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ABSTRACT

PURPOSE. To evaluate a rapid Quantitative Real-Time PCR for direct detection and quantification of pneumococcal DNA bacterial load (DBL) in patients with pneumonia and empyema.

METHODS. DBL and molecular serotype detection was determined by DNA quantification of ply gene and an additional capsular gene by Real-Time PCR. Plasma or pleural fluid samples from children and adolescents with confirmed pneumococcal pneumonia were analyzed. DBL was correlated with clinical parameters and outcomes.

RESULTS. 169 patients with pneumococcal pneumonia (145 empyema) had performed bacterial cultures and Real-Time PCR. Of them, 41 (24.3 %) had positive both, 4 (2.4%) had positive culture alone and 124 (73.3%) had positive Real-Time PCR alone. Pleural fluid DBL was lower in patients with prior antibiotics (P=.01), and higher in those with positive culture (P<.001). Pleural fluid DBL positively correlated with serum C-reactive protein (P=0.009), pleural fluid neutrophils (P<.001), and pleural fluid glucose (P<.001). Plasma and pleural fluid DBL were higher in patients with ≥ 8 days of hospital stay (P =.002), and pleural fluid DBL positively correlated with hours of pleural drainage (P <.001).

CONCLUSIONS. Quantification of pneumococcal DBL by Real-Time PCR may be helpful for the diagnosis and clinical management of pediatric patients with pneumonia and empyema

KEY WORDS: Pneumococcal pneumonia; Real-Time PCR; bacterial load; *Streptococcus pneumoniae*; serotyping

INTRODUCTION

Streptococcus pneumoniae is one of the major bacterial pathogens causing severe infections with high morbidity and mortality [1]. There are 93 pneumococcal capsular types (serotypes) but only a limited number of them cause the majority of invasive pneumococcal disease (IPD) [2].

The goal standard culture-based method for the diagnosis of IPD requires the isolation and identification of *Streptococcus pneumoniae* from normally sterile clinical specimens; it needs up to 48-72 hours to confirm the results and may have a low sensitivity [3].

It is well known that many febrile children who are seen at the Emergency Department had received prior antibiotics and it can avoid grow of *Streptococcus pneumoniae* in cultures [4]. Therefore, several episodes of infection caused by antibiotic susceptible pneumococcal serotypes may be misdiagnosed and consequently the rates of IPD can be underestimated. In order to improve the diagnosis of IPD and also to perform valuable epidemiological surveillance studies, we need additional methods with higher sensitivity and carried out in a shorter period of time than the standard culture-based method.

Molecular techniques applied to quantify viral load are widely accepted for monitoring the course of infections such as hepatitis C or human immunodeficiency virus [5 6 7 8]. However, little is known about the usefulness of direct detection and quantification of pneumococcal DNA in patients with IPD. Current molecular techniques such as pneumococcal Real-Time PCR (direct detection of pneumococcal DNA) may be a rapid method to improve the diagnosis of IPD and also for serotyping. Moreover, Real-Time PCR may allow determining the quantification of pneumococcal DNA bacterial load (DBL) in different clinical samples and improving the management of patients with IPD as occur in some viral infections [9, 10]. To our knowledge there are no reports about plasma and pleural fluid DBL in children with pneumonia and empyema.

The objectives of this study were: 1) to evaluate a rapid Quantitative Real-Time PCR for direct detection and quantification of pneumococcal DNA bacterial load in sterile clinical samples, and 2) to examine the relationship between DBL (in plasma and pleural fluid) with several clinical and microbiological variables in children with pneumonia and empyema.

PATIENTS AND METHODS

We prospectively studied all children and adolescents (less than 18 years old) with confirmed pneumococcal pneumonia who were attended at "Sant Joan de Deu Hospital", a 345-bed children´s hospital in Barcelona, Spain, from 6/2003 to 6/2008.

Confirmed pneumococcal pneumonia was defined as the presence of clinical and radiological findings of pneumonia together with isolation of *Streptococcus pneumoniae* and/or DNA detection of *S.pneumoniae* by Real-Time PCR in plasma and/ or pleural fluid. Plasma and/or pleural fluid samples were obtained when available and according to patient's symptoms. For the present study we have selected those children with confirmed pneumococcal pneumonia who had been studied by both, culture and Real-Time PCR..

As a control group, the Real-Time PCR assay was assessed with 106 plasma samples from healthy individuals, 50 of them were healthy nasopharyngeal carriers of *S.pneumoniae*. Blood samples were extracted in the outpatient's clinic prior to a minor surgical procedure (such us ocular surgery, phimosis, or non complicated hernia) and after a written informed consent was obtained..

