Streptococcus pneumoniae DNA Load in Blood as a Marker of Infection in Patients with Community-Acquired Pneumonia[▽]

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Received 2 June 2009/Returned for modification 8 July 2009/Accepted 4 August 2009

Direct detection of *Streptococcus pneumoniae* DNA in blood adds to culture results in the etiological diagnosis of patients with community-acquired pneumonia (CAP). Quantification of the amount of DNA, the bacterial DNA load (BDL), provides a measurement of DNAemia that may increase the understanding of the clinical relevance of *S. pneumoniae* DNA in blood. We evaluated the *S. pneumoniae* BDL as a diagnostic tool in adult patients with CAP. The BDL was determined in whole-blood samples collected simultaneously with blood for culture from 45 adult patients with CAP. After DNA extraction, *S. pneumoniae* DNA was detected with specific real-time PCR amplification, and the BDL was calculated with a standard curve. PCR and microbiological results were compared, and the BDL was related to clinical and laboratory parameters. *S. pneumoniae* DNA was detected in 10/13 patients with positive blood cultures and in 67% of patients with microbiologically confirmed pneumococcal pneumonia. The positive predictive values of the receiver operating characteristic curves for the BDLs for pneumococcal infection (100%) and pneumococcal bacteremia (69%) were higher than those for the level of C-reactive protein (CRP; 43% and 23%, respectively) and the white blood cell count (WBC; 42% and 35%, respectively); the negative predictive values of these three parameters were in the same range (± 90 and $\pm 97\%$, respectively). The BDL was higher in patients presenting with systemic inflammatory response syndrome and in patients with bacteremia. Positive correlations were observed for the BDL with WBC, CRP level, and length of stay. We conclude that the BDL supports the diagnosis of *S. pneumoniae* infection in patients with CAP and provides a putative marker of the severity of disease.

Streptococcus pneumoniae is the most prevalent cause of community-acquired pneumonia (CAP) in adults (1, 10). Identification of *S. pneumoniae* as a cause of CAP can be achieved with Gram stain microscopy, culture of respiratory tract samples, blood culture, PCR, and antigen detection testing (26). A positive blood culture result provides a definite diagnosis. However, blood cultures have a low sensitivity for the confirmation of the pneumococcal etiology of CAP. This is due to the low prevalence of bacteremia in CAP, especially in uncomplicated cases, and the use of antibiotics prior to drawing blood samples for culture (11, 18, 32). In addition, blood cultures usually require 1 or 2 days before results are available and thus may have limited impact on the initial choice of empirical antimicrobial treatment (6, 29). PCR and antigen detection provide non-culture-based tools for the rapid identification of *S. pneumoniae* in respiratory tract specimens, pleural aspirate samples, and blood samples (7, 8, 13). These tests can add to

culture methods in the etiological diagnosis of patients with CAP, particularly for patients who received antimicrobial treatment prior to sampling for culture (13, 26, 33).

Several studies have evaluated the use of real-time PCR assays for the detection of *S. pneumoniae* DNA in blood samples from patients suspected of infection with invasive pneumococcal disease, with variable results (5, 14, 24, 31). These studies all used blood culture as the reference standard. However, positive PCRs relate not only to bacteremia but also to detection of DNA from nonviable bacteria, i.e., within phagocytes or due to the use of antibiotics. As such, it is understandable that most studies found cases with positive *S. pneumoniae* DNAemia and negative blood cultures. In this study, we used microbiologically documented *S. pneumoniae* infection as the gold standard for evaluation of PCR results and hypothesized that the bacterial DNA load (BDL) can be used to discriminate between patients with invasive infection and those with localized infection.

The magnitude of bacteremia, as based on quantitative blood cultures, relates to the severity of *S. pneumoniae* infection (2). A high bacterial count in blood samples from children with bacteremia is associated with an increased risk of development of more-serious disease (27). Also, a shorter amount of time needed to detect positivity for blood cultures, supposedly reflecting a higher initial bacterial load, is associated with higher severity of disease (20). This relation between bacterial concentration and severity of disease may also hold true for the

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[▽] Published ahead of print on 12 August 2009.

