TABLE 1. Frequencies of CD11c$^+$ DCs and CD8, CD11b, B220, and costimulatory molecule expression by CD11c$^+$ DCs of mucosal effector and inductive tissues of mice given nasal rPspA with pFL or pORF[a,e]

Tissue source	Adjuvant given with nasal rPspA	% CD11c$^+$ total lymphocytes[b]	% CD11c$^+$ DCs expressing:						
			CD8[c]	CD11b[c]	B220[c]	CD40[d]	CD80[d]	CD86[d]	MHC II[d]
NALT	pFL	*6.6 ± 1.7	*23.5 ± 3.7	*23.1 ± 3.1	56.5 ± 6.2	**3.1 ± 0.7	10.9 ± 4.5	*19.3 ± 4.8	**68.9 ± 8.2
	pORF	1.7 ± 1.2	12.1 ± 1.6	12.3 ± 3.9	50.4 ± 0.6	0.4 ± 0.5	5.9 ± 1.6	8.9 ± 2.6	45.9 ± 1.9
Lungs	pFL	*5.7 ± 1.3	*12.4 ± 2.4	68.6 ± 4.1	24.8 ± 3.5	*4.6 ± 1.1	*15.2 ± 5.6	*20.8 ± 3.6	*51.5 ± 9.6
	pORF	3.3 ± 1.6	7.7 ± 0.9	63.9 ± 3.9	20.6 ± 4.7	1.6 ± 0.1	6.9 ± 2.4	9.5 ± 7.9	39.1 ± 0.7
NPs	pFL	*10.1 ± 2.4	*22.1 ± 2.3	*60.1 ± 6.9	19.3 ± 3.7	*11.1 ± 3.3	*23.8 ± 3.5	25.9 ± 6.2	38.5 ± 4.2
	pORF	5.3 ± 0.9	14.8 ± 3.8	31.1 ± 6.0	20.6 ± 4.7	3.6 ± 0.7	17.8 ± 0.1	23.1 ± 7.6	33.3 ± 1.9
CLNs	pFL	*2.3 ± 0.4	*32.8 ± 3.6	*31.8 ± 3.6	*45.4 ± 4.1	**3.1 ± 0.2	**24.7 ± 1.0	*43.3 ± 2.9	87.4 ± 5.9
	pORF	0.7 ± 0.2	21.1 ± 3.6	24.8 ± 3.2	33.3 ± 4.5	0.7 ± 0.9	10.6 ± 1.3	28.5 ± 8.5	85.3 ± 0.6
Spleen	pFL	*2.4 ± 0.7	*21.7 ± 2.2	*33.4 ± 5.1	44.6 ± 3.5	*4.3 ± 2.0	20.3 ± 6.4	**26.4 ± 5.3	**87.5 ± 1.5
	pORF	1.4 ± 0.5	18.2 ± 0.9	25.2 ± 3.4	39.9 ± 3.5	1.1 ± 0.1	12.9 ± 0.6	11.4 ± 2.8	75.0 ± 4.2

[a] Mononuclear cells from NALT, lungs, NPs, CLNs, and spleens of mice immunized with rPspA plus pFL or pORF were stained with a combination of anti-CD11c and the respective MAb and subjected to FACSCalibur flow cytometry analysis.
[b] Mononuclear cells were stained with PE-conjugated anti-CD11c MAb and subjected to flow cytometry analysis.
[c] Mononuclear cells were stained with FITC-conjugated anti-CD8α, -CD11b, and -B220 MAb and PE-labeled anti-CD11c.
[d] Mononuclear cells were stained with PE-labeled anti-CD40, -CD80, -CD86 or I-A^b and biotinylated anti-mouse CD11c MAb followed by CyChrome-streptavidin.
[e] *, $P < 0.05$; **, $P < 0.01$ (compared with those of mice immunized with rPspA plus pORF).

production by PspA-stimulated CD4$^+$ T cells were essentially the same between mice given pFL or pORF (Table 3). Intracellular IL-17 analysis revealed that no significant increase in the frequency of IL-17-producing CD4$^+$ T cells was seen in CLNs and spleen of mice given nasal pFL compared with results for mice given empty plasmid as a nasal adjuvant (data not shown). These results show that pFL as a nasal adjuvant preferentially induces Th2-type dominant cytokine responses in the lower respiratory mucosa when rPspA is used as an Ag for nasal vaccination.

Protection against *S. pneumoniae* infection by nasal vaccination with rPspA plus pFL. In order to determine the functional properties of nasal vaccination with rPspA plus pFL, mice were challenged with *S. pneumoniae* strain WU2 (1.8×10^7 CFU/20 μl) 1 week after the last vaccination. When the bacterial densities in the lungs, NWs, and blood were examined 48 h after nasal challenge, mice given nasal rPspA plus pFL showed significantly lower bacterial density. Conversely, lungs, NWs, and blood of mice given rPspA plus pORF contained high numbers of *S. pneumoniae* bacteria (Fig. 5). These results show that nasal rPspA plus pFL provides effective protection against *S. pneumoniae* infection at the lung and nasal mucosa.

DISCUSSION

In this study, we have investigated whether nasal pFL as a mucosal adjuvant elicits functional bacterial Ag (rPspA)-specific Ab responses for protection against *S. pneumoniae* infection. Our results clearly showed that nasal vaccination with rPspA plus pFL elicited DC-mediated Th2-type and IL-2 cytokine responses and subsequent anti-rPspA Abs for protection against pneumococcal infection at the pulmonary mucosa. Since a risk of central nervous system toxicity is one of the major issues for nasal vaccine development (6, 9, 29), we also examined pFL uptake and inflammatory cytokine synthesis in the nasal mucosa. Our results indicated that nasal pFL was taken up only by NALT and NP DCs, as well

TABLE 2. Frequencies of costimulatory molecule expression on CD8 or CD11b DCs in mucosal effector and inductive tissues of mice given nasal rPspA with pFL or pORF[a]

Tissue source	Adjuvant given with nasal rPspA	% CD11c$^+$ DCs							
		CD8$^+$ DCs				CD11b$^+$ DCs			
		CD40	CD80	CD86	MHC II	CD40	CD80	CD86	MHC II
NALT	pFL	**1.4 ± 0.1	*4.4 ± 0.6	*9.5 ± 1.4	**34.5 ± 2.2	**1.3 ± 0.1	*4.1 ± 0.3	*8.5 ± 1.7	*27.3 ± 4.2
	pORF	0.2 ± 0.1	2.0 ± 0.4	4.0 ± 0.7	12.4 ± 1.3	0.2 ± 0.1	1.7 ± 0.2	3.7 ± 1.0	14.6 ± 1.5
Lungs	pFL	*1.7 ± 0.2	**7.7 ± 0.3	**7.5 ± 0.4	**18.7 ± 3.1	*1.4 ± 0.2	*6.6 ± 0.9	*8.0 ± 0.8	*30.5 ± 3.8
	pORF	0.5 ± 0.1	1.3 ± 0.1	2.3 ± 0.2	6.2 ± 1.0	0.9 ± 0.3	3.5 ± 0.9	3.6 ± 1.0	14.5 ± 5.7
NPs	pFL	*5.8 ± 0.4	*8.2 ± 1.7	*9.8 ± 1.5	*14.8 ± 2.4	*4.5 ± 1.1	*13.1 ± 2.0	11.5 ± 3.3	*20.6 ± 3.5
	pORF	1.4 ± 0.3	4.9 ± 1.4	4.9 ± 1.0	7.3 ± 1.9	2.5 ± 1.0	7.0 ± 1.7	8.7 ± 2.0	12.0 ± 2.2

[a] CD11c-positive DCs from NALT, lungs, and NPs of mice immunized with rPspA plus pFL or pORF were purified from mononuclear cells by using the automatic cell sorter system AutoMacs and were stained with a combination of FITC-conjugated anti-mouse CD8α MAb or anti-mouse CD11b MAb and PE-labeled anti-CD40, -CD80, -CD86, or I-A^b and subjected to FACSCalibur flow cytometry analysis. *, $P < 0.05$; **, $P < 0.01$ (compared with those of mice immunized with rPspA plus pORF).

