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Immunity to pneumococcal surface proteins in children with community-acquired pneumonia: a distinct pattern of responses to pneumococcal choline-binding protein A

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Abstract

The aetiological diagnosis of community-acquired pneumonia (CAP) is challenging in children, and serological markers would be useful surrogates for epidemiological studies of pneumococcal CAP. We compared the use of anti-pneumolysin (Ply) antibody alone or with four additional pneumococcal surface proteins (PSPs) (pneumococcal histidine triad D (PhtD), pneumococcal histidine triad E (PhtE), LytB, and pneumococcal choline-binding protein A (PcpA)) as serological probes in children hospitalized with CAP. Recent pneumococcal exposure (positive blood culture for *Streptococcus pneumoniae*, Ply^+ blood PCR finding, and PSP seroresponse) was predefined as supporting the diagnosis of presumed pneumococcal CAP (P-CAP). Twenty-three of 75 (31%) children with CAP (mean age 33.7 months) had a Ply^+ PCR finding and/or a ≥2-fold increase of antibodies. Adding seroresponses to four PSPs identified 12 additional patients (35/75, 45%), increasing the sensitivity of the diagnosis of P-CAP from 0.44 (Ply alone) to 0.94. Convalescent anti-Ply and anti-PhtD antibody titres were significantly higher in P-CAP than in non P-CAP patients (446 vs. 169 ELISA Units (EU)/mL, p 0.031, and 189 vs. 66 EU/mL, p 0.044), confirming recent exposure. Acute anti-PcpA titres were three-fold lower (71 vs. 286 EU/mL, p <0.001) in P-CAP children. Regression analyses confirmed a low level of acute PcpA antibodies as the only independent predictor (p 0.002) of P-CAP. Novel PSPs facilitate the demonstration of recent pneumococcal exposure in CAP children. Low anti-PcpA antibody titres at admission distinguished children with P-CAP from those with CAP with a non-pneumococcal origin.

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Introduction

Community-acquired pneumonia (CAP) is a common paediatric infection and a leading cause of mortality worldwide in young children [1,2]. Aetiological diagnosis is particularly challenging [3]. Recent studies using modern microbiological diagnostic tools have repeatedly identified *Streptococcus pneumoniae* as a leading cause of CAP [4,5]. Recently, the diagnosis of pneumococcal CAP (P-CAP) was enhanced with new bacterial antibody and immune complex assays using capsular C-polysaccharide or type-specific polysaccharides, and pneumolysin (Ply) surface protein [6], a cytolytic toxin involved in pneumococcal pathogenesis [7]. Serodiagnosis of P-CAP remains particularly challenging in young children, who are frequently colonized by *S. pneumoniae* and in whom recent carriage acquisition may trigger serological responses [8–10]. Diagnosis of P-CAP in paediatric studies relies upon a combination of criteria showing recent *S. pneumoniae* exposure with positive blood culture or PCR findings, and anti-pneumococcal seroresponses [4–6]. Without a reference standard, these assays remain incompletely validated and useless for individual diagnosis. However, they currently constitute the best approach available to estimate pneumococcal disease burden in studies.

Recently, probing of the antibody repertoire for pneumococcal surface proteins (PSPs) identified approximately 140

antigens [11]. Their contribution to diagnosis, recovery or protection against subsequent infection remains unknown. We investigated how additional PSPs would impact on the serodiagnosis of P-CAP as compared with the use of anti-Ply antibodies alone. Four PSPs were selected, primarily on the basis of their availability, conservation across pneumococcal strains (>97.5%) [12] (sanofi Pasteur, unpublished data) and putative role(s) in bacterial pathogenesis [13]. Pneumococcal histidine triad D (PhtD) and pneumococcal histidine triad E (PhtE) are thought to be involved in laminin binding and/or zinc acquisition [14]. LytB is a choline-binding protein linked to daughter cell separation [15], and possibly contributes to bacterial spread. Pneumococcal choline-binding protein A (PcpA) may play a role in bacterial adherence to respiratory epithelium in the lower respiratory tract [16].