TABLE 1. Sequences of oligonucleotide primers and probes used for real-time PCR^a

Microorganism	Gene target	Sequence (5'–3')		
		Forward primer	Reverse primer(s)	Dye-labeled probe
<i>S. pneumoniae</i>	<i>lytA</i>	ACCGCTGGAGGAAGCACA	GCCTGTAGCCATTTTCGCCT AGCGATTTTCTTCCAGCCTGT	AGACGGCAACTGGTACT ^c
PhHV-1 ^b	gB polymerase	GGGCGAATCACAGATTGAATC	GCGGTTCCAAACGTACCAA	CGCCACCATCTGGAT ^d

^a Abbreviations: *lytA*, autolysin A gene; FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher; MGB, minor groove binding; gB, glycoprotein B; NED, 2',7',8'-benzo-5'-fluoro-2',4,7-trichloro-5-carboxyfluorescein.

^b As published by van Doornum et al. (30). The probe was modified into a minor groove binding probe.

^c Minor groove binding probe with 6-carboxyfluorescein label and nonfluorescent quencher.

^d Minor groove binding probe with 2',7',8'-benzo-5'-fluoro-2',4,7-trichloro-5-carboxyfluorescein (NED) and fluorescent quencher.

level of *S. pneumoniae* DNA in blood samples: the *S. pneumoniae* BDL was higher in human immunodeficiency virus-infected children who died from invasive pneumococcal disease than in survivors (5).

In this study, we evaluated the *S. pneumoniae* BDL as a putative diagnostic tool and clinical marker of infection in adult patients with CAP.

MATERIALS AND METHODS

Collection of patient samples and data. This study was conducted during a 16-month period at the Scheper Hospital in Emmen, which serves a regional function in the rural, northeast area of The Netherlands, and was approved by the local Medical Ethical Committee. Participating departments included the department of internal medicine, the emergency department, and the intensive care unit. When blood was drawn for culture upon clinical indication from patients admitted to these departments, a whole-blood sample was simultaneously and routinely collected and stored at –20°C. Retrospectively, the following inclusion criteria were used to select blood samples from these stored blood samples for analysis of the *S. pneumoniae* BDL: samples from all adult patients (>18 years of age) who were admitted with CAP (as defined below) and had blood samples drawn for culture and PCR within 24 h of admission.

Clinical and laboratory data, including C-reactive protein (CRP) values and white blood cell counts (WBC), were collected retrospectively from patients' charts and were included only if they had been determined within 12 h of the time point of blood sampling for the BDL in order to obtain the best possible analysis of the association among these variables.

Clinical definitions. CAP was defined as an acute respiratory illness associated with at least two of the following symptoms plus the presence of an infiltrate on a chest radiograph: cough, dyspnea, pleuritic chest pain, fever or hypothermia (temperature of <36 or >38°C, respectively), and auscultatory findings of abnormal breath sounds and rales. Pneumonia was considered to be of pneumococcal etiology when *S. pneumoniae* was isolated from a blood sample, pleural fluid sample, or respiratory tract sample. Bacteremia was defined as the isolation of *S. pneumoniae* from blood culture. The systemic inflammatory response syndrome (SIRS) was defined as described by Bone and colleagues (4). The pneumonia severity index (PSI) and CURB-65 score were used as described elsewhere (9, 15). The SIRS criteria, PSI, and CURB-65 score are well-accepted clinical measures of the severity of pneumonia infection.

Microbiological investigations. Blood samples were collected in blood culture bottles (BacT/Alert FAN) for routine culture. After automatic detection of growth, microorganisms were identified with standard microbiological techniques. Gram stain microscopy and culture of sputum samples were performed according to standard microbiological principles (25). Serological analysis for *Chlamydia pneumoniae* (sandwich enzyme-linked immunosorbent assay; Medac, Wedel, Germany) and *Mycoplasma pneumoniae* (Virotech enzyme immunoassay; Genzyme Virotech, Rüsselsheim, Germany) and the urinary antigen test for *Legionella pneumophila* (Binax NOW; Binax, Inc., Emergo Europe, The Hague, The Netherlands) were done when indicated by the clinician.

