Eur J Clin Microbiol Infect Dis (2013) 32:43–49
DOI 10.1007/s10096-012-1712-9

ARTICLE

Influence of age, social patterns and nasopharyngeal carriage on antibodies to three conserved pneumococcal surface proteins (PhtD, PcpA and PrtA) in healthy young children

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Received: 15 June 2012 / Accepted: 23 July 2012 / Published online: 18 August 2012

Abstract The acquisition of specific antibodies is paramount to protect children against pneumococcal diseases, and a better understanding of how age, ethnicity and/or *Streptococcus pneumoniae* (Spn) nasopharyngeal carriage influence the acquisition of antibodies to pneumococcal surface proteins (PSP) is important for the development of novel serodiagnostic and immunisation strategies. IgG antibody titres against three conserved PSP (PhtD, PcpA and PrtA) in the sera of 451 healthy children aged 1 to 24 months from Israel [Jewish (50.1 %) and Bedouin (49.9 %)] were measured by enzyme-linked immunosorbent assay (ELISA), while nasopharyngeal swabs from these children were assessed for the presence of Spn. Globally, anti-PhtD and anti-PrtA geometric mean concentrations (GMC; EU/ml) were high at <2.5 months of age [PhtD: 35.3, 95 % confidence interval (CI) 30.6–40.6; PrtA: 71.2, 95 % CI 60–84.5], was lower at 5–7 months of age (PhtD: 10, 95 % CI 8–12.4; PrtA: 17.9, 95 % CI 14.4–22.1) and only increased after 11 months of age. In contrast, an increase in anti-PcpA was observed at 5–7 months of age. Anti-PcpA and anti-PrtA, but not anti-PhtD, were significantly higher in Bedouin children (PcpA: 361.6 vs. 226.3, $p=0.02$; PrtA: 67.2 vs. 29.5, $p<0.001$) in whom Spn nasopharyngeal carriage was identified earlier (60 % vs. 38 % of carriers <6 months of age, $p=0.002$). Spn carriage was associated with significantly higher anti-PSP concentrations in carriers than in non-carriers ($p<0.001$ for each PSP). Thus, age, ethnicity and, essentially, nasopharyngeal carriage exert distinct cumulative influences on infant responses to PSP. These specific characteristics are worthwhile to include in the evaluation of pneumococcal seroresponses and the development of new PSP-based vaccines.

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Abbreviations

GMC	Geometric mean antibody concentrations
PcpA	Pneumococcal choline-binding protein A
PhtD	Pneumococcal histidine triad protein D
PrtA	Serine proteinase precursor A
PSP	Pneumococcal surface proteins
Spn	*Streptococcus pneumoniae*

Introduction

Streptococcus pneumoniae (Spn) is a major cause of respiratory and invasive diseases in childhood and contributes significantly to morbidity and mortality in children [1–3]. In industrialised countries, pneumococcal vaccination and antibiotic treatments have markedly reduced childhood mortality caused by invasive pneumococcal infections [1, 4, 5]. Pneumococcal polysaccharide-based glycoconjugate vaccines are immunogenic and effective, but the presence of more than 90 different Spn serotypes [1, 6–8] and serotype replacement [1, 2, 4, 6, 9] are limitations. New-generation vaccines are currently under investigation, including the use of non-polysaccharide antigens [7]. Probing the antibody repertoire of exposed or convalescent patients for pneumococcal surface proteins (PSP), Giefing et al. identified approximately 140 protein antigens, many of them surface-exposed [7]. The potential contribution of most of these antigens to the serodiagnosis of pneumococcal infections or protection against subsequent infection—including in vaccines—remains undefined. Nonetheless, little is yet known on the natural acquisition of antibodies to PSP [10–12].