TABLE 3. $CD4^+$ Th1- and Th2-type cytokine responses after *in vitro* restimulation with rPspA[a]

Tissue	Nasal adjuvant	Stimulation index[b]	Production of Th1- or Th2-type cytokine[c]					
			IFN-γ (ng/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-6 (ng/ml)	IL-10 (ng/ml)
Lungs	pFL	*5.5 ± 1.8	2.03 ± 0.40	*249.8 ± 48.8	*56.7 ± 9.4	*255.7 ± 76.9	*1.80 ± 0.49	1.19 ± 0.32
	pORF	2.5 ± 0.6	1.48 ± 0.24	130.1 ± 35.7	21.2 ± 4.4	86.6 ± 22.0	0.78 ± 0.29	0.89 ± 0.34
CLNs	pFL	*6.1 ± 1.3	1.63 ± 0.24	*201.3 ± 55.3	*55.6 ± 14.9	*314.5 ± 66.6	1.51 ± 0.68	1.43 ± 0.41
	pORF	2.4 ± 0.2	1.13 ± 0.18	78.8 ± 13.6	18.8 ± 5.40	66.5 ± 28.8	0.72 ± 0.34	1.21 ± 0.47
Spleen	pFL	*4.8 ± 1.4	1.65 ± 0.47	*391.1 ± 68.9	*45.1 ± 10.5	*286.8 ± 53.4	*1.33 ± 0.50	1.57 ± 0.39
	pORF	1.9 ± 0.4	1.22 ± 0.39	53.3 ± 19.1	16.1 ± 6.20	57.7 ± 15.1	0.89 ± 0.42	1.40 ± 0.24

[a] The $CD4^+$ T cells (4×10^6 cells/ml) from lungs, CLNs, and spleen were isolated 7 days after the last immunization with rPspA (5 μg) and pFL or pORF as a mucosal adjuvant and cultured with T cell depleted feeder cells (8×10^6 cells/ml). Values are presented as means ± SEM of data from 30 mice for each group and a total of three experiments. *, $P < 0.05$ when compared with mice given rPspA plus pORF.

[b] Proliferative responses of $CD4^+$ T cells from mice nasally immunized with rPspA plus pFL or pORF as a mucosal adjuvant were represented as the stimulation index by measuring counts per minute (cpm) of wells with or without rPspA (control). The levels of [^{3}H]TdR incorporation for each control well were between 500 and 1,000 cpm. The results show the individual values from these separate experiments of 30 mice per experimental group.

[c] The culture supernatant were harvested after 48 h of incubation and subjected to the respective cytokine ELISAs.

as nECs, but not by ON/E. Further, minimal levels of IL-1β, IL-6, and TNF-α production were induced in NWs of mice given pFL. Taken together, the current study is the first to show that nasal pFL is a safe mucosal adjuvant that effectively elicits bacterial Ag (PspA)-specific functional Ab responses that are potent for the prevention of pneumococcal pneumonia and bacteremia.

We recently showed that pFL as a nasal adjuvant elicited PspA-specific S-IgA Ab responses in the nasal cavity to prevent nasal carriage of *S. pneumoniae* (12). Although the recent study clearly indicated the potential of pFL as a nasal adjuvant for a pneumococcal vaccine, it still remained to be elucidated whether pFL can induce protection in the lower respiratory mucosa, including the lungs. In this regard, nasal pFL elicited functional rPspA-specific S-IgA Ab responses in the BALF and lungs when mice given nasal rPspA plus pFL were nasally challenged with a large amount (20 μl; 1.8×10^7 CFU) of WU2 (invasive strain), allowing bacterial exposure to the lungs. Thus, mice given nasal rPspA plus pFL showed a significantly lower number of bacteria in the BALF after being challenged fatally with WU2 than did mice given empty plasmid as a nasal adjuvant. Based upon our previous studies and those of others (12, 42), we expected that nasal immunization with rPspA plus pFL induced Ag-specific functional S-IgA Ab responses in the nasal cavity. Indeed, a remarkable anti-rPspA S-IgA Ab induction and the inhibition of bacterial growth were seen in NWs after bacterial challenge. Thus, it is possible that an effective inhibition of nasopharyngeal bacterial colonization might lead to drastic reduction of bacterial growth in the lungs and prevention of subsequent bacteremia. In any case, the presence of anti-PspA IgA Abs in the lower and upper respiratory mucosa is the fundamental factor to prevent bacterial invasion of the hosts.

The FL protein is known as a synergistic hematopoietic growth factor that has emerged as a potential immunomodulator (8, 21, 38) and can expand DC populations and enhance antigen-presenting cell (APC) activity (13, 24). In

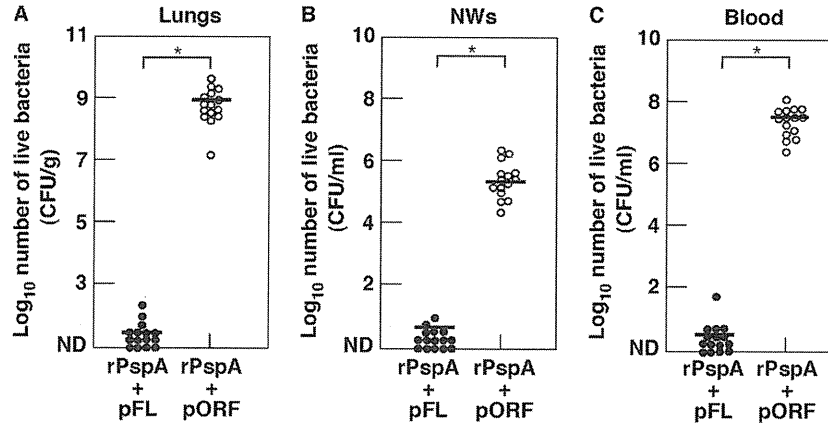

FIG. 5. Comparison of protective effects against *S. pneumoniae* infection with nasal pFL-based rPspA vaccine. One week after the last immunization with rPspA plus pFL (closed circle) or pORF (open circle), mice were challenged with 1.8×10^7 CFU of the WU2 strain. (A) Forty-eight hours after bacterial challenge, the lungs were removed aseptically and homogenized in 9 ml of sterile saline per gram of lung tissue for the culture. (B) NWs were harvested aseptically by flushing with 1 ml of PBS and cultured on agar medium. (C) Blood samples were plated on agar medium from the culture. The detection limit of bacterial cultures was 10^2 CFU/g. The values shown are the means ± SEM ($n = 15$). Each line represents the median $\log_{10}$ CFU/mouse. *, $P < 0.05$, compared with mouse group given rPspA plus pORF.

addition, it was recently reported that percutaneous administration of the FL protein regulated migration and Ag uptake of lung DCs (34, 35). In this regard, our previous studies showed that nasal pFL increases the frequency of $CD8^+$ DCs in various mucosal and systemic lymphoid tissues (11, 19). Our present study showed increased numbers of $CD8^+$ DCs, which agrees with these previous findings even though a bacterial Ag was used as a component of the nasal vaccine. Since a recent study indicated that induction of $CD8^+$ DCs promoted protection against respiratory infection (7), it is possible that induction of $CD8^+$ DCs in the mucosal and systemic compartments contributes to *S. pneumoniae* clearance in the respiratory tract and blood. Further, increased frequencies of $CD11b^+$ DCs were also seen in mice given nasal rPspA plus pFL. Recent reports showed that the interactions between $CD4^+$ T cells and DCs play a key role in the induction of pulmonary immunity (1) and that DCs polarize initial $CD4^+$ T cell activation toward Th2-type immune responses (33). Further, our previous and present studies showed that nasal pFL elicited $CD8^+$ DC-mediated Th2-type responses. In this regard, it is possible that $CD11b^+$ DCs play a role in the downregulation of Th2-prone cytokine responses for the maintenance of mucosal homeostasis. Indeed, it was suggested that FL treatment induced Th2-suppressive lung $CD11b^+$ DCs (15, 35). Further, nasal application of FL-expressing adenovirus as a mucosal adjuvant preferentially expands $CD11b^+$ DCs to produce a balanced Th1- and Th2-type cytokine response (32). The actual immunoregulatory functions of $CD11b^+$ DCs induced by nasal pFL are currently being tested in our laboratory.

In summary, the present study shows that pFL as a nasal adjuvant induces enhanced PspA-specific immunity in the nasal-pulmonary mucosa via $CD8^+$ and $CD11b^+$ DC subset-mediated Th2-type cytokines responses. Importantly, nasal vaccination with rPspA plus pFL inhibits bacterial growth in the lungs and nasal cavities of mice in order to prevent the early phases of pneumococcal pneumonia without CNS toxicity or inflammation. These findings suggest that pFL is a safe nasal adjuvant for use in the future development of vaccines that can induce enhanced specific immunity against bacterial and viral Ags.

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Genotypic Profile of *Streptococcus suis* Serotype 2 and Clinical Features of Infection in Humans, Thailand

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To examine associations between clinical features of *Streptococcus suis* serotype 2 infections in humans in Thailand and genotypic profiles of isolates, we conducted a retrospective study during 2006–2008. Of 165 patients for whom bacterial cultures of blood, cerebrospinal fluid, or both were positive for *S. suis* serotype 2, the major multilocus sequence types (STs) found were ST1 (62.4%) and ST104 (25.5%); the latter is unique to Thailand. Clinical features were examined for 158 patients. Infections were sporadic; case-fatality rate for adults was 9.5%, primarily in northern Thailand. Disease incidence peaked during the rainy season. Disease was classified as meningitis (58.9%) or nonmeningitis (41.1%, and included sepsis [35.4%] and others [5.7%]). Although ST1 strains were significantly associated with the meningitis category ($p<0.0001$), ST104 strains were significantly associated with the nonmeningitis category ($p<0.0001$). The ST1 and ST104 strains are capable of causing sepsis, but only the ST1 strains commonly cause meningitis.