Determination of the BDL. Blood samples for PCR were collected in 3 ml EDTA tubes (Becton Dickinson). Prior to DNA isolation, 3,000 copies of phocine herpesvirus 1 (PhHV-1) used as an extraction and inhibition control (equal to 300 copies/PCR) were spiked with 200 µl of blood (30). Blood samples were treated to remove hemoglobin, as described elsewhere (21). DNA was then extracted from the equivalent of 200 µl of blood with the QIAamp DNA minikit

(Qiagen, Germany), according to the manufacturer's instructions, and eluted in 100 µl of elution buffer.

DNA amplification was done using a TaqMan 7000 system (Applied Biosystems, Foster City, CA) with oligonucleotide primers and probes specific for *S. pneumoniae* and PhHV-1 DNA (Table 1). The technical specificity of these primers and probes was evaluated for a large number of *S. pneumoniae*-related species, and no cross-reactions were observed. For each reaction, we used 20 µl of DNA in a total reaction volume of 50 µl. The analytical sensitivity of this *S. pneumoniae* PCR assay was less than 5 CFU equivalents/PCR, as determined by a dilution series of *S. pneumoniae* (strain ATCC 6306) spiked with pooled, culture-negative whole-blood samples.

PCR results were defined as positive when amplification signals were observed and negative if such a signal was absent in the presence of an adequate amplification of the PhHV-1 internal control reaction. If a PhHV-1 control failed, DNA from that sample was diluted 1:1 with phosphate-buffered saline and amplified again. If the PhHV-1 amplification signal then was still insufficient, the blood samples were retested. If the PhHV-1 control reactions failed twice, the sample was considered inhibited.

The BDL was calculated with a standard curve of *S. pneumoniae* DNA, consisting of four 10-fold dilutions of a stock solution of 5,000 CFU equivalents/PCR (strain ATCC 6306). This standard curve was generated after isolation of DNA from pooled, culture-negative blood samples spiked with the relevant amount of bacteria. In case two blood samples had been obtained simultaneously from one patient, the average BDL for these samples was computed. The detection limit for the BDL in the blood samples was 5 CFU equivalents/PCR, which equals 125 CFU equivalents/ml blood.

Statistical analysis. Statistical analysis was performed with SPSS version 15.0 (SPSS Inc., Chicago, IL). Standard two-by-two tables were calculated for sensitivity and specificity. Comparison of groups was performed using the Mann-Whitney U test or Kruskal-Wallis test for continuous variables and the chi-square test or Fisher's exact test for categorical variables. Spearman rank correlation (r_s) was used to describe the relationship between continuous variables. Receiver operating characteristic (ROC) curves were designed and analyzed with MedCalc version 9.6.4.0 (MedCalc Software, Mariakerke, Belgium). The positive predictive values (PPV) and negative predictive values (NPV) of the ROC curves were calculated on the basis of an estimated disease prevalence of 25% for *S. pneumoniae* infection and 10% for pneumococcal bacteremia in patients with CAP (3, 14, 17, 28). *P* values of <0.05 were considered to be statistically significant.

RESULTS

Patient characteristics. Blood samples from 45 patients with CAP, of whom 3 (7%) were admitted to the intensive care unit, were included (Table 2). Twelve patients (27%) had taken antibiotics within 48 h of admission. Microbiologically confirmed pneumococcal pneumonia was present in 18/45 (40%) of patients, as demonstrated by positive cultures of blood ($n = 13$), sputum ($n = 10$), and throat ($n = 1$) samples. Six of the thirteen patients with pneumococcal bacteremia also had a positive culture of a sputum ($n = 5$) or throat ($n = 1$) sample; the last patient presented with classical symptoms and a chest X ray of lobar pneumonia. Nine out of forty-five patients with