Three PSP were selected primarily on their availability, their putative role(s) in bacterial pathogenesis and their conservation across pneumococcal strains (>97 %) [13]. Pneumococcal histidine triad protein D (PhtD) belongs to the family of surface-exposed pneumococcal proteins that have histidine triad motifs in their amino acid sequence [14]. PhtD has been suggested to be involved in the invasion process, and is highly conserved among various strains [14]. Anti-PhtD antibodies have been detected in the convalescent-phase sera of infants and children with pneumococcal bacteremia, confirming that this protein is exposed and recognised by the immune system during pneumococcal disease [14, 15]. Pneumococcal choline-binding protein A (PcpA) has been suggested to play a role in bacterial adherence to the respiratory epithelium in the lower respiratory tract [6]. PcpA is distinct from other pneumococcal choline-binding proteins (PspC and CbpA) [6, 14, 16]. Studies have shown that manganese concentration directly affects the presence of PcpA on the Spn surface, which may explain why its expression is possibly increased in bacteria infecting low-manganese sites, such as the lungs and blood [6]. Serine proteinase precursor A (PrtA) is a cell-wall-associated serine proteinase that contributes to pneumococcal virulence [17]. It is also regulated by manganese and zinc and, for this reason, we were interested in assessing whether it elicited a similar or distinct seroresponse pattern to PcpA.

The aim of this study was, thus, to evaluate the antibody levels produced following the natural acquisition of three immunogenic and highly conserved PSP (PhtD, PcpA and PrtA) in infants and young children, and to evaluate the influence of age, ethnicity, social status, and pneumococcal carriage on these anti-PSP responses.

Materials and methods

Patients

Healthy children less than 24 months of age were enrolled prospectively after written informed consent at Soroka University Medical Center or at maternal and children health centres in southern Israel between October 1996 and November 2006. All enrolled children came to the medical centre for reasons other than infectious diseases or fever. None of the children had been immunised against Spn and none had a clinically relevant history of pneumococcal infection. Each patient had venous blood sampling and nasopharyngeal (NP) swab culture. Carriage was defined as being culture-positive for Spn on an NP swab without clinical symptoms at the time of sampling. Serum samples were stored at −70 °C until analysis.

This study was approved by the Soroka Medical Center and the Israel Ministry of Health Ethics Committees.

Setting

In the Negev region of southern Israel, the Jewish and Bedouin populations live side by side. The Jewish population is mainly urban, whereas the Bedouin population is gradually moving away from its nomadic lifestyle [18]. Children of the two populations do not frequent the same day-care facilities or schools, and have distinct social lives. In 2004, the crude birth rate was 55.3 versus 21 births per 1,000 persons in the Bedouin and Jewish populations, respectively [19], and the mean ± standard deviation (SD) family size among the Bedouin population was 8.2±0.9 persons compared with 3.2±0.1 among the Jewish population [20]. The average monthly family income was 2-fold higher among the Jewish population [19]. Hospitalisation rates for respiratory and other infectious diseases were higher among Bedouins [21].

During the study period, the 7-valent pneumococcal conjugate vaccine (PCV7) had not yet been introduced in Israel,

and <3 % of the population had participated in clinical trials with one of the experimental pneumococcal conjugate vaccines.

Pneumococcal surface proteins

PhtD, a truncated version of PcpA and a truncated histidine-tagged version of PrtA were recombinantly expressed and purified from *Escherichia coli*.

Antibody titres were measured by indirect enzyme-linked immunosorbent assay (ELISA), as previously described [13]. Purified PSP were coated on Immulon (Thermo Labsystems) plates. To allow precise quantification, eight serial dilutions (2-fold) of serum samples were added, followed by conjugated anti-IgG antibodies (Cappel) and ABTS substrate. A common reference human antibody serum was used in each assay and its antibody concentration in ELISA units was defined by the reciprocal of its dilution at OD= 1.0. Assay results were expressed in EU/ml by interpolation to the reference serum, allowing precise quantification. A cut-off of 5 EU/ml was experimentally identified as allowing the reliable detection of serum concentrations. Anti-PSP concentrations below 5 EU/ml were given a titre of 2.5 EU/ml. The results are expressed as geometric mean antibody concentrations (GMC). The specificity of PhtD and PcpA antigen-specific ELISA was demonstrated by complete loss of binding of human antibody sera by competitive inhibition with specific antigens, and not with other PSP as the control antigen.