Streptococcus suis, an emerging zoonotic pathogen, causes invasive infections in persons who are in close contact with infected pigs or contaminated pork-derived products (*1*). On the basis of capsular polysaccharides, 33 serotypes of *S. suis* have now been identified. Of these, serotype 2 is the most prevalent type in humans infected with this pathogen (*1,2*). Since the largest outbreak of human *S. suis* infection in 2005, in Sichuan Province, People's Republic of China (*3*), this disease has been increasingly recognized worldwide. The numbers of reported cases, especially in persons from Southeast Asian countries, have increased dramatically during past few years (*4*).

In Thailand, at least 300 cases of *S. suis* infection in humans have been reported (*5–11*). Although an outbreak of *S. suis* infections was confirmed in Phayao Province during May 2007 (*9*), most cases in humans occur sporadically and are primarily located in the northern region of this country (*6–11*). A relatively low incidence of cases with *S. suis* serotype 14 has also been reported in this region (*12*). Although previous studies have reported high frequencies (59.0%–88.7%) of *S. suis* infections in persons in this area who ate raw pork products (*8–11*), the pathogenesis of this disease, including routes of transmission, is unclear.

The major clinical manifestations of the disease are bacterial meningitis and sepsis, but other manifestations have been reported (*1,4, 8,10,13*). Most cases of bacterial meningitis can be attributed to the hematogenic spread of invasive bacteria, but how circulating bacteria cross the blood–cerebrospinal fluid (CSF) barrier and cause meningitis is not clear (*14,15*). Furthermore, the overall clinical features of this disease have not been extensively and comprehensively investigated in Southeast Asian countries.

A variety of virulence factors associated with *S. suis* have been reported (*16–20*), but none have been proven to be essential for the host defense of this disease, except

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the capsular polysaccharide (*19*). In serotype 2 isolates obtained during a previous outbreak in Sichuan, China, an ≈89-kb DNA fragment, which has been associated with a pathogenicity island (89K PAI), was identified (*21*). The 89K PAI fragment encodes a 2-compartment signal transduction system, SalK-SalR, which is required for full virulence (*22*).

We report the results of a retrospective study of the clinical features of 158 cases of human infection with *S. suis* serotype 2 and the molecular epidemiology of 165 *S. suis* serotype 2 isolates. The study objective was to demonstrate associations between the clinical features of disease caused by *S. suis* serotype 2 in persons in Thailand and the genotypic profiles of the isolates. The study was reviewed and approved by the Ethics Committees of Research Institute for Microbial Diseases, Osaka University, and conducted according to the principles expressed in the Declaration of Helsinki.

Methods

Isolate Identification

From January 2006 through August 2008, a total of 1,154 unidentified streptococcal isolates from blood or CSF were collected from hospitals in all 76 provinces of Thailand. Biochemical testing of these isolates, using API Strep (bioMérieux, Durham, NC, USA) and *S. suis*–specific and *S. suis* serotype 2– or 1/2–specific PCR (*12,23*), confirmed 165 isolates from 34 hospitals in 25 provinces as *S. suis*. The final serotype of all strains was confirmed by coagglutination tests that used rabbit antiserum (Statens Serum Institute, Copenhagen, Denmark).

Genotypic Profiles of Isolates

Multilocus sequence type (MLST) testing was performed as described by King et al. (*24*), with a modification for *mutS* as described by Rehm et al (*25*). MLST alleles and the resulting sequence type (ST) were assigned by using the *S. suis* MLST database (http://ssuis.mlst.net). eBURST was used to identify the clonal complexes for these 165 serotype 2 strains within *S. suis*, and the overall structure of the population was obtained through the MLST database (*26*). Virulence-associated genes (VAG), including extracellular released protein factor (*epf*), muramidase-released protein (*mrp*), and suilysin (*sly*), and variants of *mrp* or *epf* were determined by PCR as described by Silva et al. (27), with minor modifications. Presence of the 89K PAI fragment was determined by PCR as reported by Chen et al (*21*). Pulsed-field gel electrophoresis (PFGE) was performed as described (*28*), and the pulsotypes were assigned to clusters of isolates with >80% similarity from the dendrogram. The dendrogram representing the genetic relationships between the representative pulsotypes from 165 *S. suis* serotype 2 strains was drawn by using the Cluster 3.0 software program and examined by using the TreeView program as described (*12,29*).

Clinical Features of Cases

Of the 165 patients whose culture results were positive for *S. suis* serotype 2, medical records for 158 were retrospectively reviewed by physicians at local hospitals in Thailand. Medical records for the remaining 7 patients were not available. The clinical manifestations were mostly divided into 2 categories: meningitis and nonmeningitis. The meningitis category involved confirmed meningitis, bacteremic meningitis, and probable meningitis. All patients in the meningitis category had typical meningeal signs, such as neck stiffness, and acute disease onset. Although bacteremic meningitis was defined as a case in which both CSF and blood cultures were positive, confirmed meningitis was defined as a case with a positive CSF culture only, and probable meningitis was defined as a case with a positive blood culture only. The nonmeningitis category included the clinical manifestations of sepsis and sepsis with focal signs other than meningitis (septic arthritis or spondylodiscitis, infective endocarditis, and bacteremic pneumonia). Sepsis was defined as systemic inflammatory response syndrome and a positive blood culture (*30*), and septic arthritis or septic spondylodiscitis was defined as described (*31*). Diagnosis of infectious endocarditis was based on the Duke criteria (*32*). Septic shock was also defined as described (*33*).

Statistical Analyses

Comparisons of the clinical characteristics between fatal and nonfatal cases were analyzed by using the χ^2 test or Fisher exact test with Stata version 10.0 software (StataCorp, College Station, TX, USA). Patient ages and periods of hospital admission were tested for normality of the distribution using the Kolmogorov-Smirnov test and were compared by using the Student *t* test with SPSS version 11.0 software (SPSS Inc., Chicago, IL, USA). Data were considered significant at $p<0.05$.

Results

Genotypic Profiles of Isolates

Of the 165 *S. suis* serotype 2 isolates, 123 were isolated from blood and 42 from CSF. eBURST analysis based on MLST enabled classification of these strains into 4 ST complexes: the ST1, ST27, ST29, and ST104 complexes (Table 1). ST126, a novel ST, has a single locus variant from ST1. The largest cluster of 89K PAI–carrying strains was ST1 (n = 81, 49.1%), which had the *epf*+/*sly*+/*mrp*+ genotype; these strains were isolated from blood and CSF. Another large cluster of non-89K PAI–carrying strains was

Table 1. Genotypic profiles of 165 clinical isolates of *Streptococcus suis* serotype 2, Thailand, January 2006–August 2008*

ST complex	ST	VAG†	Isolation site	89K PAI +	89K PAI –	No. (%) strains
1	1	*epf–/sly+/mrp+*	Blood	1	0	103 (62.4)
		epf+/sly+/mrp+	Blood	52	13	
			CSF	29	5	
		epf+/sly+/mrp^{s}	Blood	0	1	
			CSF	0	2	
	126	*epf+/sly+/mrp+*	Blood	1	0	3 (1.8)
			CSF	2	0	
27	28	*epf–/sly–/mrp+*	Blood	0	1	3 (1.8)
			CSF	0	2	
29	25	*epf–/sly–/mrp**	Blood	8	0	11 (6.7)
		epf–/sly–/mrp+	Blood	3	0	
	103	*epf–/sly–/mrp**	Blood	2	0	3 (1.8)
		epf–/sly–/mrp+	Blood	1	0	
104	104	*epf–/sly+/mrp–*	Blood	3	38	42 (25.5)
			CSF	0	1	
Total no. strains	NA	NA	NA	102	63	165 (100)

*ST, sequence type; VAG, virulence-associated gene; 89K PAI, an ≈89-kb pathogenicity island; CSF, cerebrospinal fluid; NA, not applicable.
†*mrp*^{s} and *mrp** are *mrp* variants that produce ≈750-bp and ≈1,800-bp fragments, respectively, by PCR (*23*,*34*).

ST104, which had the *epf–/sly+/mrp–* genotype (n = 39, 23.6%); most of these strains (n = 38) were isolated only from blood. ST103, ST104, and ST126 were found only in isolates from humans in Thailand.

PFGE of Isolates

Of the 165 serotype 2 strains, PFGE analyses identified 20 pulsotypes (Figure 1, panel A). Analysis of the dendrogram for these 20 pulsotypes revealed at least 16 clusters (I to XVI) (Figure 1, panel B). Although 5 pulsotypes of A were identified for the ST1 and ST126 strains, 2 major pulsotypes (A [n = 32] and A1 [n = 43]), A1 (n = 43), and A4 (n = 3) were grouped in 1 cluster. Pulsotype A2 (n = 21), which consisted of ST1 strains lacking the 89K PAI fragment, was classified into a distinguished cluster. PFGE showed diverse DNA patterns for strains ST25 and ST103. ST25 strains were classified into 5 clusters of I, II, III, IV, and VIII. ST103 strains were classified into 3 clusters of VI, XIV, and XV. Three ST28 strains lacking 89K PAI exhibited the unique DNA pattern of pulsotype D; these were classified into cluster XVI. Although 4 pulsotypes (H, H1, H2, and H3) were identified for ST104 strains, 2 major pulsotypes (H [n = 29] and H1 [n = 11]) in ST104 strains were classified into cluster VII. Collectively, clusters X and XI for ST1 and ST126 strains and cluster VII for ST104 strains accounted for the major 3 clusters found for cases in Thailand.