Microbiological bacterial cultures

All pneumococcal isolates were identified by standard microbiological methods as previously described [11] [12].

Serotype identification

Serotyping of strains isolated by culture was carried out by the Quellung reaction at the National Center for Microbiology (Majadahonda Madrid). Detection of pneumococcal serotypes in clinical samples with negative culture were performed in our laboratory, according to Multiplex Real-Time PCR [13].This sequential PCR approach distinguishes among 24 serotypes (1, 3, 4, 5, 6A, 6B, 7F/A, 8, 9V/A/N/L, 14, 15B/C, 18C/B, 19A, 19F/B/C, 23A, and 23F). Serotypes were classified in two groups: PCV7 serotypes (4, 6B, 9V, 14,18C,19F and 23F) and non-PCV7 serotypes including all others.

Extraction of DNA

DNA of pneumococci in sterile samples was extracted using 20 % w/v Chelex-100 resin (BioRad Laboratories,Hercules,California, USA). Plasma and pleural fluid samples required a previously preparation: 200 µl of samples were centrifugated at 4° and 25000g during 60 min. After removal of the

supernatant, samples were resuspended in 100 µl of PBS-buffer. 50 µl of cerebrospinal fluid (CSF) or prepared samples were added and vortexed with 150 µl of 20 % w/v Chelex-100; the tubes were incubated for 20 minutes at 56°C followed by a 10 minutes incubation at 99 °C. After cooling, the supernatant was used as a template in Real-Time PCR experiments.

DNA from samples collected since 2006 were extracted from 200 µl of biological fluid (plasma, pleural fluid or CSF) by an automated system for DNA extraction (EasyMag, BioMerieux Laboratories) Selected samples were performed in parallel in order to compare the recovery of bacterial DNA using both DNA extraction methods.

DNA quantification by Real Time PCR assay

TaqMan fluorescent probes and specific primers for pneumolysin *(ply)* gene of *S.pneumoniae* (GenBank accession nº MX52474) [14] that have been previously described by Corless et al were used [15]. In addition, These primer sets were tested using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) for universal conditions of amplification**.** Amplification was done in a Real-Time PCR instrument Abiprism 7000 (Applied Biosystem, Foster City, CA). The reaction volume of 25 µl contained 5-µl of DNA extract from samples or controls and 2x TaqMan Universal Master Mix (Applied Biosystems), which includes dUTP and uracil-*N*-glycosylase; each primer was used at a final concentration of 300 nM. The TaqMan probe was used at a final concentration of 150 nM. Internal controls for monitoring false negatives by PCR inhibitors were run for each sample to monitor the overall assay performance and these consisted of 1 µl of TaqMan Rnase P control reagent (VIC) (Applied Biosystem, Foster City, CA) that included human RNAse P primers and the TaqMan Probe with VIC as fluorescent reporter dye at the 5' end. Amplification was done performing universal amplification conditions: incubation for 2 min at 50°C (uracil-N-glycosylase digestion) and 10 min denaturation at 95°C, 45 cycles of two-step amplification (15 s at 95°C, 60 s at 60°C) . Amplification data were analyzed by SDS software (Applied Biosystems). The reporter dye (FAM for samples or VIC for internal control) signal was measured relative to the internal reference dye (ROX) signal to normalize for non-PCR related fluorescence fluctuations occurring from well to well. The cycle threshold (CT) value was defined as the cycle at witch the reporting dye fluorescence first exceeds the calculate background level. At low CT value thus corresponds to a high target concentration. Quantification of *S. pneumoniae*

in each sample was based on a standard curve generated by plotting the C_T value against known genomic equivalents

Determination of PCR efficiency and Calibration curve for DNA quantification

0.5 McFarland suspension was made from a serotype 19F *S. pneumoniae* strain obtained from our bacteriological laboratory and serial dilutions from 1.7 ng/µl (7.10^7 CFU/mL) to 0.6 fg/µl (7 CFU/mL) were performed. These serial dilutions were used to generate a reference standard curve which was exported and used as an external standard curve after each run. The external standard curve was calibrated with two standards controls included in each run with the clinical samples. DNA extraction of these serial dilutions was performed by using manual method and EasyMag Platt form.

The reproducibility of triplicate Ct values was assessed on all standard specimens. The mean intra-assay and inter-assay coefficient of variation were calculated from Ct values.