Here, we investigated antibody responses to four new PSPs in children, their dynamics during CAP, and how these anti-PSP responses differ between children with presumed P-CAP and those with non-pneumococcal CAP (NP-CAP).

Materials and Methods

After written consent had been obtained, 99 children aged ≤6 years were enrolled between 2003 and 2005 in a prospective descriptive study on CAP in the University Hospitals of Lausanne and Geneva (Switzerland) [17]. Children were eligible if they presented with clinical signs of pneumonia according to the WHO classification [18]; children with actively treated asthma, chronic underlying disease, immunosuppression or wheezing were excluded. Pneumonia was defined as: (i) non-severe when the child had only fever and tachypnoea; (ii) severe when the child had, in addition, increased work in breathing; and (iii) very severe when the child had difficulty in feeding. One child had a positive blood culture for *S. pneumoniae*. No child had received pneumococcal vaccination. This study was approved by both Ethical Committees.

Chest radiographs were reviewed by a senior radiologist blinded to clinical and laboratory findings. The presence of consolidation or pleural effusion with parenchymal infiltrate defined radiological pneumonia. Blood and nasopharyngeal samples were assessed for viruses and bacteria. PCR analyses on nasopharyngeal samples included 13 common respiratory viruses, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*, and acute blood samples were assessed by Ply-specific PCR [17]. Convalescent serology findings were obtained from 75/99 children, who agreed to return 3 weeks later. Children with paired samples were enrolled in the study. Samples from 58 healthy children, matched by age, and admitted for elective surgical procedures without evidence of acute infection, were used as controls.

Recombinant PhtD, PhtE, PcpA and LytB were expressed in *Escherichia coli* as soluble proteins, and purified with combinations of ion-exchange chromatography. PhtD and PhtE were full-length proteins, whereas PcpA and LytB were truncated forms without the choline-binding domain. All four were cloned from strain 14453 (serotype 6B). Each protein was obtained at ≥90% purity. Serum samples were stored at −20°C until analysis. Paired acute–convalescent samples were tested in the same run by indirect ELISA, with the use of purified proteins to coat Immulon (Thermo Labsystem Helsinki, Finland) plates, anti-IgG antibody (Cappel, Laboratory Cochran Ville; PA), and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) substrate. Eight serial dilutions (two-fold) of samples were performed to allow precise quantification of antibody titres. A common reference human AB serum was used in each assay. Its antibody concentration in ELISA Units (EU) was defined by the reciprocal of its dilution at an optical density of 1.0. Results were expressed in EU/mL by comparing individual optical densities to that of a reference serum. A cut-off of 5 EU/mL was experimentally identified as allowing reliable detection of serum concentrations. Concentrations below 5 EU/mL were given a titre of 2.5 EU/mL. Antibody concentrations were log transformed to allow comparisons of geometric mean concentrations (GMCs). A significant response in antibody titres was conservatively predefined as a ≥2-fold increase between acute-phase and convalescent-phase samples. Children admitted for CAP with a positive blood culture for *S. pneumoniae*, Ply^+ PCR and/or significant anti-PSP responses to any PSP were considered to have P-CAP, and were compared with those lacking such evidence (NP-CAP). Additional analyses used a more stringent criterion of a ≥4-fold increase to define seroresponses. Nasopharyngeal carriage was not used to define the origin of CAP.

Variables were compared with Student's *t*-test, chi-square tests, or Fisher's exact test when appropriate. Serological responses among groups were compared by using analysis of variance. Logistic regression analysis was used to calculate adjusted ORs and 95% CIs, controlling for any statistically significant demographic variable that might function as a confounder. Differences were considered significant at $p < 0.05$, or when the 95% CI did not include 1.0. SPSS version 15.0 was used for analyses.

