Research paper

Serological diagnosis of pneumococcal infection in children with pneumonia using protein antigens: A study of cut-offs with positive and negative controls

Dafne Carvalho Andrade [a],[*],[1], Igor Carmo Borges [a],[1], Lauri Ivaska [b], Ville Peltola [b], Andreas Meinke [c], Aldina Barral [a],[d], Helena Käyhty [e], Olli Ruuskanen [b], Cristiana Maria Nascimento-Carvalho [a],[f]

[a] Postgraduate Programme in Health Sciences, Federal University of Bahia School of Medicine, Salvador, Bahia, Brazil
[b] Department of Pediatrics and Adolescent Medicine, Turku University Hospital and University of Turku, Turku, Finland
[c] Valneva Austria GmbH, Campus Vienna Biocenter 3, Vienna, Austria
[d] Department of Pathology, Federal University of Bahia School of Medicine and Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Bahia, Brazil
[e] National Institute for Health and Welfare, Helsinki, Finland
[f] Department of Pediatrics, Federal University of Bahia School of Medicine, Salvador, Bahia, Brazil

ABSTRACT

The etiological diagnosis of infection by *Streptococcus pneumoniae* in children is difficult, and the use of indirect techniques is frequently warranted. We aimed to study the use of pneumococcal proteins for the serological diagnosis of pneumococcal infection in children with pneumonia. We analyzed paired serum samples from 13 Brazilian children with invasive pneumococcal pneumonia (positive control group) and 23 Finnish children with viral pharyngitis (negative control group), all aged <5 years-old. Children with pharyngitis were evaluated for oropharyngeal colonization, and none of them carried *S. pneumoniae*. We used a multiplex bead-based assay with eight proteins: Ply, CbpA, PspA1 and 2, PcpA, PhtD, StkP and PcsB. The optimal cut-off for increase in antibody level for the diagnosis of pneumococcal infection was determined for each antigen by ROC curve analysis. The positive control group had a significantly higher rate of ≥2-fold rise in antibody levels against all pneumococcal proteins, except Ply, compared to the negative controls. The cut-off of ≥2-fold increase in antibody levels was accurate for pneumococcal infection diagnosis for all investigated antigens. However, there was a substantial increase in the accuracy of the test with a cut-off of ≥1.52-fold rise in antibody levels for PcpA. When using the investigated protein antigens for the diagnosis of pneumococcal infection, the detection of response against at least one antigen was highly sensitive (92.31%) and specific (91.30%). The use of serology with pneumococcal proteins is a promising method for the diagnosis of pneumococcal infection in children with pneumonia. The use of a ≥2-fold increase cut-off is adequate for most pneumococcal proteins.

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1. Introduction

Streptococcus pneumoniae infection is an important cause of morbidity and mortality in children, causing both invasive and non-invasive diseases (O'Brien et al., 2009). The diagnosis of infection by *S. pneumoniae* is difficult, particularly in children with community acquired pneumonia (CAP), as the gold standard for the etiological diagnosis is isolation of the bacterium from sterile tissues. This diagnostic strategy is highly specific, but poorly sensitive, because the minority of CAP patients has positive blood cultures for pneumococcus (Shah et al., 2010) and this bacterium cannot always be isolated in pleural effusion analysis (Nascimento-Carvalho et al., 2013).

Furthermore, although molecular diagnostic tests are a promising method (De Schutter et al., 2014), they are not commonly available. In this scenario, indirect techniques have been developed to identify the cases of infection caused by *S. pneumoniae.*

Serological techniques are a suitable alternative for the diagnosis of invasive pneumococcal infection, especially in research or epidemiological surveillance. The rational for these tests relies on the microbe-specific immune responses elicited by the individual upon contact with *S. pneumoniae.* However, the use of serology in children has been criticized, mainly due to insufficient validation with bacteremic samples (Korppi et al., 2008). Furthermore, as many serological tests detect antibodies to serotype-specific polysaccharides, the sensitivity of such assays has been questioned. The inclusion of pneumococcal proteins in serological assays represents an option to improve the performance of the diagnostic tests, as proteins are more immunogenic than polysaccharides (Korppi et al., 2008). Also, many pneumococcal proteins are highly specific for *S. pneumoniae*, and have a ubiquitous distribution among the various pneumococcal strains (Tai, 2006).