Statistical analysis

Statistical analysis was performed with the PASW software package (version 17.0). pneumococcal DNA bacterial load (DBL) data were log transformed to assume a normal distribution. Continuous variables were compared using the t test (for approximately normally distributed data) or the Mann-Whitney U test (for skewed data) and described as mean values and standard deviations or median and interquartile range P25-P75 (IQR) according to the presence of normal distribution. Chi-square test or Fisher's exact test (two-tailed) was used to compare categorical variables. Associations between routine biochemical variables and DBL were examined using Spearman correlation coefficient. Comparison between groups was performed by Kruskal-Wallis test. Logistic regression models were used to examine the independent effect of DBL on length of hospital stay after adjusting for other variables. Statistical significance was set at a *P* value of <.05.

RESULTS

Overall there were 206 children with confirmed pneumococcal pneumonia and we included in the study 169 children (145 with empyema and 24 without empyema) who had performed both bacterial cultures and Real-Time PCR. Of them, 41 (24.3 %) had both positive culture and Real-Time PCR, 4

(2.4%) had positive culture and negative Real-Time PCR and 124 (73.3%) had positive Real-Time PCR and negative cultures.

Reproducibility and Specificity of Real-Time PCR

The Real-Time PCR assay detected correctly all serial dilutions (range 7 to 7.10^7 CFU copies/mL). There was a lineal correlation between the log of the standards and the threshold cycles (Ct), with a regression line showing a slope of - 3.5 with Pearson correlation coefficient of 0.99 when DNA extraction was made by Chelex-100 method or by EasyMag method. The amount of PCR product formed was derived from the function $y = - 3.5x + 29.4$ for Chelex method and $y = - 3.5x + 32.2$ for EasyMag method. Reproducibility was calculated using the standard curve that was amplified in three consecutive runs using manual method and EasyMag method. The standard deviation of the mean Ct was always less than 1 cycle for the two procedures.

Real-time PCR was highly specific; only two of the 106 plasma samples from healthy children were positive by Real-time PCR, and they had a low DNA pneumococcal load (DBL) (5 and 2 CFU copies/mL, respectively). Of note, one of these two children was a pneumococcal nasopharyngeal carrier and the other was presumably a non-identified carrier**.**

Pneumococcal DNA bacterial load (DBL) and clinical characteristics

There were 165 children with pneumonia and positive detection of pneumococcal DNA in sterile samples (one or more). Pleural fluid was positive in 126 of 129 samples collected (97.6%) and plasma was positive in 85 of 137 (62%) samples. Among 145 children with empyema, plasma and pleural fluid were analyzed in 101 of them, and 52 (51.5%) were positive for pleural fluid only, 3 (3%) were positive for plasma only and 46 (45.5%) were positive for both.

Overall, there were 89 males (53.9%), and the mean age was 54.2; SD 38.8 (months) (range 1 month - 17 years). According to the criteria of the American Academy of Pediatrics [16], 9 patients (6.2%) had risk factors for IPD: chronic pulmonary disease 6, chronic cardiac disease 2, and measles coinfection 1. Prior vaccination with PCV7 was detected in 42 of 150 patients (28%) who had available information, and all of them were infected by non-vaccine serotypes. Mean hours of fever before admission (in 161 cases) were 107.5; SD 64 (hours). Prior antibiotics were identified in 84 of 153 patients (54.9%) who had available information. Of the 165 pneumococcal pneumonia cases, 126 were serotyped

(76.3%) (41 by conventional serotyping in culture positive samples and 85 by Multiplex Real-Time PCR in culture negative samples); lack of enough material for serotyping was not available in 39 patients. Of the 126 episodes with serotype information 10 (7.9%) were caused by vaccine serotypes and 116 (92.1%) by non-vaccine serotypes. The main serotypes detected were serotype 1 (53 patients;42%), serotype 19A (16 patients; 12%) , serotype 3 (11 patients ; 8.7%), 7F (11 patients; 8.7%) and serotype 5 (6 patients;4.7%). 14 of 165 patients (8.5%) were admitted to the PICU (Pediatric Intensive Care Unit). The mean length of hospital stay was 11.4; SD 6.5 (days). No patient died.

As shown in Figure 1, Pneumococcal DNA bacterial Load (DBL) was much higher in pleural fluid than in plasma (Mann-Whitney test, *P*<.001*).* According to the clinical syndrome, plasma DBL was slightly higher in patients with pneumonia and empyema (n=65) than in patients with non-complicated pneumonia (without empyema) (n=20): bacterial load median $\log_{10}$ CFU copies/mL (interquartile range): 1.14 (0.44-1.59) vs. 0.97 (0.55-1.15), but it did not reach statistical significance (Mann-Whitney test, *P*=0.4).