Results

Among 75 children (mean age 33.7 months) hospitalized with CAP, 15 (20%) were Ply^+ by PCR (Table 1). Rising anti-Ply

TABLE 1. Seroresponses to pneumococcal surface protein (PSP) in children with community-acquired pneumonia (CAP) according to their pneumolysin (Ply) PCR status

	Anti-Ply seroresponse ≥2 fold *N* (%)	Any PSP seroresponse ≥2 fold *N* (%)	Any PSP seroresponse ≥4 fold *N* (%)
Ply^+ PCR (*N* = 15)	8 (53)	13 (87)	11 (73)
Ply^- PCR (*N* = 60)	8 (13)	20 (33)	13 (22)

IgG responses identified 16 (21%, ≥2-fold) or eight (11%, ≥4-fold) children with P-CAP (Table 2). Consideration of Ply^+ PCR and/or anti-Ply seroresponses as markers of P-CAP increased this proportion to 31% (23/75, ≥2-fold) or 24% (18/75, ≥4-fold) [17]. *S. pneumoniae* nasopharyngeal carriage was detected in 24 (32%).

We first compared the proportions of CAP children with acute seroresponses to each of four additional PSPs. Responses to LytB were rare (five, 7%). In contrast, responses to PhtD, PhtE or PcpA were observed in 20% (≥4 fold) to 32% (≥2 fold) of children (Table 2). A rising response to one or more PSPs was observed in 44% (≥2-fold) and 31% (≥4-fold), respectively. Such responses were frequently directed against several PSPs (≥2 PSPs, 30%; ≥3 PSPs, 25%; ≥4 PSPs, 14%; ≥5 PSPs, 1%), providing strong evidence of recent pneumococcal exposure. These fold changes were marked for anti-PhtD (mean: 4.22), anti-PhtE (mean: 6.88), and anti-PcpA (mean: 5.62). Use of ≥2-fold or ≥4-fold definitions for seroresponses had little impact on the patient's attribution to a P-CAP vs. an NP-CAP group (not shown). Age did not influence fold changes of anti-PSP IgG ($R^2 < 0.162$ for all).

We then compared the contribution of anti-Ply alone vs. anti-PSP for the diagnosis of P-CAP children with Ply^+ PCR findings in blood. Among 15 Ply^+ PCR patients, eight (53%) had ≥2 fold anti-Ply responses, yielding a negative predictive value (NPV) of 0.88 and a positive predictive value (PPV) of 0.50. Single anti-PSP responses resulted in higher NPVs, except for LytB, without improving PPVs (Table 2). Most Ply^+ PCR children (13, 87%) responded (≥2 fold) to one or more of the PSPs tested (Table 1). Two non-responders were a 2.5-month-old boy, presumably too young to rapidly raise infection-driven B-cell responses, and a 43-month-old girl admitted with >1-week history of cough and fever, who already had high serum titres against the five tested PSPs when admitted with lobar pneumonia. Thus, adding PhtD, PhtE and PcpA to anti-Ply antibody markedly increased the NPV (0.96) of serodiagnosis in P-CAP children.

Relying on acute/convalescent seroresponses provides the demonstration of recent pneumococcal exposure. However, such seroresponses may be elicited prior to admission. Therefore, many clinical studies add a criterion of 'high titres at admission', derived from comparisons with control children, in their diagnostic algorithm [19,20]. Comparison of the GMCs of the 38 CAP children older than 24 months (mean age 43.1 months) at enrolment with those of 58 healthy control children (mean age 43.6 months) indicated that anti-PSP IgG titres at admission were higher in control children than in CAP children (in EU/mL: Ply, 370 vs. 200; PthD, 185 vs. 93; PhtE, 345 vs. 215; PcpA, 500 vs. 170). This remained true when only the 15 children with evidence of P-CAP were included (not shown). Thus, reliance on 'high' anti-Ply titres at admission for the diagnosis of P-CAP was not considered to be optimal in this study.

