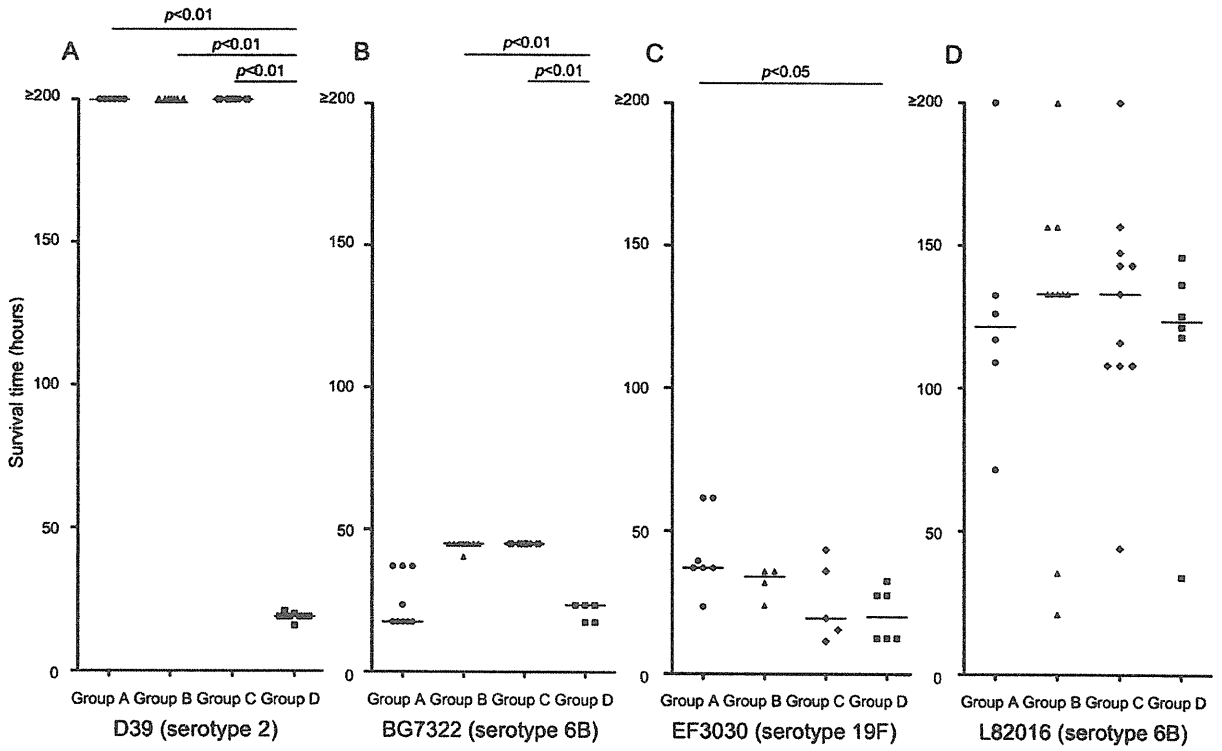

Figure 8. Cross-protection against fatal infections with pneumococcal strains expressing family 1 PspA. As in the prior studies the mother mice were immunized with a rPspA2 of strain TIGR4. Offspring at 10-days of age were intraperitoneally challenged with D39 (PspA1, serotype 2; 50 CFUs/mouse), BG7322 (PspA1, serotype 6B; 20 CFUs/mouse), EF3030 (PspA1, serotype 19F; 5×10^6 CFUs/mouse), and L82016 (PspA1 serotype 6B, 5×10^6 CFUs/mouse) in 100 µl with anesthesia. After challenge, the mice were monitored for 10 days to determine the day of death. Group A mice were offspring delivered from PspA-immunized mothers and breast-fed by the same mothers (n = 6 for D39, n = 10 for BG7322, n = 7 for EF3030, n = 6 for L82016). Group B mice were offspring from sham-immunized mothers and breast-fed by a PspA-immunized mothers (n = 7 for D39, n = 11 for BG7322, n = 4 for EF3030, n = 10 for L82016). Group C were offspring from PspA-immunized mothers and breast-fed by sham-immunized mothers (n = 9 for D39, n = 11 for BG7322, n = 5 for EF3030, n = 11 for L82016). Group D mice were offspring from sham-immunized mothers and breast-fed by the same mothers (n = 9 for D39, n = 5 for BG7322, n = 6 for EF3030, n = 6 for L82016). $p<0.05$ and $p<0.01$ for the indicated comparisons with offspring in Group D by Kruskal-Wallis test with Dunn's multiple comparison test are shown.
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pneumococci [69], SIgA make little sense for protecting against pneumococci once they have invaded the nasal tissues [69].

Ferreira et al reported that the reduction of nasal colonization was strongly associated with increased levels of IgG2a complement fixing antibody and lower levels of IgG1 antibody which had less complement fixing activity [70,71]. As a consequence, lower IgG1/IgG2a ratios were also correlated with lower levels of colonization [70,71]. In this study, IgG1/IgG2a ratio was decreased on day 0 to 14 in both mother's sera and breast milk. As for the offspring's sera in our study, the anti-PspA specific IgG2a antibodies were increased in sera from offspring in Group A where immunized mothers nursed their own pups. A balanced IgG1/IgG2a antibody response was maintained in the Group A offspring over time. The fact that the only group that showed even a hint of protection against colonization was group A, which had the highest relative concentrations of IgG2a as compared to IgG1, is consistent with the earlier published observations from experiments with adult mice [71]. Our failure to see stronger protection against carriage, may in retrospect be due the fact that our mice were sacrificed only 2 days post challenge. In most adult mouse studies sacrifice at 5-day, 7-day, or later time points, which may have permit the cumulative actions of immunity over time may have had larger effects on colonization.

As compared to colonization, where the protective effects were quite modest if at all, maternal immunization was clearly protectected the offspring against pneumococcal lung infections and fatal sepsis. The absence of bacteremia and sepsis in the aspiration-pneumonia infection model might suggest that the observed protection against lung infection results from direct protection in the lung, rather than just being realized through a protection against septicemia. Offspring delivered from mother mice immunized intranasally with PspA were protected from systemic pneumococcal infections. Intranasal immunization with PspA has been shown to protect adult mice against pneumonia and fatal sepsis and occasionally against nasal carriage [72,73]. The present studies have shown that similar immunizations of mother mice can protect progeny in these models and that the protection is to some extent independent of the PspA family of the challenge strain.

While significant protection was seen against infection of TIGR4 PspA2 strain by maternal immunization with homologous PspA2, we observed strong portection against invasive infection with only one of four PspA1 challenge strains. The current findings caution that for protein vaccines one must take care to include PspAs representative of both major PspA families: PspA1 and PspA2. Darrieux M. et al. demonstrated that a chimeric fusion