Table 1 shows plasma and pleural fluid DBL according to several parameters. Most clinical and microbiological variables were not associated with differences in plasma DBL. However, plasma DBL was significantly higher in patients with ≥ 8 days of hospital stay as compared with those with <8 days (Table 1).

As shown in Table 1, pleural fluid DBL was lower in patients with prior antibiotic therapy (as compared with no prior antibiotics) (*P*=.01), and was higher in those with positive culture for *S.pneumoniae* (as compared with negative cultures) (*P*<.001). In addition, as occurred in plasma DBL, there were higher levels of pleural fluid DBL in patients with ≥ 8 days of hospital stay as compared with those with ≤ 7 days. Using multiple logistic regression models we found that plasma DBL (adjusted OR 3.53; 95% CI : 1.43-8.70; *P*=.006) and pleural fluid PDL (adjusted OR 1.46; 95% CI : 1.09-1.96; *P*=.01) were independently associated with prolonged hospital stay (≥ 8 days) after adjusting for age, hours of fever before admission and positive bacterial culture.

Figure 2 shows a positive correlation between pleural fluid DBL and days of hospital stay (Spearman's rho: 0,4; *P*<.001) and total hours of pleural drainage (spearman rho: 0,4; *P*<.001).

As shown in Figure 3, pleural fluid DBL showed a positive correlation with plasma DBL (Spearman's rho: 0.3; *P*<.01). In addition, pleural fluid DBL had a significant correlation with several biochemical markers of infection such as serum C reactive protein (CRP) (Spearman's rho: 0.2 ; *P*=.009),

pleural fluid neutrophils (spearman rho:0.4; *P*<.001), and pleural fluid glucose levels (Spearman's rho: -0.8; *P*<.001).

DISCUSSION

DNA detection and quantification may provide useful epidemiological and clinical information and improve our knowledge about dynamics of pneumococcal replication. Two major findings are pointed out in this study. First, the significant increase of detecting pneumococcal pneumonia and empyema in children and subsequent serotyping when adding Real-Time PCR for diagnosis to traditional culture-based methods. And second, a positive correlation of pleural fluid pneumococcal DNA bacterial load (DBL) with several clinical and outcome variables.

Up to date, the reported studies about the use of PCR for diagnosis of invasive pneumococcal disease (IPD) have shown controversial results. Recently, Avni et. al. [17] have published an accurate meta-analysis and found that the studies were highly heterogeneously including different type of PCR methods[18,19], different target genes [20], most used stored frozen [21] and different samples (i.e. whole blood or plasma) [22], as well as different patients' characteristics and different clinical syndromes [23,24]. The authors concluded that "the lack of appropriate reference standard might have caused underestimation of the performance of the PCR" and that "currently available methods for PCR with blood samples for the diagnosis of IPD lack the sensitivity and specificity necessary for clinical practice".

In our study we used fresh plasma and pleural fluid samples for the detection and quantification of the DNA and in most cases we have added the identification of serotypes by a multiplex Real Time PCR, which allowed us to increase significantly the number of confirmed episodes with respect to the culture-based method, as occurred in the study reported by Azzari et al [19]. Moreover, in the meta-analysis by Avni et al [17] the rate of diagnosis by positive PCR was also higher than the rate by positive blood culture in 16 of 25 studies.

Some authors have alerted of reporting IPD only by PCR because of the possibility of detecting false positive results in blood samples of healthy children [25]. In our study, we used plasma samples and found a good specificity (only two samples of 106 tested from healthy controls). Of note, the worst specificity was found when using buffy-coat as blood sample [26] instead of plasma samples. One limitation of our study is that we used *ply* gene of pneumococcal for detection (diagnosis) and quantification of pneumococcal DNA bacterial load (DBL). *Ply* gene may be unspecific because some

other strains closely related with *S.pneumoniae* may share the virulent genes encoding by *S.pneumoniae* such as *ply* or *lytA* genes [27]. Carvalho et al [20] tested two *ply* based PCR assays against 10 *Streptococcus pseudopneumoniae* isolates and 11 isolates of pneumococcus-like viridans group streptococci (P-LVS) and found a positive reaction in all of them. However, we may consider that in our study the consecutively serotyping by Real-Time PCR (which included tested with capsular genes of pneumococci) and the detection of a virulent gene (*ply* gene) of pneumococci in sterile sample of patients with clinical symptoms and radiologically confirmed pneumonia and empyema may have significant clinical value. Up to date, the clinical role of *S.pseudopneumoniae* and others quasi-pneumococcus in invasive disease is unknown, but some authors have alerted about the increase of these strains in the nasopharynx [28]. So, the clinical role of these closely related pneumococcal strains isolated in sterile samples need to be clarified.