Geographic and Seasonal Distribution

Of the 165 isolates, 136 (82.4%) were from the northern region, 19 (11.5%) from the central region, 7 (4.2%) from the northeast region, and 3 (1.8%) from the eastern region (Table 2; Figure 2, panel A). No strains were isolated from the southern region. The dates of isolation suggest that human cases occur more frequently during the rainy season, June–August of each year (Figure 2, panel B).

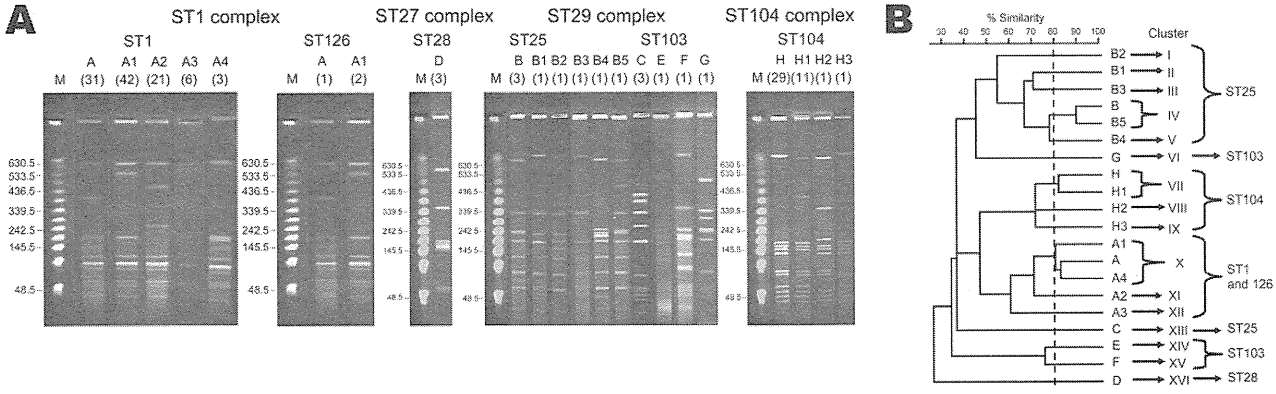

Figure 1. A) Pulsed-field gel electrophoresis profiles of 165 human isolates of *Streptococcus suis* serotype 2, after *Sma*I digestion. Numbers of isolates are indicated in parentheses below pulsotype numbers. B) Dendrogram generated from the pulsed-field gel electrophoresis profiles. ST, sequence type.

Table 2. Distribution of sequence types of 165 clinical isolates of *Streptococcus suis* serotype 2, by region, Thailand

Sequence type	North	Northeast	East	Central	South
1	85	6	1	11	0
25	11	0	0	0	0
28	3	0	0	0	0
103	1	0	1	1	0
104	33	1	1	7	0
126	3	0	0	0	0
Total	136	7	3	19	0

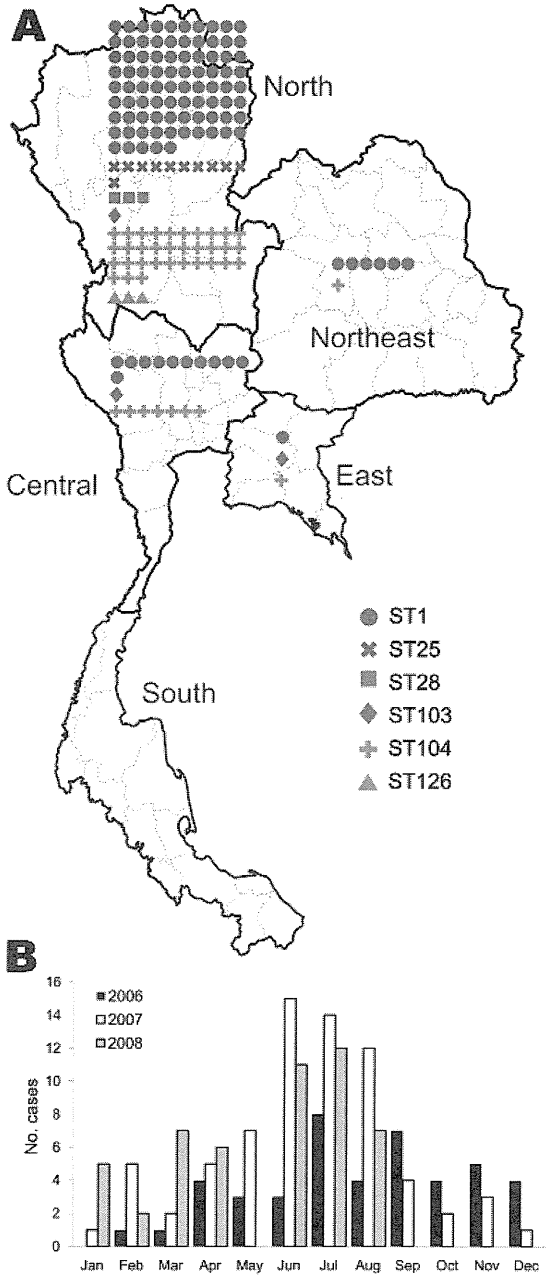

Figure 2. Distribution and sequence types (STs) of 165 human isolates of *Streptococcus suis* serotype 2, January 2006–August 2008, Thailand. A) Regions of isolation; B) monthly distribution of isolations.

Clinical Features of Cases

The clinical features of the 158 human cases of *S. suis* serotype 2 infection are summarized in Table 3. The median age (range) of the 155 patients for whom age was known was 55.0 (18–93) years; 72.8% were male. No cases in children were identified in this study. All 158 patients had been hospitalized; median duration (range) of hospitalization for the 158 patients was 11 (1–45) days; 15 (9.5%) patients died. No significant differences were found between the fatal and nonfatal cases with respect to patient age or period of admission.

The meningitis category (n = 93) included 22 cases of confirmed meningitis, 44 cases of bacteremic meningitis, and 27 cases of probable meningitis (Figure 3). The nonmeningitis category (n = 65) included sepsis with focal signs other than meningitis (n = 9) and sepsis (n = 56). Sepsis with focal signs other than meningitis included septic arthritis (n = 5), infective endocarditis (n = 3), and bacteremic pneumonia (n = 1). Of the 15 fatal cases, 8 were assigned to the meningitis category (probable meningitis [n = 6], meningitis [n = 1], bacteremic meningitis [n = 1]), 6 cases were sepsis, and 1 case was infective endocarditis (Table 3). Although the cases of bacteremic meningitis were significantly associated with a nonfatal outcome ($p = 0.043$), the probable meningitis cases were significantly associated with a fatal outcome ($p = 0.013$). The combined frequencies for the recent consumption of raw pork products and exposure to pigs were 39.9%. None of the clinical signs or possible risk factors, including recent exposure to pigs or raw pork products, or alcohol abuse, was significantly associated with a fatal outcome. Of the 158 patients, 154 parenterally received antimicrobial drugs, such as ceftriaxone, and data concerning antimicrobial drug treatment were not available for 4. Corticosteroids, such as dexamethasone, were used for only 4 patients.

Clinical Features and Genotype Profiles

The distributions of STs for the 158 human isolates for the meningitis and nonmeningitis categories are shown in Table 4. Although the ST1 strains were significantly associated with the meningitis category ($p<0.0001$), the ST104 strains were significantly associated with the nonmeningitis category ($p<0.0001$). The VAG profile of *epf+/sly+/mrp+*, which was dominant in the ST1 strains,

Table 3. Demographic and clinical features of 158 human cases of *Streptococcus suis* serotype 2 infections, Thailand, January 2006–August 2008*

Characteristic	All, n = 158	Fatal, n = 15; 9.5%	Nonfatal, n = 143; 90.5%	p value
Demographic				
Male sex, %	72.8	66.7	73.4	0.386
Mean (median) age, y†	56.6 (55.0)	53.9 (52.5)	57.0 (56.0)	0.264
Period of admission, d, mean (median)	12.5 (11)	10.1 (6)	12.9 (12)	0.737
Meningitis category, no. (%) cases				
Confirmed meningitis	22 (13.9)	1 (6.7)	21 (14.7)	0.348
Bacteremic meningitis	44 (27.8)	1 (6.7)	43 (30.1)‡	0.043
Probable meningitis	27 (17.1)	6 (40.0)	21 (14.7)§	0.013
Nonmeningitis category, no. (%) cases				
Septic arthritis	5 (3.2)	0	5 (3.2)	1
Infective endocarditis	3 (1.9)	1 (6.7)	2 (1.4)	0.905
Bacteremic pneumonia	1 (0.6)	0	1 (0.7)	1
Sepsis	56 (35.4)	6 (40.0)	50 (35.0)	0.698
Signs and symptoms, no. (%) cases				
Diarrhea	28 (17.1)	5 (33.3)	23 (16.1)	0.1
Hearing loss	34 (21.5)	4 (26.7)	30 (21.0)	0.409
Altered consciousness	35 (22.2)	4 (26.7)	31 (21.7)	0.434
Shock	9 (5.7)	2 (13.3)	7 (4.9)	0.205
Possible risk factors, no. (%) cases				
Recent consumption of raw pork products	52 (32.9)	5 (33.3)	47 (32.9)	0.589
Recent exposure to pigs	11 (7.0)	2 (13.3)	9 (6.3)	0.28
Alcohol abuse	33 (21.0)	5 (33.3)	28 (19.6)	0.178