However, many pneumococcal proteins have only been recently described and identified as antigens, and they have not been validated for use in serology. Thus far, most studies have used the cut-off of ≥2-fold increase in antibody levels to recognize an antibody response. This cut-off was determined for pneumolysin (Ply), a pneumococcal cytotoxin, using data from a group of healthy children (Nohynek et al., 1995). However, there was no validation performed with true-positive controls. Scott et al. (2005) evaluated the use of pneumococcal surface adhesin A (PsaA) for serology in children with invasive pneumococcal disease (IPD) and healthy Kenyan children, and found that the cut-off of ≥2.7-fold increase in antibody levels against this antigen was adequate for pneumococcal infection diagnosis (Scott et al., 2005). Therefore, distinct protein antigens may have different thresholds for the detection of an antibody response when evaluated by distinct serological methods. We aimed to study the use of pneumococcal proteins for the serological diagnosis of pneumococcal infection in children with pneumonia using a multiplexed bead based assay.

2. Methods

2.1. Study participants

2.1.1. Positive controls

The positive control group comprised 13 Brazilian children 2–59 months old with invasive pneumococcal pneumonia who were enrolled in a prospective study for the etiological diagnosis of community-acquired pneumonia carried out at the Professor Hosannah de Oliveira Pediatric Centre, Federal University of Bahia, Salvador, Northeast Brazil, from September 2003 to May 2005 (Nascimento-Carvalho et al., 2008). All children were hospitalized and had serum samples collected at admission and 2–4 weeks later. None of the children were vaccinated against *S. pneumoniae*.

Invasive pneumococcal pneumonia was diagnosed by the isolation of *S. pneumoniae* on blood culture (9 patients) or by the detection of pneumococcal DNA in the buffy-coat (4 patients) using PCR with the *ply* primers. Data about this study group have been previously published elsewhere (Nascimento-Carvalho et al., 2008; Borges et al., 2015).

Blood culture testing was carried out at the Federal University of Bahia. From each of the collected blood specimens, 0.5–4.0 mL were immediately inoculated in 20 mL of supplemented BHI and incubated in OrganonBact/Alert equipment at 35 °C, for seven days. After a positive result was informed by the equipment, the inoculated medium was subjected to sub-culture on Columbia agar with 5% lamb blood and on agar-chocolate, and was again incubated for 18–24 h at 35 °C with 5% CO_2. *S. pneumoniae* was distinguished from other alpha-hemolytic streptococci by means of tests for solubility in bile and optochin. The identification of pneumococcal isolates was confirmed at Adolfo Lutz Institute (National Reference Laboratory, Brazilian Ministry of Health).PCR assays were carried out at the National Public Health Institute, Oulu, Finland. Extraction of DNA was performed using QIamp DNA Blood Mini-Kit (Qiagen, Hilden, Germany) (Nascimento-Carvalho et al., 2008).

2.1.2. Negative controls

The negative control group included 23 Finnish children 12–59 months old with pharyngitis evaluated at the Turku University Hospital, Turku, Finland, from January 2014 to February 2015. Children included in this study were previously healthy. At admission, all children had oropharyngeal samples collected for culture, and none presented current colonization by *S. pneumoniae*. Eighty-seven percent of children with pharyngitis had viral infection. Acute and convalescent blood samples were collected at admission and 2–5 weeks later. Data on pneumococcal vaccination were not collected; however, vaccination coverage with 10-valent pneumococcal conjugate vaccine has been estimated to be 95% after it was implemented in Finland in 2010 (http://www.thl.fi/roko/rokotusrekisteri/kattavuusraportit2014, n.d.).

2.2. Reagents

The presence of antibodies against *S. pneumoniae* was investigated using eight distinct pneumococcal proteins: pneumolysin [Ply], choline binding protein A [CbpA], pneumococcal surface protein A families 1 and 2 [PspA 1 and PspA2], pneumococcal choline binding protein A [PcpA], pneumococcal histidine triad protein D [PhtD], serine/threonine protein kinase [StkP-C, SP1732-3, a C-terminal fragment of StkP], and protein required for cell wall separation of group B streptococcus [PcsB-N, SP2216-1, a N-terminal fragment of PcsB]). Hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide-HCl (EDC) were provided by Thermo Fisher Scientific, Rockford, IL, USA. R-phycoerythryn (R-PE)-conjugated AffiniPure goat anti-human IgG, Fc$_\gamma$ Fragment specific was obtained from Jackson ImmunoResearch Laboratories Inc. (Westgrove, PA, USA).