In our study, pleural fluid DBL was higher than plasma DBL and there was a positive correlation between both of them. Plasma DBL levels were not significantly associated with age, sex, underlying conditions and other clinical and microbiological variables, but was higher in children with > 8 days of hospital stay (Table 1). A low levels of plasma DBL was also found in other studies performed by culture-based methods [29] or by Real-Time PCR [30,31], and this may explain, at least in part, the low rate of positive blood cultures detected and the poor correlation of blood culture results with DBL (plasma DBL was similar in children with positive and negative blood cultures). It is probably that the main factor for detecting pneumococci in blood culture-based methods (or by PCR based methods) is related to the volume of sample processed, and in younger children the blood volume is usually very low (approximately us 1-2 ml in children less than 6 months or 2-5 ml in children between 6months and 5 years). On the contrary, pleural fluid DBL was highly correlated with pleural fluid culture results (pleural fluid DBL was significantly higher in cases with positive pleural fluid culture than in negative samples). It may be related to the inoculated volume for pleural fluid culture (approximately 5-10 ml of pleural fluid sample) which is usually higher than in blood cultures. However, pleural fluid DBL levels were still high in patients with negative pleural fluid culture, and this may suggest that other factors should explain the low rate of positive culture in this type of samples. One may speculate that pleural fluid samples are usually sent to the laboratory in a sterile tube without transport medium, and this may be critical to reach a viable number of bacteria for culture. Our data suggest the need of improving the transport of pleural fluid samples for bacteriological culture.

Some studies have reported that prior antibiotics may affect the sensitivity of PCR [23, 32] while others did not [22, 33]. In our study we have found that plasma DBL was very low and not significantly different in those with prior and no prior antibiotics, but pleural fluid DBL was higher in those with no prior antibiotics (table 1). There were no significant differences of plasma and pleural DBL levels between patients infected with PCV7 serotypes vs. non vaccine serotypes. However, it should be noted that several serotypes are included in each group and the low number of each specific serotype precluded further analyses. Positive correlations were observed for the pleural fluid DBL with plasma DBL as well as with several routine haematological and biochemical parameters such as serum C reactive protein (CRP), pleural fluid neutrophils, and pleural fluid glucose levels.

Regarding the outcomes, both plasma and pleural fluid DBL levels were higher in patients with >8 days of hospital stay (table 1). We could not correlate DBL with mortality because none of our children died. Our data are in accordance with previous reports showing a relationship between DBL and worse outcome [34, 10] and prolonged length of hospital stay [9] in patients with severe pneumococcal infections such as pneumonia and meningitis; as well as occurred in patients with meningococcal meningitis in whom there was an association between bacterial load and mortality, complications, sequelae and length of hospital stay [35].

We found that in our children the pleural fluid DBL was correlated with the number of hours of drainage (length of pleural drainage) (figure 1). The treatment of pneumococcal empyema in children may be controversial, and recent studies have suggested that an early use of surgery with video-assisted thoracoscopy is associated with lower in-hospital mortality, length of hospital stay and duration of antibiotic therapy, compared with a non-surgical group [36]. However, a paediatric surgeon may not always be available in all hospitals**.** These data suggest that pleural fluid DBL (high levels) could help to the management of children with pleural pneumococcal empyema, and further studies are needed to evaluate this hypothesis.

In conclusion quantification of pneumococcal DBL by Real-Time PCR may be helpful for the diagnosis and clinical management of pediatric patients with pneumonia and empyema