*Statistical analyses were performed by using the χ^2 or Fisher exact test.
†Ages were not available for 3 patients.
‡One case of bacteremic meningitis was associated with pneumonia.
§Two cases of probable meningitis were associated with spondylodiscitis.

was also significantly associated with the meningitis category (p<0.0001). The VAG profile of *epf–/sly+/mrp–*, which was observed only in the ST104 strains, was also significantly associated with the nonmeningitis category (p<0.0001). Because the largest cluster of 89K PAI–carrying strains was associated with the VAG profile of *epf+/sly+/mrp+*, the presence of 89K PAI was also significantly associated with the meningitis category (p<0.0001). None of the genotypic profiles that included STs, VAG, and presence of 89K PAI were significantly associated with fatal or nonfatal outcomes (data not shown).

Discussion

Our finding that isolated *S. suis* serotype 2 strains peaked during the rainy season of 2006–2008 confirmed conclusions reached in previous small-scale studies

Table 4. Genotypic features of *Streptococcus suis* serotype 2 as risk factor for meningitis*

Feature	Clinical category, no. (%) strains			p value
	All, n = 158	Meningitis, n = 93	Nonmeningitis, n = 65	
Sequence type				
1	98 (62.0)	73 (78.5)	25 (38.5)	<0.0001†
104	40 (25.3)	6 (6.5)	34 (52.3)	<0.0001‡
25	11 (7.0)	7 (7.5)	4 (6.2)	0.478
28	3 (1.9)	2 (2.2)	1 (1.5)	0.632
103	3 (1.9)	2 (2.2)	1 (1.5)	0.655
126	3 (1.9)	2 (2.2)	0	0.201
VAG profile				
epf+/sly+/mrp+	97 (61.4)	72 (79.6)	25 (35.4)	<0.0001†
epf+/sly+/mrp^s	3 (25.3)	3 (3.2)	0 (0)	0.201
epf–/sly+/mrp–	40 (25.3)	6 (6.5)	34 (52.3)	<0.0001‡
*epf– /sly–/mrp**	10 (6.3)	6 (6.5)	4(6.2)	0.607
epf–/sly–/mrp+	7 (4.4)	5 (5.3)	2 (3.1)	0.392
epf–/sly+/mrp+	1 (1.0)	1 (1.1)	0 (0)	1
89K PAI profile, 89K PAI+	98 (62.0)	70 (75.3)	28 (43.1)	<0.0001†

*Statistical analyses were performed by using the χ^2 or Fisher exact test. VAG, virulence-associated gene; 89K PAI, ≈89-kb pathogenicity island.
†Significant association with the meningitis category.
‡Significant association with the nonmeningitis category.

conducted in northern Vietnam and Hong Kong (*35,36*). The predominant distribution of these isolates in northern Thailand is also in accordance with previous reports (*6–11*). However, why no human cases were identified in southern Thailand remains uncertain. A recent study from Hong Kong reported heavy contamination of *S. suis* in raw pork meat at local supermarkets or wet markets; therefore, a hot and humid climate may facilitate the growth of *S. suis* in raw pork products in those markets (*36*) and increase the risk for *S. suis* infections in humans in northern Thailand. The finding of no cases in children suggests that the routes of transmission are associated with adult behavior.

A recent study from northern Thailand, based on 20 human isolates collected during 1998–2002, reported that the most common isolates of *S. suis* serotype 2 were ST25 (40%), followed by ST1 (15%) and ST103 (15%) (*34*). By contrast, the MLST and PFGE results in this study clearly demonstrated that ST1 strains with major pulsotypes of A, A1 and A2, and ST104 with major pulsotypes of H and H1 were currently circulating in the same region of Thailand during 2006–2008. Collectively, these data suggest dynamic replacement of STs from ST25 to ST1 and ST104 among serotype 2 strains during recent years in this region.

Although *S. suis* serotype 2 has been reported to be the most frequent cause of bacterial meningitis in adults in Vietnam (*13,35*), other clinical manifestations, such as sepsis and infectious endocarditis, have also been found to be common in Thailand (*6,8,11*). Of the 158 human cases in the study reported here, ≈60% were assigned to the meningitis category and ≈35% were sepsis. Other clinical manifestations, including infective endocarditis, were rare. The findings reported here demonstrate significant associations between the ST1 strains and the meningitis category and between the ST104 strains and the nonmeningitis category. These findings indicate that both the ST1 and ST104 strains cause bacteremia and sepsis but that the ST1 strains are more likely to cross the blood–CSF barrier and subsequently result in meningitis. Because ≈80% of the cases in the meningitis category were caused by strains with ST1, as evidenced by a VAG profile of *epf*+/*sly*+/*mrp*+ and 89K PAI, these genotypic profiles of *S. suis* serotype 2 may favor bacterial survival and multiplication in the bloodstream, which would result in high levels of bacteremia, crossing of the blood–CSF barrier, and invasion of the meninges and the central nervous system (*15*). Our PFGE data showed that the pulsotype A1 found in serotype 2 strains with ST1 was identical to pulsotype 11 of serotype 2 strains with ST1 from Vietnam and pulsotype I of the serotype 2 strains with ST1 from Hong Kong (*13,28*). These isolates from Vietnam and Hong Kong were associated with a VAG profile of *epf*+/*sly*+/*mrp*+, and the strains from Vietnam were also the cause of meningitis in adults. A unique DNA pattern of pulsotype D, classified into cluster XVI, was found for 3 strains with ST28 isolated from nonfatal cases in this study. Previous studies also reported 1 nonfatal case caused by the ST28 strain from Thailand and Japan (*34,38*).

Associations for bacteremic meningitis cases with nonfatal outcomes and probable meningitis cases with fatal outcomes contrasted strikingly in this study. Of 6 fatal cases of probable meningitis, 2 were caused by ST1, 2 by ST25, and 2 by ST104 strains. The extent to which the virulence of each ST strain contributed to these deaths remains uncertain. Another possible explanation may be a frequent involvement of critically ill patients, for whom lumbar puncture was not possible; these patients had probable meningitis and typical meningeal signs, acute disease onset, and positive blood culture only.

Because the clinical charts were retrospectively reviewed and the etiologic diagnosis of *S. suis* infection might not have been readily reported to the attending physicians during the hospitalization of the patients in this study, the extent of investigations of clinical manifestations, possible risk factors, and causes of death might have been limited. Because different physicians were involved in the assessment of different patients in this study, the possibility of misdiagnosis for clinical categories cannot be completely excluded even though meningeal signs and acute disease onset are clinical indicators of meningitis.

In conclusion, this study of the clinical features of 158 cases of *S. suis* seotype 2 infection in humans in Thailand showed that the disease occurs sporadically in adults and results in a mortality rate of ≈9.5%; the major clinical manifestations include meningitis and sepsis. MLST analyses of 165 isolates from humans indicated that the major STs were ST1 followed by ST104. Although both ST1 and ST104 strains cause sepsis, it is likely that only the ST1 strain causes meningitis. Further studies are needed to elucidate the pathogenesis of the human *S. suis* infections that are prevalent in Southeast Asian countries.

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Case Report

Sepsis and spontaneous bacterial peritonitis in Thailand

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In June, 2007, a 66-year-old man (case 1), an alcohol misuser with alcoholic liver cirrhosis who habitually ate raw pork, was referred to Uttaradit Hospital, northern Thailand. He had a fever and massive ascites. His leucocyte count was $4{\cdot}4\times10^9$/L, and total bilirubin and albumin concentrations were 23·7 mg/L, and 26 g/L, respectively. Polymorphonuclear leucocyte count of ascitic fluid was $4{\cdot}1\times10^8$/L and culture was positive despite a negative blood culture. This patient was diagnosed with spontaneous bacterial peritonitis,[1] and successfully treated with ceftriaxone. Testing of this isolate with the API 20 Strep Kit (BioMérieux, Marcy l'Etoile, France) suggested *Streptococcus equi* subspecies *zooepidemicus* with 91·8% identification. However, there was 99% similarity of the 16S rDNA sequence with known *S suis* strains. Confirmation that this isolate belonged to this species was further supported by a positive reaction for *S suis*-specific PCR amplification of the *S suis* 16S rRNA gene.[2]

In May, 2007, a 62-year-old woman (case 2), with liver cirrhosis who had had repeated episodes of spontaneous bacterial peritonitis in 2006, was admitted to Phetchabul Hospital, northern Thailand, with a fever. Physical examination showed cutaneous jaundice and ascites. Her leucocyte cell count, total bilirubin, and albumin were $15{\cdot}1\times10^9$/L, 108·2 mg/L, and 18 g/L, respectively. Culture of ascitic fluid was negative, blood culture was positive, and she was diagnosed with sepsis. The isolate was identified as *S suis* by the API 20 Strep Kit. This patient also improved on treatment with ceftriaxone.