Statistical analyses were carried out using SPSS (PASW Statistics 18.0.0; IBM Corporation, Somers, NY). Standard descriptive statistics were used to describe socio-demographic characteristics. Categorical data were compared using Chi-square tests or Fisher's exact tests, when appropriate. Serologies among groups were compared by using Student's *t*-test, linear regression or the Mann–Whitney *U*-test, according to the type of independent variable. Univariate statistical analyses were performed for each variable to determine its relationship to the dependent variable, being a case patient or not. A multivariate linear regression analysis model was used to assess the goodness of fit, calculate adjusted odds ratio (OR) and 95 % confidence interval (CI) for variables that had p-values<0.25 in the univariate analyses. For all statistical tests, differences were considered to be significant at $p<0.05$ or when the 95 % CI did not include 1.0.

Results

Four hundred and fifty-one serum samples from children aged 1 to 24 months were included in this study. The children were divided into five age groups, each including at least 75 children (Table 1), with a similar distribution among the two ethnic groups. Boys, which were slightly more numerous overall (53.7 %), were similarly represented in the two ethnic groups.

Pneumococcal carriage

Spn carriage was identified in 58.8 % of children overall, equally distributed among genders (Table 1). There was a wide distribution among Spn serotypes (>40 identified), without a significantly predominant serotype (not shown). No association between PSP antibody levels and pneumococcal serotype was observed. The proportion of Spn carriers increased with age, and carriage was present earlier ($p=0.005$) and more frequently ($p<0.001$) in Bedouin children (Table 1).

Anti-PSP antibodies

Antibodies to all three PSP were detectable in each age group. Anti-PhtD and anti-PrtA GMC were 2–3-fold higher in 1–2.5-month-old infants than in 2.5–4- or 5–7-month-old infants, in whom the nadir was observed (Table 2), and they were only significantly higher in the second year of life. In contrast, anti-PcpA GMC remained similar throughout infancy, reflecting their earlier increase (5–7 months) to markedly higher levels (Table 2).

Comparing anti-PSP concentrations in Bedouin and Jewish children indicated similar anti-PhtD antibodies in both ethnic groups (Table 2). In contrast, anti-PcpA ($p<0.05$) and anti-PrtA ($p<0.001$) antibodies were significantly higher in Bedouin infants. This difference was similar for anti-PrtA in the second year of life, as these antibodies were higher later in Jewish children. Spn acquisition is known to trigger antibody responses; we assessed the influence of Spn carriage at the time of sampling on anti-PSP concentrations. Overall, anti-PSP antibodies were significantly higher in Spn-positive children ($p<0.001$ for each PSP; Table 2 and Fig. 1). This influence was statistically significant ($p=0.002$) in 11–15-month-old children for anti-PhtD antibodies. For anti-PcpA antibodies, significant differences were already present in 2.5–4-month- ($p=0.043$) and 5–7-month-old ($p=0.002$) carrier infants, in whom anti-PrtA antibodies were also higher (PrtA, $p=0.025$) (Table 2 and Fig. 1). After the age of 17 months, the influence of Spn carriage at sampling was not statistically significant.

Multivariate analyses including age, ethnicity and Spn carriage indicated significantly higher anti-PSP GMC in carrier than non-carrier Jewish children (PhtD: 42.6 vs. 17.9; PcpA: 542.8 vs. 105.9; PrtA: 43.1 vs. 21.2; $p\leq0.001$ for all). Interestingly, however, similarly high GMC were observed in carrier and non-carrier Bedouin children (PhtD: 30.7 vs. 29.8; PcpA: 398.8 vs. 284; PrtA: 67.2 vs. 66.9).