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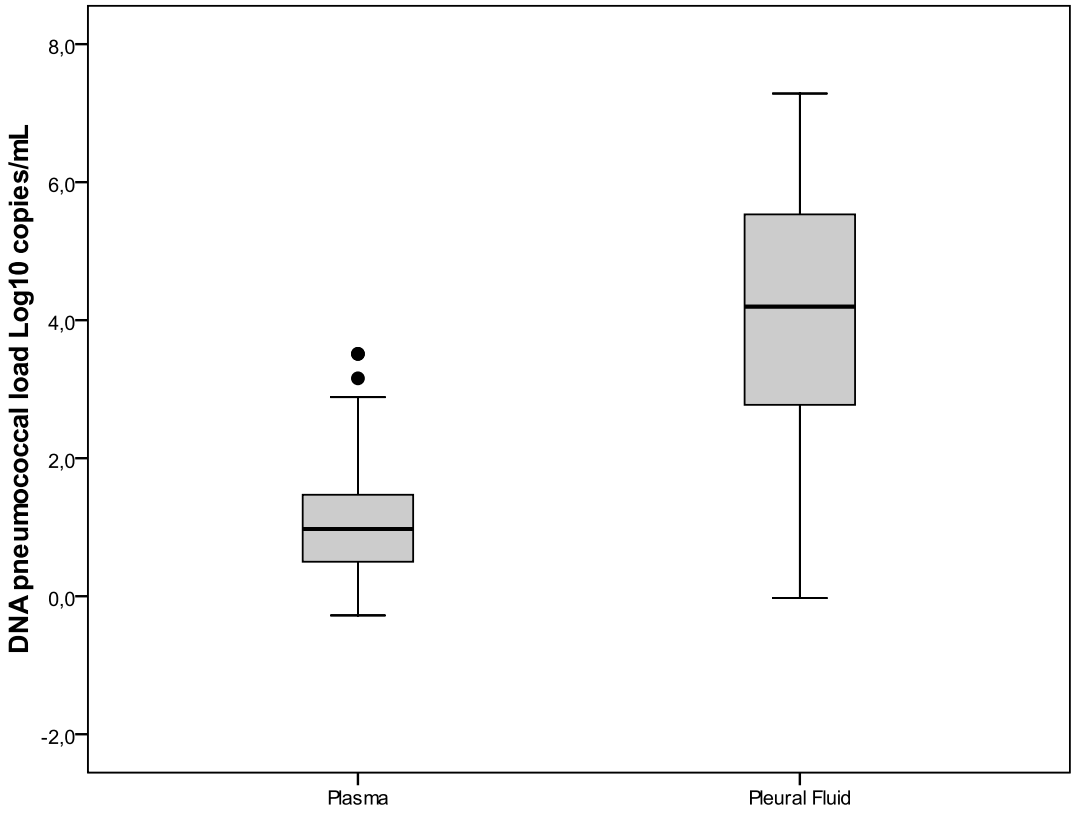
POTENTIAL CONFLICT OF INTEREST:

D.T. has been an Advisor of GlaxoSmithKline Biologicals and Wyeth Vaccines. All other authors: no conflict of interest.

Figure 1. Pneumococcal DNA bacterial load (DBL) in plasma and pleural fluid samples



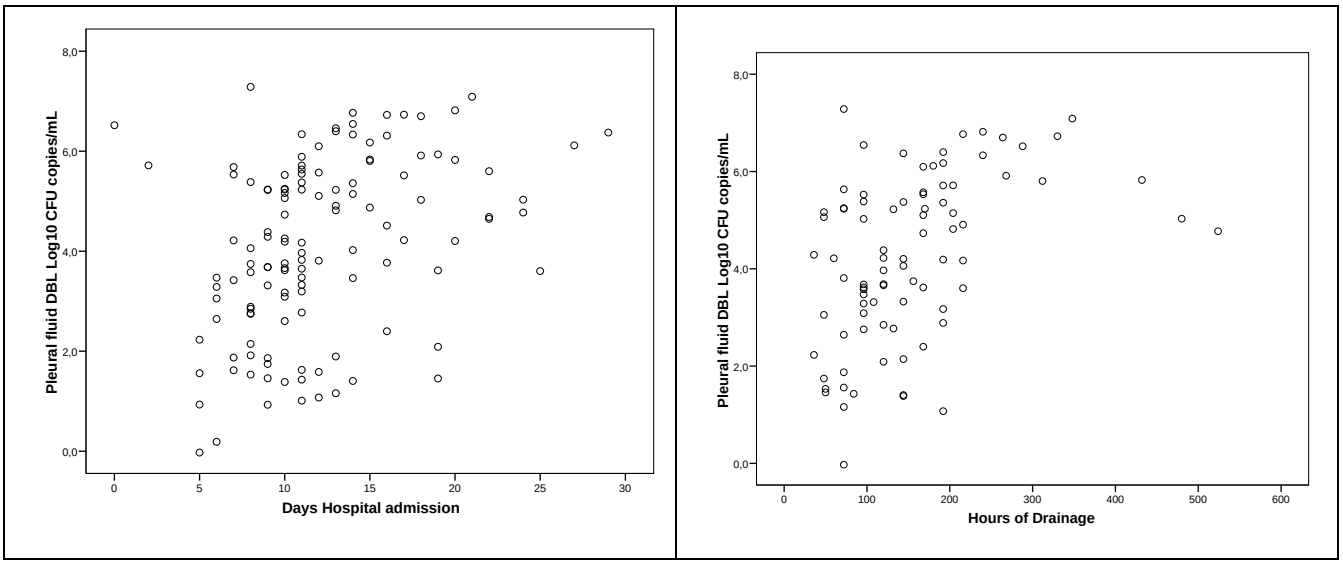
Pleural Fluid; n=126
DBL median log_{10} CFU copies/mL (interquartile range): 4.07 (2.76-5.53)