Our P-CAP definition identified 35 children with evidence of acute pneumococcal exposure, of whom 15 (42%) had Ply^+ PCR findings, and defined 40 NP-CAP children. Radiological consolidation was present in 31/35 P-CAP (89%) vs. 19/40 (48%) NP-CAP patients ($p < 0.001$). As expected from the natural history of pneumococcal exposure and disease, *S. pneumoniae* carriage was more frequent in P-CAP (46%) than in NP-CAP children (20%, p 0.017). A viral agent was identified in the respiratory secretions of 21/35 (60%) P-CAP vs. 29/40 (73%, p not significant) NP-CAP children. Univariate analyses indicated that neither age, gender, clinical severity of pneumonia, duration of cough or fever nor antibiotic

TABLE 2. Seroresponses to and diagnostic performance of a panel of surface pneumococcal proteins in 75 children with community-acquired pneumonia

	Seroresponders *N* (%)			Comparison with children with Ply^+ PCR findings in the blood			
	≥2-fold	≥4-fold	GMC (EU/mL, 95% CI)	Sensitivity	Specificity	NPV	PPV
Ply	16 (21)	8 (11)	154	0.53	0.87	0.88	0.50
PhtD	20 (27)	15 (20)	108	0.67	0.84	0.91	0.50
PhtE	24 (32)	14 (19)	239	0.80	0.79	0.95	0.46
PcpA	23 (31)	14 (15)	279	0.60	0.78	0.90	0.38
LytB	5 (7)	3 (4)	24	0.13	0.95	0.80	0.40
Any	33 (44)	23 (31)	NA	0.87	0.72	0.96	0.39

EU, ELISA Units; GMC, geometric mean concentration; NA not applicable; NPV, negative predictive value; PcpA, pneumococcal choline-binding protein A; PhtD, pneumococcal histidine triad D; PhtE, pneumococcal histidine triad E; Ply, pneumolysin; PPV, positive predictive value.

TABLE 3. Sensitivities of the combination of two anti-pneumococcal surface protein (PSP) seroresponses for the diagnosis of pneumococcal community-acquired pneumonia (P-CAP)

	Ply	PhtD	PhtE	PcpA	LytB
Ply	0.46	0.60	0.69	0.89	0.49
PhtD	0.60	0.57	0.69	0.91	0.63
PhtE	0.69	0.69	0.66	0.94	0.69
PcpA	0.89	0.91	0.94	0.66	0.72
LytB	0.49	0.63	0.67	0.69	0.14

PcpA, pneumococcal choline-binding protein A; PhtD, pneumococcal histidine triad D; PhtE, pneumococcal histidine triad E; Ply, pneumolysin.
Sensitivities are calculated from the number of P-CAP patients with ≥2-fold responses to one or two PSPs among all P-CAP patients.

use within 30 days of admission differed between children hospitalized for P-CAP and those hospitalized for NP-CAP. This confirms that clinical patterns alone may not reliably identify children with P-CAP.

Assessment of the capacity of anti-PSP responses to identify P-CAP children indicated that anti-Ply IgG responses would have missed 19/35 (47%) patients. Combining two PSPs significantly increased assay sensitivity (Table 3). The combination of anti-Ply, anti-PcpA and anti-PhtE responses yielded the optimal result of 0.97 for sensitivity and 0.98 for NPVs. Unexpectedly, the maximal sensitivity of any combination of PSPs without PcpA remained below 0.70, suggesting its importance in the serological diagnosis of P-CAP.

As pre-existing anti-pneumococcal antibodies may contribute to protection against pneumococcal disease, PSP-specific immunity at admission was assessed in P-CAP and NP-CAP children. Most patients had detectable acute antibodies to several PSPs (≥2 PSPs, 96%; ≥3 PSPs, 92%; ≥4 PSPs, 89%; ≥5 PSPs, 86%), indicating past exposure/carriage. Correlations between age and GMCs were strong for Ply (R^2 = 0.63434), PhtD (R^2 = 0.63297), and PhtE (R^2 = 0.59359), and weaker, although significant, for LytB (R^2 = 0.45824) and PcpA (R^2 = 0.31625). Exposure-driven anti-PSP antibodies in children indicated that anti-PcpA antibodies were present at high titres in some children younger than 18 months (Fig. 1), their increase with age being significant (p 0.022) but less marked than for other PSPs (p <0.001).