The isolates from these two cases were confirmed by a co-agglutination test as serotype 5 for case 1 and serotype 24 for case 2 (table), and were assigned to the novel sequence types by multilocus sequence typing.[2]

We report the first human cases of *S suis* infection with serotypes 5 and 24. *S suis* is a zoonotic pathogen that can cause invasive infections in human beings who consume raw pork products or are in close contact with infected pigs.[3] Although serotype 2 is the most prevalent in human beings, cases with serotypes 1, 4, 14, and 16 have been reported.[2–4] In Thailand between 2006 and 2008, 179 human isolates of *S suis* were collected from sterile sites eg, blood, cerebrospinal fluid. Of these isolates, 165 (92·2%) were serotype 2, and 12 (6·7%) were serotype 14.[2] The differential diagnosis of our two cases includes melioidosis and leptospirosis. Bacterial translocation has an important role in the pathogenesis of spontaneous bacterial peritonitis in cirrhosis, and the most common pathogens are enterobacteriaceae.[1] Previous reports on human infections after recent consumption of raw pork products suggested that the gastrointestinal tract is a major route of entry in cases of *S suis* infections in Thailand and Vietnam.[2,3,5] In this region, the occurrence of spontaneous bacterial peritonitis through bacterial translocation of *S suis* after consumption of raw pork products is possible in patients with liver cirrhosis. A similar case of spontaneous bacterial peritonitis caused by serotype 16 strain of *S suis* in a patient with alcoholic liver cirrhosis was reported from Vietnam.[4] Although the isolation rates for serotypes 5 and 24 are low (2/179 cases; 1·1%), *S suis*-specific PCR is recommended for identification of streptococcal isolates from sterile sites, and a serious caution against eating raw pork products should be given to patients with liver cirrhosis, especially in southeast Asian countries.

	Case 1	Case 2
Source of isolate	Ascites	Blood
Clinical diagnosis	Spontaneous bacterial peritonitis	Sepsis
Comorbid illness	Alcholic liver cirrhosis	Liver cirrhosis
Identification with API 20 Strep	*S equi* subspecies *zooepidemicus*	*S suis*
S suis-specific PCR	Positive	Positive
Sequencing of 16S rRNA gene	*S suis* (99%)	*S suis* (99%)
Serotype	5	24
Multilocus sequence typing	ST181	ST221

ST: sequence type.

Table: **Microbiological features of two human cases of *Streptococcus suis* infection**

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Patient care: AB, SS. Microbiology: AK, WN, SC, YA. Serotyping: MG. Study coordination: SD. Writing: PS, DS, KO. Written consent to publish was obtained.

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CASE REPORT

Recurrent bacterial meningitis by three different pathogens in an isolated asplenic child

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Abstract Isolated congenital asplenia (ICA) is a rare condition at risk for overwhelming infection. When complicated by invasive infection, the mortality remains high, at greater than 60%. We describe a girl with ICA who developed recurrent meningitis by three different pathogens. The first, meningitis by *Escherichia coli*, occurred 4 days after premature birth. The other two pathogens were serotype 6B *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib), at 18 and 25 months of age, respectively. The patient was successfully treated with prompt antimicrobial therapy in all episodes. Serum anti-polyribosylribitol phosphate (PRP) and anti-6B-type pneumococcal antibodies were below the levels for protective activity after natural infections. Although anti-PRP antibody was significantly increased after Hib vaccination, two (6B and 19F) of seven serotype-specific pneumococcal antibodies were not elevated to protective levels after the second 7-valent pneumococcal conjugate vaccine (PCV7). We, therefore, added a third PCV7. To our knowledge, this is the first neonatal ICA patient with invasive infection and the first case of bacterial meningitis occurring three times. Our findings indicate that monitoring of immune responses after natural infections and vaccinations, and reevaluations of vaccine schedule, are important for ICA patients to prevent subsequent invasive infections.

Keywords Isolated congenital asplenia · Bacterial meningitis · Immunological response · Recurrence · Neonate · Vaccine

Introduction

Congenital asplenia often occurs as part of a recognized malformation syndrome with anomalies of the heart, great vessels, and viscera [1]. The best known among these syndromes is the asplenia/polysplenia syndrome associated with visceroarterial heterotaxy, and its incidence is estimated at approximately 1/10,000 to 1/40,000 live births [2]. In contrast, isolated congenital asplenia (ICA) occurs fairly more infrequently. A recent French nationwide study indicated that the prevalence is 0.51 per million births [2]. Both conditions have an increased susceptibility to overwhelming invasive infections, carrying considerable mortality. However, the diagnosis of ICA is sometimes difficult because of the lack of other anomalies; therefore, such individuals may be unrecognized until postmortem autopsy.

Practice guidelines for the prevention of life-threatening infections in children with hyposplenia and asplenia advocate antibiotic prophylaxis and immunizations against *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib), the most common causative organisms for

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these patients [3]. However, given that several asplenic cases of overwhelming infections that could be considered as vaccine failures have been documented [4, 5], the immunogenicity of vaccination for asplenic patients is still an important concern.

We present here a girl with ICA who developed multiple episodes of meningitis caused by three different pathogens, namely, *Escherichia coli*, *S. pneumoniae* (serotype 6B), and Hib. She was successfully treated with prompt initiation of antibiotics in all episodes. We also present the details of immune responses to natural infections by Hib and serotype 6B *S. pneumoniae* and those to immunizations of Hib conjugate vaccine and 7-valent pneumococcal conjugate vaccine (PCV7).

Case report

A 4-day-old girl, who was born of nonconsanguineous parents as their first child, weighing 1,742 g at the 34th week of gestation, presented with repetitive apnea during admission because of prematurity. Physical examination showed that heart rate was 135/min and body temperature was 37.2°C. Laboratory data showed WBC of 5.8×10^9/l with 28.5% neutrophils, C-reactive protein (CRP) of 4.3 mg/dl, and blood glucose of 95 mg/dl. Cerebrospinal fluid (CSF) examination showed 3,947 cells/μl with 96% polymorphonuclear cells, 197 mg/dl protein, and 44 mg/dl glucose. Two days later, isolates from the CSF and blood were identified as *E. coli* OX:K1:H–, and the same bacterium was also subsequently isolated from the stool of her asymptomatic mother. The patient was diagnosed as having early-onset *E. coli* meningitis that was vertically transmitted. We treated the patient with cefotaxime (CTX) for 21 days. Auditory brainstem response examination at 28 days of age revealed profound hearing impairment at the right ear. The patient was discharged at 38 days of age. Genetic analysis [6, 7] showed that the strain harbored virulence factor genes such as *iroN*, *papG3*, *afa*, and *kps*, but not *cnf1*, *sfa*, or *ibeA*.

At 18 months old, the patient was rehospitalized because of a 6-h history of fever and generalized tonic-clonic convulsion lasting 3 min. On admission, 30 min after the convulsion, heart rate was 170/min and body temperature was 39.4°C. Her consciousness had become clear. Laboratory findings showed WBC of 21.7×10^9/l and CRP of 6.0 mg/dl. CSF examination showed no pleocytosis, with normal concentrations of protein (10 mg/dl) and glucose (85 mg/dl). Treatment with intravenous CTX was empirically initiated under the tentative diagnosis of occult bacteremia. The day after admission, serotype 6B *S. pneumoniae* was isolated from the blood but not from the CSF. Resistance to penicillin was established by microbiological [minimum inhibitory concentration (MIC), 2 μg/ml] and genotypic (mutations in *pbp1a*, *pbp2X*, and *pbp2b* [8]) analyses, and CTX was substituted with panipenem–betamipron. On day 3, prolonged fever and frequent vomiting led us to perform a second CSF examination, showing 14,500 cells/μl, protein of 58 mg/dl, and glucose of 63 mg/dl. The CSF was positive for *S. pneumoniae* antigen test (Binax NOW *S. pneumoniae*; Binax), but yielded no organisms in culture. The blood WBC and CRP were elevated to 21.7×10^9/l and 22.1 mg/dl, respectively. We diagnosed her disease as pneumococcal meningitis following bacteremia and increased the doses of panipenem–betamipron with good clinical response. She received antimicrobial therapy for 14 days and was discharged without any additional sequelae.