Plasma; n=85
DBL median log_{10} CFU copies/mL (interquartile range): 1.10 (0.50-1.47)

Mann-Whitney-test DNA pneumococcal load in plasma vs pleural fluid; P <.001**.**

Dots means outlier's values.

Figure 2. Pneumococcal DNA bacterial Load (DBL) in pleural fluid samples in relation to outcomes

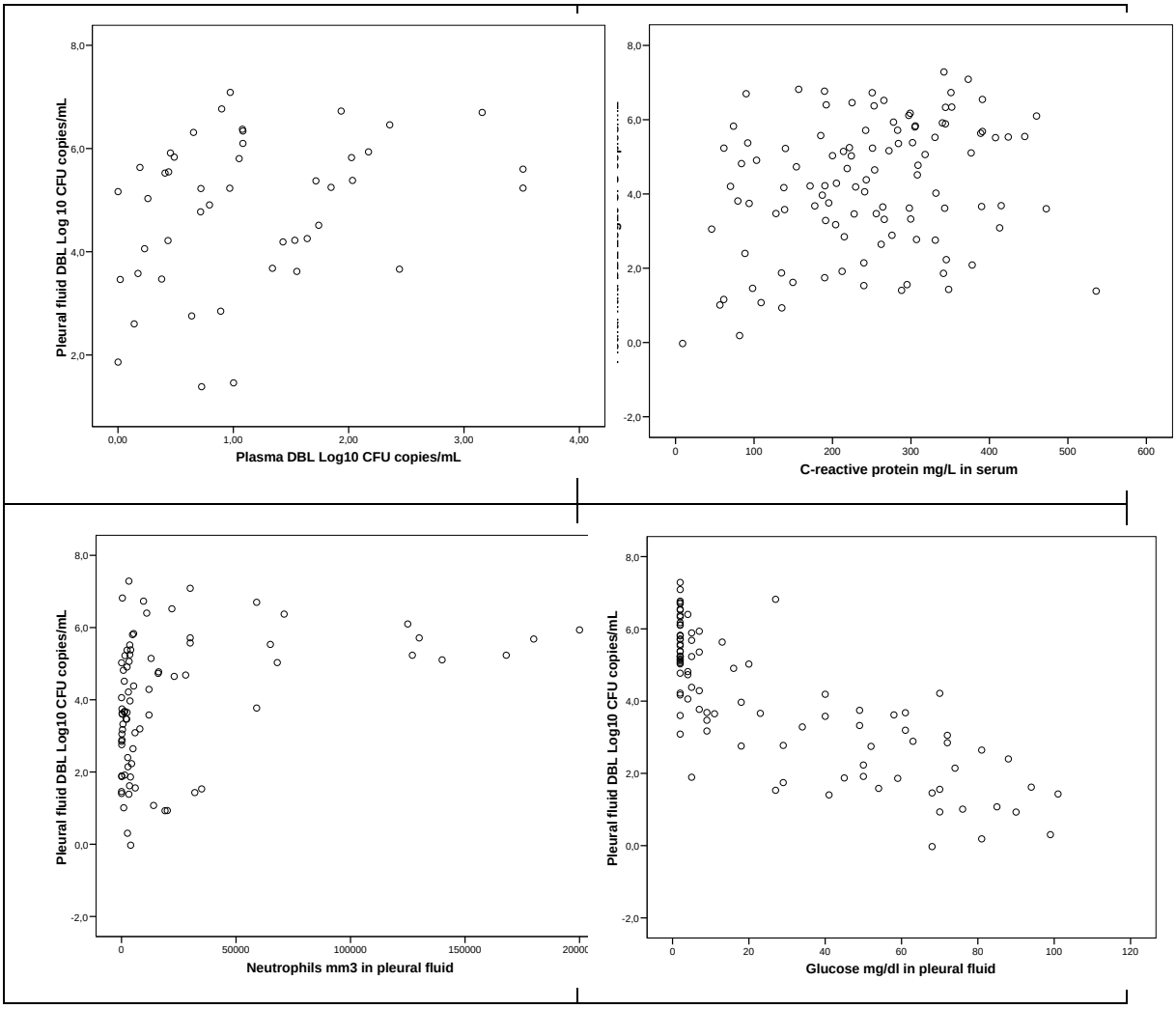


Outcomes:

Pleural fluid DBL and days of Hospital stay, spearman rho: 0,4; *P* <.001

Pleural fluid DBL and hours of pleural drainage, spearman rho: 0,4;*P* <.001

Figure 3. Pneumococcal DNA Load (DBL) in pleural fluid samples in relation to Plasma DBL, and routine haematological and biochemical markers



Pleural fluid DBL and Plasma DPL, spearman rho: 0.3; *P = 0.01*

Pleural fluid DBL and C-reactive protein in serum, spearman rho: 0.2; *P* = 0.009

Pleural fluid DBL and Neutrophils counts in pleural fluid, spearman rho: 0.4 ;*P* <.001

Pleural fluid DBL and Glucose levels in pleural fluid, spearman rho: -0.8; *P* <0.001

Table 1. Plasma and Pleural fluid pneumococcal DNA bacterial load (DBL) according to different parameters in children with pneumonia

	Plasma DBL			Pleural fluid DBL		
	Nº patients studied	**median log10 CFU copies/mL (IQR)**	***P***	**Nº patients studied**	**median log10 CFU copies/mL (IQR)**	***P***
Sex						
Male	**49**	**1.15 (0.67-1.53)**	**.4**	**63**	**4.01 (2.75-5.35)**	**.7**
Female	**36**	**1.03 (0.43-1.47)**		**63**	**4.04 (3.05-5.60)**	
Age group						
0-23 months	**18**	**1.22 (0.64-2.02)**	**.4**	**21**	**4.20 (3.13-5.45)**	**.7**
≥24 months	**67**	**1.07 (0.48-1.40)**		**105**	**4.04 (2.69-5.61)**	
PCV7 vaccination						
Yes	**21**	**1.02 (0.37-1.83)**	**.7**	**35**	**3.94 (2.74-5.22)**	**.7**
No	**55**	**1.09 (0.59-1.43)**		**80**	**4.04 (2.76-5.65)**	
Prior antibiotic exposure						
Yes	**42**	**1.25 (0.68-1.74)**	**.1**	**68**	**3.71 (2.61-5.00)**	.01
No	**36**	**0.94 (0.41-1.20)**		**51**	**4.52 (3.08-6.31)**	
Hours of fever before admission						
0-72 hours	**28**	**1.00 (0.48-1.26)**	**.4**	**40**	**3.86 (2.21-5.47)**	**.3**
>72 hours	**57**	**1.15 (0.49-1.59)**		**86**	**4.17 (2.87-5.54)**	
Presence of underlying diseases (risk factors)						
Yes	**5**	**1.04 (0.28-1.26)**	**.8**	**4**	**3.50 (0.75-5.96)**	**.5**
No	**80**	**1.11 (0.51-1.47)**		**122**	**4.09 (2.82-5.53)**	
Microbiological Data						
Positive culture	**25**	**1.11 (0.48-1.80)**	**.9**	**36**	**5.57 (5.18-6.39)**	<.001
Negative culture	**60**	**1.10 (0.55-1.45)**		**90**	**3.47 (2.04-4.78)**	
PCV7 serotypes	**8**	**1.64 (0.97-2.08)**	**.1**	**7**	**4.46 (3.61-5.82)**	**.9**
Non-PCV7 serotypes	**60**	**1.12 (0.45-1.59)**		**92**	**4.41 (3.34-5.70)**	
Outcomes						
PICU Admission						
Yes	**7**	**1.54 (0.71-2.17)**	**.3**	**12**	**4.04 (2.59-5.68)**	**.9**
No	**78**	**1.06 (0.46-1.44)**		**114**	**4.07 (2.76-5.52)**	
Days of hospital stay						
0-7 days	**28**	**0.78 (0.38-0.99)**	.002	**29**	**3.20 (1.85-4.13)**	.002
≥8 days	**57**	**1.28 (0.71-1.76)**		**97**	**4.33 (3.39-5.61)**	

DBL in plasma was studied in 85 of the 165 patients with pneumonia with and without empyema and DBL in pleural fluid was studied in 126 of the 145 patients with empyema

IQR, interquartile range; Mann-Whitney U test for skewed data, unpaired Student's t test for approximately normally distributed data

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