At admission, antibodies to Ply, PhtD, PhtE and LytB were similar in P-CAP and NP-CAP children (Table 4 and Fig. 2), suggesting comparable past exposure to *S. pneumoniae* and B-cell response capacity. In striking contrast, anti-PcpA GMCs were four-fold lower (71 vs. 286 EU/mL, p <0.001) in P-CAP children, reflecting a greater proportion of NP-CAP children with anti-PcpA titres at any value >10 EU/mL (Fig. 2). At convalescence, anti-PcpA titres were similar in both groups, whereas anti-Ply and anti-PhtD titres were

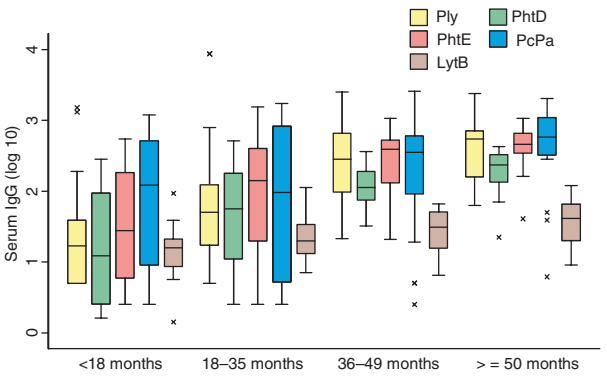

FIG. 1. Anti-pneumococcal surface protein serum IgG antibodies at admission in children with community-acquired pneumonia (CAP). PcpA, pneumococcal choline-binding protein A; PhtD, pneumococcal histidine triad D; PhtE, pneumococcal histidine triad E; Ply, pneumolysin.

TABLE 4. Comparison of anti-pneumococcal surface protein (PSP) seroresponses in children with non-pneumococcal community-acquired pneumonia (NP-CAP) vs. those with pneumococcal community-acquired pneumonia (P-CAP)

	Stage	NP-CAP (*n* = 40) (EU/mL, 95% CI)	P-CAP (*n* = 35) (EU/mL, 95% CI)	p-Value[a]
Ply GMC	Acute	104	119	0.777
Ply GMC	Convalescent	103	246	0.033
PhtD GMC	Acute	65	54	0.146
PhtD GMC	Convalescent	66	189	0.044
PhtE GMC	Acute	144	115	0.187
PhtE GMC	Convalescent	141	448	0.06
PcpA GMC	Acute	286	71	<0.001
PcpA GMC	Convalescent	283	279	0.178
LytB GMC	Acute	25	19	0.052
LytB GMC	Convalescent	23	25	0.419

GMC, geometric mean concentration; PcpA, pneumococcal choline-binding protein A; PhtD, pneumococcal histidine triad D; PhtE, pneumococcal histidine triad E; Ply, pneumolysin.
[a]P-CAP vs. NP-CAP.

significantly (Table 4) higher in P-CAP patients (Ply, 246 vs. 103 EU/mL; PhtD, 189 vs. 66 EU/L). Multivariate analyses adjusting for gender, age and clinical scores confirmed low anti-PcpA titres (p 0.002) at admission as the only significant predictors of P-CAP vs. NP-CAP in our patients.

Discussion

The identification of new PSPs has led to renewed interest in the serological diagnosis of respiratory infections [6]. The early recognition that *S. pneumoniae* is the causative agent of CAP could have an important impact on targeted antimicrobial therapy. Furthermore, use of a PSP combination may markedly improve the sensitivity of diagnosis of P-CAP in epidemiological studies.