2.3. Serology

2.3.1. Conjugation of pneumococcal proteins to the beads

The levels of antibodies against pneumococcal proteins were determined as described (Andrade et al., 2014) with a few modifications using a multiplexed bead based serological assay with Luminex xMAP® technology. Pneumococcal proteins were conjugated to Microplex beads using a 2-step carbodiimide reaction (Andrade et al., 2014), creating 7 distinct bead sets (one for each protein, except for PspA 1 and 2, which were conjugated together on the same bead). Beads were initially activated using a solution containing 5 mg/mL of Sulpho-NHS and EDC. After a 20-minute incubation, the beads were washed twice with phosphate buffered saline (PBS) and submitted to incubation with protein solution for 1.5 h in the dark. Subsequently, beads were washed twice with PBS and stored in a solution of PBS containing 10% fetal bovine serum (F-PBS) and 0.01% sodium azide.

2.3.2. Luminex assay

Sera were diluted 1:400 in F-PBS, and 25 μL of the diluted serum sample and 25 μL of bead solution were transferred to each well of a 96 well plate (Millipore MSHVN4510, Merck KGaA, Darmstadt, Germany). The mixture was then incubated for 1 h in the dark at 600 rpm. The plate was then washed with F-PBS using a vacuum washer, and 50 μL of a 1:100 solution of diluted RPE was added to each well. The plate was incubated for 30 min in the dark at 600 rpm and washed with F-PBS using a vacuum washer. Finally, 80 μL of F-PBS was added to each well (Andrade et al., 2014).

The pneumococcal reference serum 007 (Goldblatt et al., 2011) was included on each plate as a standard and was assigned an arbitrary antibody concentration of 1000 U/mL for each antigen. Patient sera antibody concentration was determined in relation to the amount of antibodies assigned in the 007 serum. Control sera with high and low antibody concentrations were analyzed on each plate to ensure good batch to batch consistency, and presented a coefficient of variation <20% for all proteins. Samples were assayed in duplicate and the results averaged. Acute and convalescent samples were always tested on the same plate. Samples were analyzed using a Luminex 200 (xMAP® Technology, Luminex Corporation, Austin, TX, USA) device. All tests were performed by DCA and ICB from October 2014 to February 2015. The samples used in this serological test were immediately processed after collection and were stored at −20 °C until the moment of analysis.

2.4. Ethical committee approval

The use of the samples from Brazilian children was approved by the Ethics Committee of the Federal University of Bahia, and the use of the samples from Finnish children was approved by the Ethics Committee of the Hospital District of Southwest Finland. All legal guardians provided written informed consent before enrolment in the investigation.

2.5. Statistical analysis

Categorical variables were presented as absolute number (percentage) and continuous variables as median (25th–75th percentiles) as they showed non-parametric distribution. Categorical variables were compared using chi-square or Fisher's exact test as appropriate and continuous variables were evaluated using Mann–Whitney U test. In order to determine the optimal parameters of antibody responses between the acute and convalescent serum samples for the diagnosis of pneumococcal infection, the sensitivity and specificity of different cut-offs were evaluated. Receiver operating characteristic (ROC) curves were plotted to determine cut-offs of fold-increase in antibody levels, using the ratio between acute and convalescent samples as the continuous variable and the origin of the sample (from either positive or negative controls) as the dichotomous variable. We determined the optimal cut-off points based on the accuracy of each cut-off. Sensitivity and specificity of the usual cut-off of ≥2-fold increase in antibody levels were also evaluated for each of the studied antigens for comparison with the cut-offs determined herein. The optimal number of antibody responses against different proteins needed for the diagnosis of invasive pneumococcal infection was also determined by ROC curve analysis. Logistic regression was performed using the presence of antibody response against each antigen considering the cut-off defined herein as the dependent variable, and the study group and the levels of the respective antibodies in the acute serum sample as independent variables. The software Stata (version 13.0) was used for the analysis.