At 25 months of ages, the patient was referred to the emergency department in another hospital with a 2-h history of fever, vomiting, and tonic–clonic convulsion of 2-min duration. At arrival, heart rate was 180/min and body temperature was 39.4°C. Her consciousness soon became clear. Laboratory examination showed WBC of 3.5×10^9/l and CRP of 0.6 mg/dl. After blood culture was obtained, the patient received intravenous sulbactam/ABPC. On day 3, the blood culture yielded β-lactamase-non-producing ABPC-resistant (BLNAR) Hib, and the laboratory examinations showed marked deterioration: WBC of 26.6×10^9/l and CRP of 21.5 mg/dl. CSF examination showed 4,992 cells/μl, 164 mg/dl protein, and 34 mg/dl glucose with positive culture for Hib. Thus, the diagnosis of a third bacterial meningitis was made. The patient thereafter received intravenous meropenem for 14 days and was discharged on day 16 after onset without any additional sequelae. Molecular analysis of the strain identified three amino acid substitutions: His-517, Thr-385, and Ile-377, in *ftsI* [9]. This substitution pattern was classified as subgroup III BLNAR by a recent nationwide study of childhood meningitis in Japan [9].

The multiple episodes of meningitis prompted us to evaluate immunological functions. The results after the second episode of meningitis showed that serum levels of IgG (639 mg/dl), IgA (65 mg/dl), IgM (97 mg/dl), IgG_2 (80 mg/dl), C3 (140 mg/dl), C4 (24 mg/dl), and CH50 (36.1 U/ml) were within normal limits. T/B-cell subsets (65/28%), CD3/CD4/CD8 lymphocyte subsets (61%/44%/14%), natural killer cell activity (25%), neutrophil phagocytic activity using fluorescence bead test by flow cytometry (70.0%), and neutrophil bacteriocidal activity (93.4%) were also normal. Computed tomography (CT) of the skull and inner ears did not show any deformity or defects. To screen interleukin-1 receptor-associated kinase 4 deficiency and myeloid differentiation primary response protein 88 deficiency, we performed flow cytometric analysis [10], resulting in normal intracellular tumor necrosis factor-α

production of monocytes after lipopolysaccharide stimulation. After the third meningitis, ultrasonography and CT of the abdomen finally revealed asplenia without visceroarterial anomalies. Howell–Jolly body-containing RBCs were exceedingly rarely found (<0.1% of RBCs) in peripheral blood. Ultrasonographic examinations of her parents detected normal size and normal position of the spleen.

Since the diagnosis of ICA at 26 months of age, chemoprophylaxis with amoxicillin of 20 mg/kg/day was introduced as well as vaccinations of Hib vaccine and PCV7. Subsequent to the introduction of these strategies, the patient has not suffered from any invasive infections for more than 2 years. At 36 months of age, we assessed her neurodevelopmental status using the New Edition of the Kyoto Scale of Psychological Development, indicating a normal developmental quotient of 88 (normal range, >80).

We evaluated immune responses to natural infections with Hib and serotype 6B pneumococcus and those to immunizations of Hib vaccine and PCV7 (Table 1). Despite natural infections, serum anti-polyribosylribitol phosphate (PRP) (0.60 μg/ml) and anti-serotype 6B (0.191 μg/ml) antibodies were below the levels of long-term protective activity (1.0 μg/ml [11] and 0.34 μg/ml [12, 13], respectively) 4 and 6 months after each infection, respectively. At 1 month after administration of the second Hib and PCV7 vaccination, anti-PRP antibody was significantly elevated to 3.15 μg/ml, but two (6B and 19F) of seven serotype-specific pneumococcal antibodies were still below the protection levels. We therefore added a third PCV7. Because antibodies to pneumococcal capsular polysaccharide protect the host by opsonizing pneumococci for phagocytosis, we concomitantly performed the opsonophagocytic killing assay (OPA) [14] after the third PCV7. Table 1 shows significantly high OPA titers against types 6B and 19F were observed, findings inconsistent with the low anti-6B and anti-19F IgG antibody levels. OPA titers against five other types were also elevated to the levels for protection (>8) [12, 13].

Discussion

We report a girl with non-familial ICA with recurrent bacterial meningitis. ICA is a rare anomaly. Mahlaoui et al. [2] recently documented 20 ICA cases in France and reviewed the literature. In addition to the 65 cases in their report and references therein [2], we found reports of 5 other ICA patients [5, 15] in the literature between January 1960 and April 2011 using the Medline database. Thus, we can here review 70 ICA cases in total. Compared with these patients [2, 5, 15], our case is informative and interesting in several respects.

First are the multiple episodes of meningitis caused by three different pathogens. Of the previous 70 cases, 48 (69%) experienced invasive bacterial infection at least once. Of these 48 patients, only 8 had multiple episodes of invasive bacterial infections, two times in 5 cases and three times in 3 cases (Table 2) [2, 16–20]. Our patient is the first described for whom all three episodes were bacterial meningitis. To better understand the underlying pathogenesis, we characterized the causative pathogens by molecular analysis. Penicillin-resistant serotype 6B pneumococcus and BLNAR Hib subgroup III were among the most prevalent strains causing childhood meningitis in Japan [8, 9]. In contrast, *E. coli* is extremely rare among ICA patients, and we are aware of only one such case, which resulted in death at 4 months of age [21]. *E. coli* in our case possessed capsular antigen K1 and the siderophore receptor gene, *iroN*, which contribute to the bacteremic step in *E. coli* neonatal meningitis [7, 22]. Because the same strain was isolated from the stool of her asymptomatic mother, we confirmed the route of contagion. Besides asplenia, prematurity of the host and high pathogenic factors of the *E. coli* strain might have contributed to this infection.

Second is the good prognosis, despite our patient developing meningitis three times, one of which occurred 4 days after premature birth. Our neonatal case is the youngest at the first invasive infection among the previously reported ICA patients. There have been only 3 ICA patients

Table 1 Serum serotype-specific IgG antibody concentrations and opsonophagocytic killing assay titer before and after 7-valent pneumococcal conjugate vaccine

Serotype	4		6B		9V		14		18C		19F		23F	
	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA
Before PCV7 (6 months after natural infection)	0.132	NA	0.191	NA	0.062	NA	0.366	NA	4.229	NA	0.295	NA	0.14	NA
1 month after 2-dose PCV7	2.809	NA	0.263	NA	4.040	NA	6.767	NA	3.949	NA	0.356	NA	0.233	NA
1 month after 3-dose PCV7	1.37	536	0.137	557	1.199	326	5.075	2367	1.89	210	0.295	192	0.471	769

PCV7 7-valent pneumococcal conjugate vaccine, *IgG conc.* anti-serotype-specific IgG antibody concentration (μg/ml), *OPA* opsonophagocytic killing assay (titer), *NA* not assessed (under treatment with antimicrobial agents)

Table 2 Isolated congenital asplenia patients with multiple episodes of invasive bacterial infections

Patient number	Gender	Infectious episodes	Age at onset	Type of infection	Organisms	Outcome	Reference
1	F	1	6 months	Meningitis	*Streptococcus pneumoniae*	Survived	[2]
		2	11 months	Meningitis, purpura fulminans	*S. pneumoniae*	Died	
2	M	1	10 months	Meningitis	*S. pneumoniae*	Survived	[2]
		2	11 months	Purpura fulminans	*S. pneumoniae*	Survived	
		3	1 year 7 months	Purpura fulminans	*S. pneumoniae*	Survived	
3	M	1	1 year 9 months	Meningitis	*S. pneumoniae*	Survived	[16]
		2	2 years 3 months	Meningitis	*S. pneumoniae*	Survived	
4	M	1	1 year 2 months	Meningitis	*S. pneumoniae*	Survived	[17]
		2	15 years	Meningitis	Not available	Died	
5	M	1	1 year	Meningitis	*S. pneumoniae*	Survived	[18]
		2	1 year	Meningitis	*S. pneumoniae*	Survived	
		3	1 year	Osteomyelitis	Culture negative	Survived	
6	F	1	6 months	Meningitis	*S. pneumoniae*	Survived	[19]
		2	2 years 6 months	Sepsis	Not available	Died	
7	F	1	1 year 6 months	Arthritis	*S. pneumoniae*	Survived	[19]
		2	1 year 9 months	Arthritis	*Haemophilus influenzae* type b	Survived	
		3	10 years	Sepsis	*S. pneumoniae*	Died	
8	M	1	5 years	Sepsis	*S. pneumoniae*	Survived	[20]
		2	9 years	Meningitis	*S. pneumoniae*	Died	
9	F	1	0 month (4 days)	Meningitis	*Escherichia coli*	Survived	Present case
		2	1 year 6 months	Meningitis	*S. pneumoniae*	Survived	
		3	2 years 1 month	Meningitis	*H. influenzae* type b	Survived	

who had overt infections under 3 months of age, which include 1 fatal case [21] and 2 with major sequelae (central nervous system deficit [23] or loss of foot and fingers [24]). Of the 45 childhood and adult patients with invasive infections whose outcomes were known, 29 (64%) died and 3 (7%) had serious sequelae [2, 5, 23, 24]. In contrast, our patient showed normal neurological development under non-serious sequelae of unilateral hearing loss. Such favorable outcome may be attributable to the early recognition and hospitalization. Fortunately, the first episode developed during the period of hospitalization under close monitoring because of prematurity. In addition, at both second and third infectious episodes, she could receive immediate antimicrobial treatment.