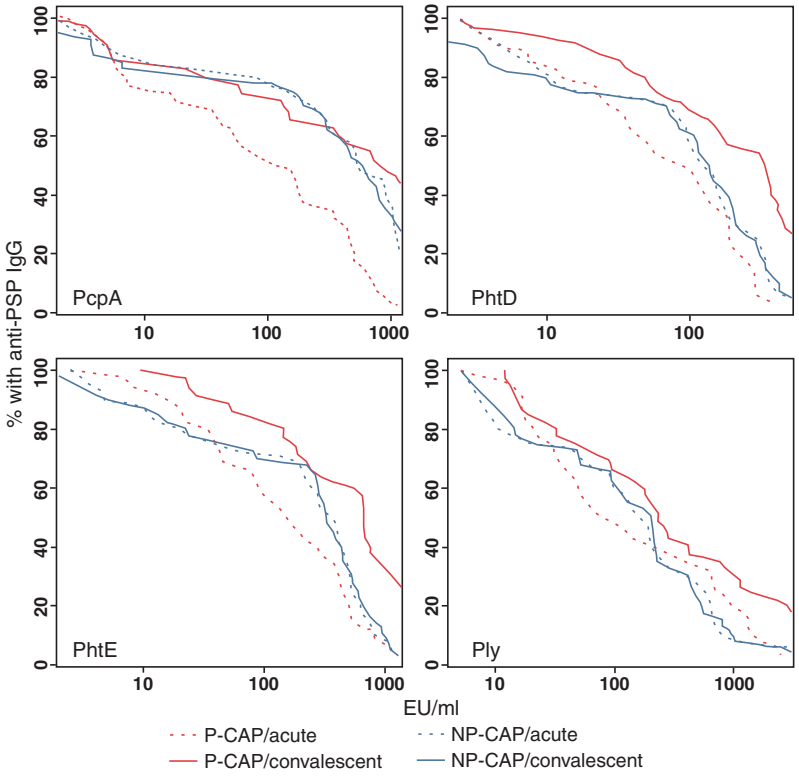

FIG. 2. Distribution of anti-pneumococcal surface protein (PSP) IgG antibodies in children with community-acquired pneumonia with recent pneumococcal exposure (P-CAP) or community-acquired pneumonia without evidence of recent pneumococcal exposure (NP-CAP). EU, ELISA Units; PcpA, pneumococcal choline-binding protein A; PhtD, pneumococcal histidine triad D; PhtE, pneumococcal histidine triad E; Ply, pneumolysin.

A sharp rise in serum antibodies within a few weeks is a validated marker of recent microbial exposure in healthy individuals. These antibody assays perform well in bacteraemic children [8]; however, they are less satisfactory for non-bacteraemic P-CAP, which is frequent in children. A pneumococcal surface adhesin A-based ELISA reached a specificity of 0.98 and a sensitivity of 0.70 in adult Kenyans with pneumonia [9], whereas its sensitivity declined to 0.42 in children [10]. Other studies have reported similarly low sensitivities [21]. These poor performances were ascribed to a higher prevalence of colonization in children. In general, nasopharyngeal *S. pneumoniae* carriage has little effect on antibody levels, and acute seroresponses are seen in <3% of healthy young children [20]. In contrast, recent carriage acquisition or otitis media may elicit antibody responses [8,22–24] and reduce the specificity of serological diagnosis of CAP in children. Notably, the specificity of PSP-based assays remains much better than their sensitivity in children with CAP [10]. This suggests that colonization essentially confounds the serological diagnosis of CAP through higher baseline antibody levels, limiting the ability to detect significant antibody rises, and thus assay sensitivity.