3. Results

This study included 36 children whose median age was 28 months ([25th–75th percentile]: 15.25–42.5 months) and 54.1% were males. The median interval of serum sample collection was 19 days ([25th–75th percentile]: 16–24 days). The positive control group was younger than the negative controls (median [25th–75th percentile]: 14 [9.5–24.5] vs.37 [25–48] months; $P < 0.001$), and there was no statistical difference on serum sample collection interval between the two groups (median [25th–75th percentile]: 20 [16.5–25.5] vs.18 (Turner and Turner, 2013; Olaya-Abril et al., 2015; van der Poll and Opal, 2009; Giefing et al., 2008; Jiménez-Munguía et al., 2015; Holmlund et al., 2009; Lebon et al., 2011; Simell et al., 2009; Hagerman et al., 2013) days; $P = 0.6$) and on the gender (76.9% vs. 43.5% of males, $P = 0.052$). The baseline antibody levels were higher in the negative control group for Ply, CbpA, PcpA, PhtD and StkP-C(Table 1). Twelve out of the thirteen children in the positive control group had a ≥2-fold increase in antibody levels to at least one pneumococcal protein, while only two out of 23 children had ≥2-fold responses in the negative control group. The patient from the positive control group who failed to respond against S. pneumoniae had invasive disease diagnosed by PCR and was 28 months old. The two patients from the negative control group who responded against S. pneumoniae had other agents detected: one patient had Group A Streptococcus cultured from her throat; the second patient had infection by Epstein–Barr Virus (EBV) based on the detection of specific IgM. The significant higher rates of ≥2-fold antibody responses in the positive controls when compared to negative controls were seen against all the studied pneumococcal antigens, except Ply (Table 1).

The ROC curves for each of the studied pneumococcal antigens are shown in Fig. 1. Table 2 shows the area under the curve and optimal cut-offs for each antigen for the diagnosis of pneumococcal infection, as well as the sensitivity, specificity and accuracy of the determined cut-off and of the ≥2-fold increase cut-off. Overall, the use of pneumococcal proteins exhibited good accuracy. The use of the optimal cut-off points demonstrated a substantial increase in the accuracy for the detection of antibody responses against PcpA and a modest increase in the accuracy for the detection of responses against Ply and PspA as compared to the use of a cut-off of ≥2-fold increase in antibody levels.

We also evaluated the sensitivity and specificity of antibody responses (using the optimal cut-offs from Table 2) against different numbers of pneumococcal proteins for the diagnosis of S. pneumoniae infection (Table 3). The detection of antibody response against only one antigen yields good sensitivity and specificity for the diagnosis of infection by S. pneumoniae, when using a serological assay with all the antigens studied herein.

Finally, the effect of different levels of antibodies in the acute serum samples and of the study group on the detection of antibody responses (using the cut-offs defined in this study) was investigated using logistic regression (Table 4). There was no interference of the baseline levels of antibodies on the detection of responses for PspA, PcpA, PhtD and PcsB-N. There was no difference in the sensitivity and specificity of the assay including only the four aforementioned proteins and those of the assay with all the studied proteins (data not shown).

4. Discussion

Our observations suggest that the quantitation of specific IgG against pneumococcal proteins in paired serum samples is accurate in detecting invasive pneumococcal disease. Overall, ≥2-fold increase in antibody level is useful. However, the use of different cut-off points of antibody level increases might improve the accuracy of the test for some antigens, especially for PcpA.

The role of serology for the diagnosis of pneumococcal infection has been largely debated over the past decades (Korppi et al., 2008). S. pneumoniae frequently colonizes the nasopharyngeal tract in pediatric patients (Bogaert et al., 2004), and this form of contact with pneumococcus may promote an increase in the level of antigen-specific antibodies (Prevaes et al., 2012). It is possible that, if the negative controls had a new carriage of pneumococcus, a higher number of antibody responses would be detected. However, a previous study demonstrated

Table 1
Comparison of baseline antibody levels and of the frequency of ≥2-fold increases in antibody levels between the positive and negative control groups.