Finally, we meticulously investigated the immunological responses to natural infections with *S. pneumoniae* and Hib and those to vaccinations. Of the 70 cases we can review [2, 5, 15], there has been no report addressing this issue. The spleen is a pivotal organ for the phagocytosis of encapsulated bacteria and for the production of immunoglobulins against these pathogens [3]. Even after natural invasive infections of Hib and serotype 6B pneumococcus, serum antibody levels were not elevated to the levels of long-term protection against the pathogens, which may reflect the immunocompromised status of asplenia. This concept is supported by findings from Mikoluc et al. [25] that the congenital asplenic patients had significantly lower concentrations of serum anti-pneumococcal antibodies and reduced responses to PCV7, especially to serotypes 6B and 23F. Similar findings were also observed in adult asplenic patients with overwhelming infection caused by *S. pneumoniae*, representing vaccine failures [4, 5]. Serum antibody concentrations against 6B and 19F in our patient were significantly lower than those against five other serotypes. In contrast, when we evaluated OPA titers after the third PCV7 vaccination, they were at sufficient levels for protection against all serotypes including types 6B and 19F. OPA might be a more important indicator for protection against *S. pneumoniae* [13].

In conclusion, we described a girl with a rare case of ICA, who presented with recurrent meningitis caused by three different pathogens, and was successfully treated without severe sequelae. Exact determination of serum antibody concentrations of encapsulated bacteria and reevaluation of vaccine schedules should be important to protect against relevant infections in ICA patients.

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MAJOR ARTICLE

Platelet Apoptosis and Apoptotic Platelet Clearance by Macrophages in Secondary Dengue Virus Infections

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Background. The mechanisms of thrombocytopenia and platelet phagocytosis in dengue illness are not fully understood.

Methods. A prospective hospital-based study was conducted to examine the relationships between platelet counts, serum thrombopoietin (TPO) levels, and platelet apoptosis and phagocytosis in 81 patients with secondary dengue virus (DV) infections and 38 healthy volunteers. The apoptosis and phagocytosis of cultured platelets after exposure to DV were also examined.

Results. Platelet apoptosis, platelet phagocytosis, and serum TPO levels were increased significantly in patients during the acute and early convalescence phases compared with levels observed in patients during the convalescence phase and in healthy volunteers. A significant correlation between platelet apoptosis and platelet phagocytosis was also observed in these patients. Platelet phagocytosis was inhibited significantly by the D89E mutant, which carries a point mutation in the RGD motif of milk fat globule–epidermal growth factor 8, a phosphatidylserine-recognizing bridge molecule. DV-induced platelet apoptosis and increased phagocytosis of DV-induced apoptotic platelets was confirmed using in vitro assays.

Conclusions. Our data suggest an increased phagocytosis of DV-induced apoptotic platelets by macrophages via a phosphatidylserine-recognizing pathway in secondary DV infection. Accelerated platelet clearance, however, was overcome by TPO-induced enhanced thrombopoiesis in these patients.

Clinical Trials Registration. UMIN000004835.

Dengue virus (DV) is a mosquito-borne human viral pathogen with 4 distinct serotypes: DV1, DV2, DV3, and DV4 [1, 2]. The rapid increase in the number of human cases of DV infection has become a major public health concern during the past 3 decades in tropical and subtropical countries. Transient thrombocytopenia associated with acute febrile illness and hemorrhagic manifestations is a hallmark of DV infections. The common severe form, dengue hemorrhagic fever (DHF), is characterized by a sudden increase in vascular permeability. Secondary DV infections, which are observed commonly in dengue-endemic areas, are more likely to constitute a risk factor for DHF than are primary DV infections [3].

Studies undertaken >40 years ago suggested that the suppression of megakaryocytopoiesis in bone marrow was responsible for the thrombocytopenia and hemorrhagic manifestations in severe dengue illness [4, 5]. However, the precise mechanisms underlying

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DV-induced bone marrow suppression during the acute phase of this disease remain unclear. Thrombopoietin (TPO) is a cytokine that specifically regulates megakaryocytopoiesis and platelet production by activating c-MPL, the receptor of TPO [6, 7]. Because TPO is elevated when platelet production is reduced, serum TPO levels may be a useful indicator of megakaryocytopoiesis in this disease.

In contrast, our previous observations using flow cytometric analysis demonstrated an in vitro increase in the phagocytosis of platelets by macrophages in patients with secondary DV infections [8]. However, no significant inhibition of platelet phagocytosis was observed using antibodies directed against Fcγ receptors or complement receptor 3. Furthermore, we reported previously that high-dose intravenous immunoglobulin treatment had no effect on the severe thrombocytopenia of patients with secondary DV infections [9]. Therefore, the mechanisms of accelerated platelet phagocytosis in this disease remain uncertain.

Previous in vitro and in vivo studies suggest that apoptotic cell death is involved in the cytopathological mechanism of DV infections [10, 11]. Platelets undergo an apoptotic program that regulates their normal circulatory lifespan [12] and is induced after platelet activation by agonists such as thrombin [13]. Apoptotic platelets express phosphatidylserine (PS) on their surfaces and also include activation of caspase 3, a key effector of apoptosis. A previous study also reported morphological changes in aged platelets, as assessed using electron microscopy, similar to those observed during granulocyte apoptosis [14]. The apoptotic cells are recognized by phagocytes through "tethering" ligands such as the milk fat globule–epidermal growth factor 8 (MFG-E8), PS receptors such as TIMD4, and scavenger receptors such as CD36 [15, 16]. The apoptotic platelets are then degraded in the phagocytes.

In this study, we hypothesized that DV induces platelet apoptosis during the acute phase of secondary DV infection, with subsequent acceleration of phagocytosis of the apoptotic platelets mediated by a PS-recognizing pathway.

MATERIALS AND METHODS

Patients and Study Design

A prospective hospital-based study of DV infection was conducted at San Lazaro Hospital, Manila, Philippines, between September 2009 and December 2010. Ninety-seven patients aged ≥10 years and with a clinical suspicion of DV infection were enrolled during the study period. The age restriction was imposed because 10 mL of blood was required for the analyses of platelets of each patient in this study. Among the patients enrolled, 86 were confirmed as having acute-phase DV infection (3−7 days after the onset of illness) on the basis of results of reverse-transcription polymerase chain reaction (RT-PCR) or dengue immunoglobulin M (IgM)–capture enzyme-linked immunosorbent assay (ELISA) for DV. Subsequently, 81 patients were found to have secondary DV infection based on a dengue hemagglutination-inhibition assay [2, 17]. DF or DHF was diagnosed according to the World Health Organization (WHO) guidelines [18]. The patients with grade III or grade IV DHF and patients requiring platelet transfusion were excluded. Because most patients had secondary DV infection, we decided to examine patients with secondary infection, and not primary infection, in this study.

Platelet-rich plasma and serum from the patients enrolled were prepared. Peripheral platelet counts, platelet apoptosis, serum TPO levels, and platelet phagocytosis were determined in these patients at the time of enrollment (acute phase), 4 days after the first blood collection (early convalescence phase), and ≥4 days after the second blood collection (convalescence phase). Thirty-eight age-matched healthy control subjects were enrolled during the same period at St Luke's Medical Center, and the same assays were performed as those performed for the patients. Serum TPO levels were measured using a sandwich ELISA (R&D Systems) in 79 patients and 27 controls only because of insufficient volume of serum samples.

The research proposal for this study was approved by the Bioethics Committee of the San Lazaro Hospital and the Institutional Ethics Review Board of St Luke's Medical Center. All patients and controls provided written informed consent.

Platelet Phagocytosis and Inhibition Assay

Platelet phagocytosis by differentiated THP-1 cells was examined in platelet samples from 81 patients and 38 controls, as described previously [8, 19]. In brief, washed platelets were stained with CellTracker Orange CMTMR (CTO; Molecular Probes), and the frequency of platelet phagocytosis was determined by counting the CTO-positive, CD61 (a platelet-specific marker)–negative cells using flow cytometry. As a positive control for each assay, the apoptotic pathway was activated in platelets obtained from healthy donors by incubation with human thrombin (5 NIH units/mL; Sigma-Aldrich) [13]. The mean frequency (SD) of phagocytosis of thrombin-treated apoptotic platelets was 50.47 (6.15). The frequency of platelet phagocytosis was expressed as a percentage, as follows: [(the frequency of phagocytosis of the test platelets/the frequency of thrombin-treated apoptotic platelets) × 100]. Assays of inhibition of platelet phagocytosis were performed in 7 patients with DHF, 17 patients with DF, and 20 controls. FLAG-tagged recombinant D89E of the MFG-E8 protein, carrying a point mutation in the RGD motif, was prepared for an inhibition assay of platelet phagocytosis, as described previously [20]. The D89E mutant protein inhibits the phagocytosis of apoptotic cells in vitro and in vivo.

CTO-stained platelets were pretreated with phosphate-buffered saline (PBS) or D89E (1.0 μg/mL) in PBS for 30 minutes at 37°C.