Table 1 Characteristics of the 451 healthy children enrolled in the study

	Age group	Total, *n* (median, IQR)	Bedouin children, *n* (%)	Jewish children, *n* (%)	*p*-value
Age group distribution in months	All	451 (6, 10)	225 (49.9)	226 (50.1)	NS
	<2.5[b]	91 (2, 1)	48 (21.3)	43 (19)	NS
	2.5–4[b]	75 (4, 1)	38 (16.9)	37 (16.4)	NS
	5–7[b]	97 (6, 1)	49 (21.8)	48 (21.2)	NS
	11–15[b]	90 (11, 1)	40 (17.8)	50 (22.2)	NS
	17–24[b]	98 (18, 0.3)	50 (22.2)	48 (21.2)	NS
Male gender, *n* (%)		242 (53.7)	128 (56.9)	114 (50.4)	NS
Streptococcus pneumoniae	All	265 (58.8)	160 (60.4)	105 (39.6)	<0.001
Carrier by age group[a], *n* (%)	<2.5[c]	35 (38.5)	25 (52.1)	10 (23.3)	0.005
	2.5–4[c]	42 (56)	26 (68.4)	16 (43.2)	0.028
	5–7[c]	61 (62.9)	37 (75.5)	24 (50)	0.009
	11–15[c]	56 (62.2)	33 (82.5)	23 (46)	<0.001
	17–24[c]	71 (72.4)	39 (78)	32 (66.7)	NS

IQR interquartile range; *NS* not significant

[a] Carriage at time of sampling

[b] % within ethnicity

[c] % within age group

Table 2 Anti-PSP GMC of 451 healthy children by age group, ethnicity and carrier state

Age group (in months)	*n*	Overall (95 % CI)	Bedouin children	Jewish children	Ethnicity *p*-value	Carrier	Non-carrier	Carrier *p*-value
		PhtD GMC (EU/ml)						
All	451	28.6 (25.3–32.2)	30.5	26.8	NS	35	21.4	<0.001
<2.5	91	35.3 (30.6–40.6)	36.6	33.8	NS	32.4	37.2	NS
2.5–4	75	17.3 (14.4–20.7)	16.1	18.7	NS	19.6	14.8	NS
5–7	97	10 (8–12.4)	10.1	9.8	NS	11.1	8.3	NS
11–15	90	34.7 (26.1–46.1)	46.7	27.3	NS	50.7	18.6	0.002
17–24	98	81.6 (63.2–105.5)	86.9	76.5	NS	102.7	44.7	NS
		PcpA GMC (EU/ml)						
All	451	285.9 (247.5–330.2)	361.6	226.3	0.02	450.6	149.5	<0.001
<2.5	91	166.7 (143.8–193.1)	194.3	140.4	0.036	179.5	159.1	NS
2.5–4	75	146.3 (115.5–185.1)	183.4	116	0.044	177.4	114.4	0.043
5–7	97	202.8 (144.3–285.1)	255	160.6	NS	345.8	82.1	0.002
11–15	90	386.7 (266.4–561.2)	591	275.4	NS	689.7	149.1	0.013
17–24	98	838.7 (627.7–1,120.8)	1,045.3	666.8	NS	1,104.9	406.3	NS
		PrtA GMC (EU/ml)						
All	451	44.5 (39.2–50.4)	67.2	29.5	<0.001	56.4	31.7	<0.001
<2.5	91	71.2 (60–84.5)	94.7	51.8	0.001	71	71.4	NS
2.5–4	75	33 (27.2–40.1)	43.2	25.1	0.006	35.4	30.3	NS
5–7	97	17.9 (14.4–22.1)	26.1	12.1	<0.001	21.8	12.7	0.025
11–15	90	38.6 (28–53.2)	80	21.5	<0.001	68.5	15.0	<0.001
17–24	98	101.2 (74.7–137.1)	148.4	67.9	0.025	128.4	54.1	NS

PSP pneumococcal surface proteins; *GMC* geometric mean antibody concentrations; *PcpA* pneumococcal choline-binding protein A; *PhtD* pneumococcal histidine triad protein D, *PrtA* serine proteinase precursor A; *NS* not significant