| Protein antigen	Antibody levels in the first serum sample (U/mL)[a]		P[d]	Frequency of ≥2-fold increase in antibody levels (n [%])		P[e]
	Positive control group (n = 13)	Negative control group (n = 23)		Positive control group (n = 13)	Negative control group (n = 23)	
Ply	189 (53–501)	773 (251–1622)	0.005	3 (23.1%)	1 (4.3%)	0.124
CbpA	140 (10.5–880)	1505 (429–8053)	<0.001	7 (53.8%)	2 (8.7%)	0.005
PspA[b]	76 (17.5–422.5)	244 (63–859)	0.115	4 (30.8%)	0 (0%)	0.012
PcpA	1221 (76.5–3232)	2417 (1025–13,645)	0.047	7 (53.8%)	2 (8.7%)	0.005
PhtD	88 (11.5–615)	1097 (404–2973)	0.001	7 (53.8%)	2 (8.7%)	0.005
StkP-C	35 (20.5–80)	422 (132–742)	<0.001	3 (23.1%)	0 (0%)	0.040
PcsB-N	433 (69–2544.5)	1357 (295–6105)	0.081	10 (76.9%)	0 (0%)	<0.001
S. pneumoniae[c]	–	–	–	12 (92.3%)	2 (8.7%)	<0.001

[a] Median [25th–75th percentile].
[b] PspA 1 and 2 were conjugated on the same bead set.
[c] Frequency of antibody response detection to at least one pneumococcal protein.
[d] Statistical value calculated with Mann Whitney U test.
[e] Statistical value calculated with Fisher exact test.
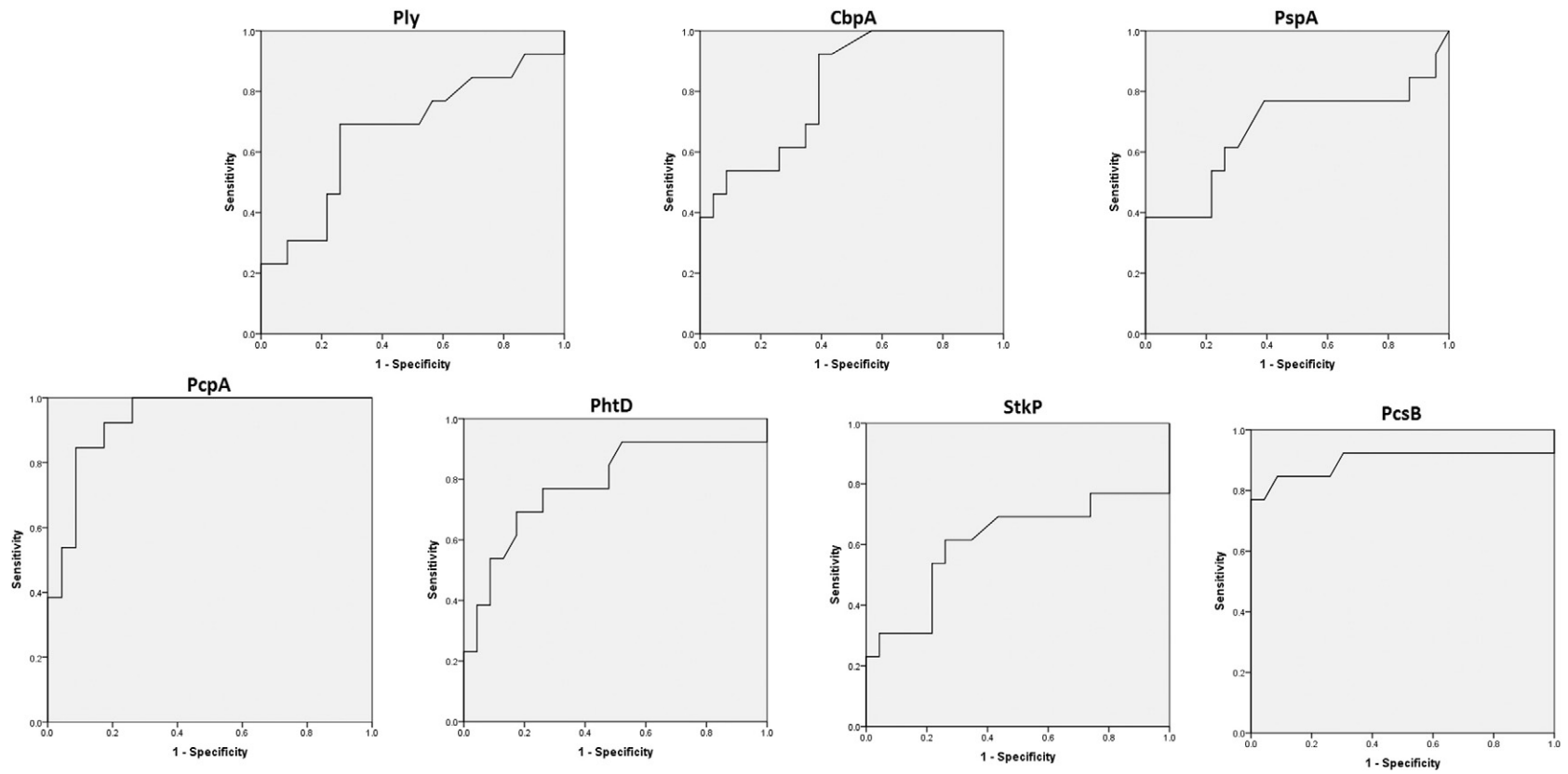
Fig. 1. Receiver operating characteristic (ROC) curve using antibody level increases for each pneumococcal antigen for the detection of pneumococcal infection in children with pneumonia. Children with invasive pneumococcal pneumonia and children with pharyngitis were used as positive and negative control groups, respectively.