Here, we postulated that the sensitivity of PSP-based serological assays could be improved by using several immunogenic proteins and/or a combination of them. This was largely confirmed: past exposure did not elicit antibodies to all PSPs, enabling seroresponses to other antigens. Consequently, a combination of anti-PcpA, anti-PhtE and anti-Ply responses identified children with evidence of recent pneumococcal exposure, and increased assay sensitivity. In addition, PcpA was unexpectedly identified as a key diagnostic marker of P-CAP: in its absence, assay sensitivity reached a maximal value of 0.67, as compared with 0.92 for a PcpA-containing PSP combination. The combined use of five immunoprobes identified 34/75 (45%) CAP children with evidence of acute pneumococcal responses, a proportion in accordance with the presumed contribution of *S. pneumoniae* to the aetiology of CAP in hospitalized children [5].

Severity scores were similar in our P-CAP and NP-CAP children, despite more frequent lung consolidation in P-CAP children. One could hypothesize that some NP-CAP children had severe acute viral disease with diffuse lung disease that did not present evidence of pneumococcal exposure. Furthermore, we acknowledge the fact that our serology-based diagnosis is not 100% sensitive and/or specific, and that this could explain some misclassification. However, in the absence of a reference standard, we conservatively relied upon the most frequently used definition to show recent pneumococcal exposure and to identify patients with P-CAP. Concerning PCR, Nakayama *et al.* [25] found, in a similar clinical setting, that a comparable PCR method had high sensitivity and specificity (respectively, 95% and 98%). Our

definition may underestimate the proportion of children with P-CAP, particularly in young infants, who may fail to raise acute seroresponses. Our low rate of positive blood cultures for *S. pneumoniae* in children with CAP is nevertheless higher than that reported by others [4,21,26]. However, we cannot exclude the possibility that some anti-PSP responses resulted from recent carriage acquisition in children with NP-CAP. Indeed, *S. pneumoniae* was more frequently identified in the nasopharynx of P-CAP than of NP-CAP patients, in accordance with other results [27] and the physiopathology of P-CAP. However, anti-PSP responses were very strong and were obtained against several PSPs, and most included anti-PcpA antibodies that are not readily elicited through carriage alone. Altogether, this supports the use of several PSPs rather than a single PSP for the serodiagnosis of P-CAP.

Titres of antibodies to Ply, PhtD, PhtE and LytB at admission were similar in both groups, confirming that these patients were otherwise healthy children previously exposed to *S. pneumoniae*. In striking contrast, anti-PcpA antibody titres were three-fold lower in children admitted for P-CAP. PcpA was first characterized in 1998 as a protein with a putative adhesion role [28]. PcpA mutant strains efficiently colonize the murine nasopharynx but fail to adhere to the lungs [29]. PcpA expression is downregulated by a metal-dependent regulator and thus by high concentrations of Mn^{2+} [29]. This provides a biological explanation for the unique role of PcpA identified here in human pneumonia. Saliva has the highest *in vivo* concentration of Mn^{2+} (36 μM). Therefore, PcpA expression is repressed unless pneumococci invade the lung or bloodstream, where the levels of Mn^{2+} are 1000-fold lower (20 nM) [30,31]. Consequently, anti-PcpA responses result from pneumococcal disease rather than colonization. Low anti-PcpA antibody titres at admission suggest a primary episode of pneumococcal disease. Whether PcpA antibodies are merely markers of protective immunity induced by prior pneumococcal disease or play a protective role against recurrence is not yet known. In summary, PcpA appears to play a critical role in pneumococcal pneumonia, which should be validated in a larger study; such a role would warrant its assessment as a diagnostic tool in various settings, and as a potential vaccine antigen.

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Transparency Declaration

Klara M. Posfay-Barbe, Annick Galetto-Lacour, Stéphane Grillet, Manon Cevey-Macherel, Jean Daniel Kraehenbuhl and Mario Gehri have nothing to declare. Martina Ochs and Roger Brookes are employees of sanofi pasteur. Claire-Anne Siegrist and Alain Gervaix have received research grants and honoraria for participation to scientific advisory boards of various vaccine manufacturers including sanofi pasteur, GlaxoSmithkline and Wyeth.

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