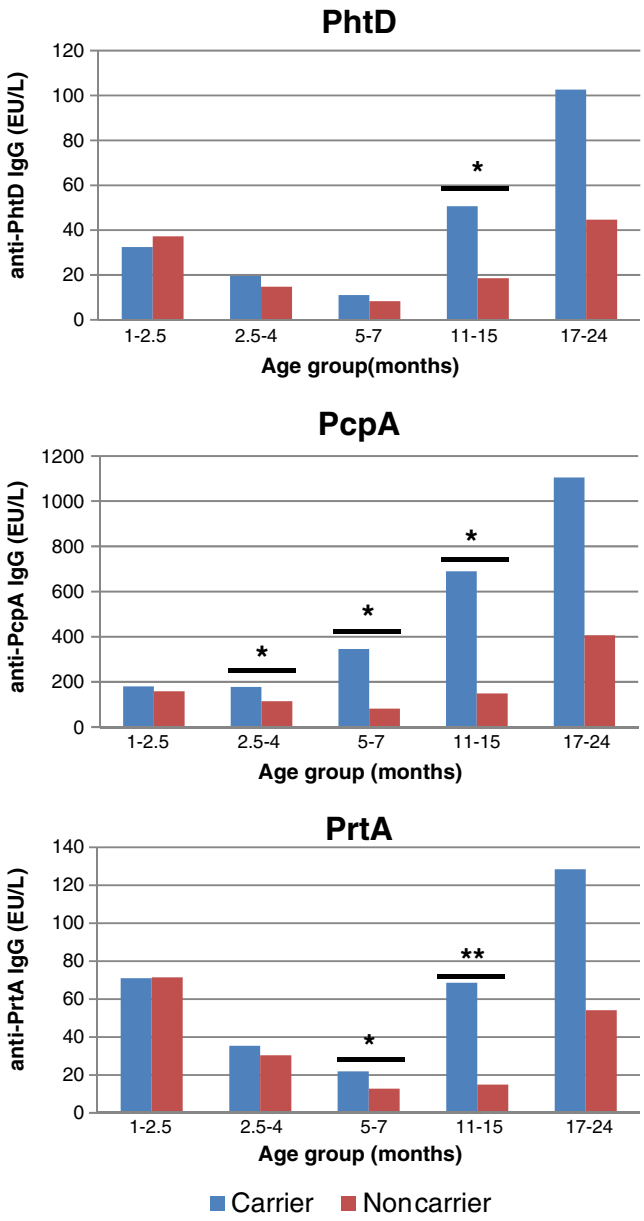

Fig. 1 Anti-PSP GMC in 451 healthy children by *Streptococcus pneumoniae* carrier state and age group. *p-value <0.05 (adjusted for ethnicity). **p-value <0.001 (adjusted for ethnicity). *PSP* pneumococcal surface proteins; *GMC* geometric mean antibody concentrations, *PcpA* pneumococcal choline-binding protein A, *PhtD* pneumococcal histidine triad protein D, *PrtA* serine proteinase precursor A

Discussion

This study shows that natural exposure to Spn generates antibody responses to three highly conserved PSP in infants, and the pattern and magnitude of these antibody responses differ for each of the three PSP assessed.

In very young infants, anti-PSP IgG antibodies represent passively transmitted maternal antibodies [14]. These maternal antibodies decrease over time, and active antibody production increases with immune maturation in response to antigenic exposure. This results in a characteristic U-type pattern, which was previously identified for several PSP antibodies in healthy or sick children [12, 14, 22–26]. In our study, anti-PhtD and anti-PrtA antibodies, indeed, follow this U-curve. The pattern of anti-PcpA antibodies was different: relatively low concentrations were present in the youngest age group as compared to toddlers, which could result from low anti-PcpA IgG concentrations in mothers. The high manganese (Mn^{++}) environment in the NP does not trigger PcpA expression by Spn [6, 27], such that maternal NP carriage may not efficiently elicit PcpA antibodies. This may explain why anti-PcpA maternal antibodies were not transferred passively to young infants and were, therefore, not detected in these young infants. However, conversely, PrtA, which was primarily selected because its expression is also down-regulated by Mn^{++}, generated sufficient anti-PrtA maternal antibodies to result in a characteristic U-curve in infants. This suggests the existence of additional levels of complexity, such as distinct placental transfer, which are possibly distinct for specific antibodies.

Another difference between anti-PcpA and anti-PhtD/anti-PrtA antibodies was that anti-PcpA antibodies peaked faster and reached much higher antibody concentrations. Anti-PcpA and anti-PrtA GMC were higher in Bedouin than in Jewish children in all age groups, probably as a result of their earlier Spn exposure. The number of children in a family, lower economic income and different access to a "hygienic environment" in each ethnic group could also have an influence on exposure to Spn and, therefore, on antibody responses.