Table 2
Area under the curve of the receiver operating characteristic [ROC] curves evaluating the use of fold-increases in antibody levels against each antigen for the diagnosis of pneumococcal infection, the sensitivity, specificity and accuracy of the optimal cut-off of each of these curves and of the ≥2-fold increase cut-off.

Antigen	Area under the curve	Optimal fold-increase	≥Optimal cut-off[a]			≥2-Fold increase		
			Sensitivity	Specificity	Accuracy of the cut-off	Sensitivity	Specificity	Accuracy of the cut-off
Ply	0.666	≥3.16	23.08%	100%	72.22%	23.08%	95.65%	69.44%
CbpA	0.814	≥2.85	46.15%	95.65%	77.78%	53.85%	91.30%	77.78%
PspA	0.677	≥1.97	38.46%	100%	77.78%	38.46%	95.65%	75.00%
PcpA	0.933	≥1.52	84.62%	91.30%	88.89%	53.85%	91.30%	77.78%
PhtD	0.783	≥2.04	53.85%	91.30%	77.78%	53.85%	91.30%	77.78%
StkP-C	0.609	≥1.77	30.77%	95.65%	72.22%	30.77%	95.65%	72.22%
PcsB-N	0.896	≥2.24	76.92%	100%	91.67%	76.92%	100%	91.67%

[a] Cut-offs defined in this investigation based on ROC curve.

that recent acquisition of a new pneumococcal colonization was not significantly associated with a ≥2-fold increase in the antibody levels to 27 pneumococcal proteins, including the ones studied herein (Turner and Turner, 2013). We have shown that the response against pneumococcal antigens was highly specific when comparing children with IPD and non-colonized controls. Although the requirement of paired samples presents an obstacle for the use of serology in clinical practice, it is still a viable option for etiology research purpose. Finally, serological techniques have been increasingly developed and overcame previous limitations on the number and type of antigens included in each assay (Andrade et al., 2014).

Many pneumococcal proteins have been described over the past decade, and their use in serological assays may have advantages compared to the commonly used capsular polysaccharides when serotype-specific data is not required. For instance, Olaya-Abril et al. recently described a protein array using 95 recombinantly produced pneumococcal proteins described via proteomics technique, representing the versatility of protein antigens for use in serodiagnostics (Olaya-Abril et al., 2015). All of the antigens included in our assay have been described as highly specific to *S. pneumoniae* and widely distributed among its strains (Tai, 2006; van der Poll and Opal, 2009; Giefing et al., 2008). Therefore, the use of pneumococcal proteins allows the identification of responses against *S. pneumoniae* regardless of the serotype of the infecting strain. Furthermore, the development of multiplex techniques has allowed the test of multiple antigens in the same assay (Andrade et al., 2014), representing a more economic option when a combination of antigens is used to improve the overall sensitivity of the test. Of note, multiplex assays including up to 64 pneumococcal antigens have been described, with good robustness and no effect from the multiplexing, representing a resourceful tool for the measurement of IgG against pneumococcal proteins (Jiménez-Munguía et al., 2015).

The differences found for the levels of antibodies in the acute phase sera may be explained by the difference in age between the study groups. The production of antibodies against protein antigens from *S. pneumoniae* starts at different ages for each specific antigen (Prevaes et al., 2012; Holmlund et al., 2009; Lebon et al., 2011; Simell et al., 2009; Hagerman et al., 2013; Holmlund et al., 2006; Rapola et al., 2000; Zhang et al., 2006) and, therefore, the age of the children directly influences the level of antibodies generated against such antigens.

It is important to emphasize, however, that the antibody levels in the acute phase sera did not affect the association between the study group and the detection of an antibody response against PspA, PcpA, PhtD and PscB (Table 4). Indeed, the serological test composed only of PspA, PcpA, PhtD and PcsB had the same sensitivity and specificity as the test with all the studied proteins (data not shown). An independent association between study group and detection of antibody responses was not found for Ply, CbpA and StkP, and we hypothesize this was due to the small sample size in this study.

The optimal cut-offs of antibody increase against each antigen herein defined were based on the maximum accuracy for pneumococcal infection diagnosis. The accuracy of these cut-offs was overall similar to the use of a cut-off of ≥2-fold increase. However, the cut-off of ≥1.52-fold increase in antibody levels against PcpA demonstrated substantially higher accuracy compared to ≥2-fold increase in antibodies against the same antigen (88.89% vs. 77.78%). There was only a modest increase in the accuracy of the test with the use of a cut-off of ≥3.16-fold increase in antibodies against Ply and a cut-off of ≥1.97-fold increase in antibodies against PspA compared to the cut-off of ≥2-fold rise in antibodies against these same antigens (72.22% vs. 69.44% and 77.78% vs. 75%, respectively). Therefore, the use of ≥2-fold increase in antibody levels may be a useful diagnostic criterion of pneumococcal infection when using the protein antigens investigated herein, but the use of distinct cut-offs can further improve the accuracy of the test, especially for antibody responses against PcpA. The use of a ≥2-fold increase in antibody levels had already been validated for Ply by measuring the levels of antibodies in healthy Finnish children (Nohynek et al., 1995), which contrasts with the higher optimal cut-off of ≥3.16 found herein. However, when comparing the specificity of the optimal cut-off with the accuracy of the ≥2-fold increase cut-off (100% vs. 95.65%, respectively), there was only a small increase in the specificity of the test by using the optimal cut-off. Therefore, the use of a ≥2-fold increase cut-off still represents a reasonable option for the diagnosis of pneumococcal pneumonia using this protein.

The use of combinations of pneumococcal protein antigens represents an interesting strategy to improve the sensitivity of a serological assay. We have shown that when using all the antigens included in this study, the detection of a response against at least one antigen is highly sensitive and specific for the presence of pneumococcal infection.

Table 3
Sensitivity, specificity and accuracy of antibody responses against different numbers of pneumococcal proteins for the diagnosis of *Streptococcus pneumoniae* infection (considering the cut-offs from Table 2 and the gold-standard of ≥2 fold increase).

Antibody responses to number of pneumococcal antigens	≥Cut-off of higher accuracy			≥2 Fold		
	Sensitivity	Specificity	Accuracy of the cut-off	Sensitivity	Specificity	Accuracy of the cut-off
≥1	92.31%	91.30%	91.67%	92.31%	91.30%	91.67%
≥2	84.62%	91.30%	88.89%	84.62%	91.30%	88.89%
≥3	69.23%	91.30%	83.33%	53.85%	91.30%	77.78%
≥4	46.15%	91.30%	75.00%	38.46%	91.30%	72.22%
≥5	30.77%	95.65%	72.22%	23.08%	95.65%	69.44%
≥6	23.08%	95.65%	69.44%	15.38%	95.65%	69.44%
≥7	15.38%	100%	69.44%	15.38%	100%	69.44%

Table 4
Effect of the study group on the frequency of antibody response detection using the cut-offs defined in this study, evaluated by logistic regression, adjusted by the antibody levels in the first serum samples.