Spn carrier state has been reported as a determinant factor for the antibody levels to several PSP [12, 14, 22–24, 26]. Obaro et al. showed lower anti-PSP titres in colonised compared to not colonised infants, raising the question that some anti-PSP antibodies may protect against Spn carriage [28]. In a recent study with other PSP, only certain PSP were immunogenic in colonised subjects [29]. In our study, carriage at sampling was usually associated with higher antibody titres. This data suggest that antibodies do not reduce nasopharyngeal carriage, because the children with the highest antibody levels had the highest carriage state. However, significant GMC differences between carrier and non-carrier children were only found in one ethnic group (Jewish children) or in certain age groups. One could hypothesise that population-associated genetic polymorphisms may have an impact on the capacity to respond to Spn exposure or that maternal antibodies exert an inhibitory effect on infant responses. A simpler explanation would be that the observation of higher anti-PSP titres in Jewish—but not in Bedouin—carriers than non-carriers resulted from differences in cumulative past Spn exposure: with age, a progressive decline of the influence of recent carriage on antibody

responses is, indeed, expected. However, the frequency of past exposure to Spn is virtually impossible to assess in children and one can only hypothesise about different exposures in different ethnic groups due to several factors, which include the number of persons in a household.

Our study has several limitations. Pairs of maternal–infant sera were not available and children were sampled only once. The study design was cross-sectional; a longitudinal cohort study would have provided information on the individual impact of age and/or carriage. However, this limitation is compensated, at least in part, by the inclusion of a larger number of children in each age group than the number of subjects which could be followed longitudinally without major resources. Another limitation is that Spn carriage was only assessed at the time of blood sampling. Reassuringly, however, carriage rates were similar to those reported in other studies [30, 31]. In this population with highly prevalent Spn, it is highly likely that most, if not all, children carried Spn at least once before being tested for anti-PSP, even in the absence of a positive culture at the time of sampling. Our study design did not allow us to distinguish the impact of individual social or economic status on antibody response. We, therefore, used only ethnicity to differentiate the two groups, which, according to the Central Bureau of Statistics of Israel, indeed, have very different lifestyles and status. It is, therefore, possible that some "atypical" families did not belong to the "typical" environment of their ethnic community.

PhtD, PcpA and PrtA elicit significant anti-PSP responses in (very) young children, with significantly distinct patterns. Young age delays responses to PhtD and PrtA for many months, possibly because of inhibition due to higher maternal antibodies; in contrast, anti-PcpA responses are elicited earlier in life. PcpA responses are significantly higher in pneumococcal carriers than non-carriers. Differences in social patterns result in distinct patterns of anti-PSP responses. Defining the influence of age and prior pneumococcal exposure on specific anti-PSP responses in healthy children of a given community is, thus, necessary for the development of new pneumococcal vaccines based on PSP.

Acknowledgments This study was funded, in part, by a research grant from sanofi pasteur.

We would like to thank all the children and their parents for participating in this study.

This work was presented, in part, at the 7th International Symposium of Pneumococci and Pneumococcal Diseases, Tel-Aviv, Israel, 14–18 March, 2010.

Conflict of interest Andrés Hagerman, Stéphane Grillet, David Greenberg, Noga Givon-Lavi and Klara Posfay-Barbe have nothing to declare.

Martina Ochs and Roger Brookes are employees of sanofi pasteur and have no other disclosure or conflict of interest to report.

Ron Dagan has received, in the last 5 years, grants/research support from Berna/Crucell, Wyeth/Pfizer, MSD and Protea.

Ron Dagan has been a scientific consultant for Berna/Crucell, GlaxoSmithKline, Novartis, Wyeth/Pfizer, Protea and MSD.

Ron Dagan was a speaker for Berna/Crucell, GlaxoSmithKline and Wyeth/Pfizer.

Ron Dagan is a shareholder of Protea.

Claire-Anne Siegrist has received, in the last 5 years, grants/research support from sanofi pasteur, GlaxoSmithKline, NasVax Ltd. and DBV Technologies.

Claire-Anne Siegrist received from sanofi pasteur, GlaxoSmithKline and Wyeth a reimbursement of travel costs to scientific meetings.

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