Protein antigen	Group	
	Non adjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Ply		
Study group	6.6 (0.609–71.557)	1.906 (0.123–29.528)
Antibody level on the first serum sample	1.009 (0.998–1.02)	1.008 (0.996–1.02)
CbpA		
Study group	12.25 (1.996–75.196)	6.801 (0.836–44.253)
Antibody level on the first serum sample	1.001 (1–1.002)	1.0004 (0.999–1.002)
PspA[a]		
Study group	13.75 (1.386–136.387)	12.188 (1.183–125.611)
Antibody level on the first serum sample	1.002 (0.998–1.005)	1.001 (0.998–1.004)
PcpA		
Study group	57.75 (7.134–467.519)	45.566 (5.505–377.177)
Antibody level on the first serum sample	1.001 (0.999–1.0003)	1.001 (0.999–1.0003)
PhtD		
Study group	12.25 (1.996–75.196)	7.385 (1.01–54.01)
Antibody level on the first serum sample	1.001 (1–1.003)	1.0004 (0.999–1.002)
StkP-C		
Study group	9.778 (0.957–99.943)	1.937 (0.12–31.319)
Antibody level on the first serum sample	1.016 (0.993–1.039)	1.013 (0.988–1.038)
PcsB-N		
Study group	73.333 (6.764–795.076)	65.906 (5.825–745.67)
Antibody level on the first serum sample	1.001 (0.999–1.004)	1 (0.9996–1.0004)

[a] PspA 1 and 2 were conjugated on the same bead set.

It is important to highlight that, among the studied antigens, PcpA and PcsB-N have shown the highest accuracy rates, and their inclusion should be considered when developing a serological assay against *S. pneumoniae*. Indeed, Posfay-Barbe et al. showed that the use of multiple antigens markedly improves the sensitivity of antibody response detection against *S. pneumoniae*, particularly if PcpA was included in the combination (Posfay-Barbe et al., 2011). Furthermore, Jiménez-Munguía et al. also described that the levels of specific IgG against PcpA were three times lower in sera of children with pneumonia collected up to 10 days of disease, when compared to that of negative controls (Jiménez-Munguía et al., 2015). Similar results were found for the evaluation of antibody responses in a cohort of 690 children with non-severe community-acquired pneumonia, in which PcpA and PcsB were the antigens with the highest rate of antibody response detection (Borges et al., 2015).

The limitations of this study should be noted. First, there were important differences between the positive and negative controls, *i.e.*, nationality, age, vaccination status and different levels of antibodies in the first serum samples. However, we demonstrated that these differences did not affect the detection of antibody responses to at least four pneumococcal proteins, including PcpA and PcsB-N (Table 4). In addition, a recent study has demonstrated that vaccination with PCV10 did not affect the rate of antibody responders in a cohort of children with non-severe CAP (Andrade et al., in press). Second, the positive and negative control groups included in this study had few participants. It is possible that the low number of participants in this study may have compromised the independent association between the study group and the detection of antibody responses against Ply, CbpA and StkP. This is due to the difficulty in acquiring paired serum samples from pediatric patients. Furthermore, less than 5% of the pediatric cases of community-acquired pneumonia develop bacteremia (Shah et al., 2010). The negative control group was composed of children with pharyngitis, who were not strictly healthy children. However, it is important

to recall that pharyngitis in children aged under 5 years is predominantly caused by viruses, and *S. pneumoniae* is not recognized as a bacterial causative agent in this setting (Hsieh et al., 2011). In fact, it is possible that the antibody responses against *S. pneumoniae* detected in the two patients with pharyngitis was caused by a polyclonal response elicited by EBV (Freijd and Rosén, 1984) or by cross reactive antibodies against Group A Streptococcus. Secondly, colonization was not evaluated at the collection of the second serum samples in the negative control group. Consequently, it is possible that the two children from the negative control group who had seroresponses against *S. pneumoniae* presented a new colonization by this bacterium between the collection of serum samples. Finally, the evaluation of colonization by *S. pneumoniae* by oropharyngeal swabs is not the most sensitive method to identify this type of contact with the pneumococcus (Satzke et al., 2013).

In conclusion, we demonstrated that serology using multiple pneumococcal proteins is a promising method for the diagnosis of pneumococcal infection in children with pneumonia. Although the cut-off of ≥2-fold increase in antibody levels is adequate for most antigens, different cut-offs may be optimal for some antigens, such as ≥1.52-fold increase in antibody levels against PcpA. Furthermore, when using the antigens studied herein, the detection of antibody response against at least one antigen is highly sensitive and specific for the diagnosis of infection by *S. pneumoniae.*

Conflict of interests

Andreas Meinke is an employee at Valneva Austria GmbH.

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