TABLE 2. Clinical characteristics of the study population

Characteristic	Value for patients with:	
	Pneumococcal pneumonia (n = 18)	Nonpneumococcal pneumonia (n = 27)
Gender (males/females)	12/6	17/10
Age (yr)	69 (24–84)	71 (32–85)
Length of hospitalization (days)	12 (1–60)	9 (2–19)
No. of mortalities	2 (11)	3 (11)
No. with COPD	8 (44)	12 (44)
No. with history of pneumonia (<5 yr)	5 (28)	6 (22)
No. with cough	16 (89)	21 (78)
No. with dyspnea	13 (72)	19 (70)
No. with chest pain	6 (33)	7 (26)
No. with cold chills reported	6 (33)	11 (41)
No. receiving antibiotic treatment before admission	2 (11)	10 (37)
Body temp (°C)	39.1 (36.4–40.6)	39.0 (35.5–40.3)
Heart rate (beats/min)	109 (64–150)	100 (60–130)
Systolic blood pressure (mm Hg)	130 (90–170)	130 (80–195)
No. with abnormalities on auscultation	17 (95)	19 (76)
No. with mental status alteration	4 (22)	3 (11)
No. with SIRS	17 (100)	17 (63)
PSI (class)	3 (1–4)	3 (1–5)
CURB-65 score	2 (0–3)	2 (0–5)

^a Data are presented as numbers (percentages) for dichotomous variables and as median values (ranges) for continuous variables. No significant statistical difference was observed for clinical characteristics between patients with and without a pneumococcal origin for CAP. COPD, chronic obstructive pulmonary disease.

CAP (20%) had another presumed etiological diagnosis: six patients had other bacteria isolated from sputum samples, including *Pseudomonas aeruginosa* (n = 3), *Haemophilus influenzae* (n = 2), *Serratia marcescens* (n = 1), and *Moraxella catarrhalis* (n = 1), and one patient had pneumonia related to urosepsis caused by *Enterococcus* species. Six patients were evaluated for *C. pneumoniae* and *M. pneumoniae* infection, and in nine cases, urinary antigen testing for *L. pneumophila* was done. Two patients had *C. pneumoniae* infection, as suggested

by serological reaction, but no cases of *M. pneumoniae* or *L. pneumophila* were identified. The microbiological etiology of CAP remained unknown for 18 patients (40%).

PCR amplification results. PCR amplification results were generated with blood samples from 44 patients with CAP; the control reactions failed for both blood samples from one patient who was excluded from further analysis. PCR amplification results generated from blood samples were positive for 12/18 (67%) of patients with pneumococcal pneumonia and for 10/13 (77%) of patients with *S. pneumoniae* bacteremia. Of the five patients with nonbacteremic pneumococcal pneumonia, two had positive PCR results. Both patients had received antibiotics <48 h before admission, while no other patient with pneumococcal pneumonia had received antibiotics prior to sampling. PCR results for all blood samples from patients with pneumonia of nonpneumococcal or unknown etiology were negative, resulting in a PCR specificity of 100% for the *S. pneumoniae* etiology of CAP.

BDL for prediction of *S. pneumoniae* infection. BDLs were determined for 43/44 patients with PCR amplification signals. Despite a positive amplification result, the BDL could not be determined for one patient with bacteremic pneumococcal pneumonia because of partial inhibition of PCR (a positive amplification result but with an inadequate inhibition control reaction); this patient was excluded from further quantitative analysis. The median BDL was 325 CFU equivalents/ml (range, <125 to 2,350 CFU equivalents/ml) for patients with pneumococcal pneumonia and <125 CFU equivalents/ml for those with pneumonia with another cause. ROC curves were generated for the BDL, WBC, and CRP values for the prediction of *S. pneumoniae* etiology of CAP and for the presence of *S. pneumoniae* bacteremia (Table 3). No difference was observed in the discriminative power for the BDL compared with those for the CRP level ($P = 0.3$) and the WBC ($P = 0.7$) for *S. pneumoniae* etiology and that for the BDL for prediction of *S. pneumoniae* bacteremia (P of 0.6 compared to the CRP level and the WBC). The PPV of the BDL for *S. pneumoniae* infection (100%) and for *S. pneumoniae* bacteremia (69%) were higher than those of the WBC (42 and 35%, respectively) and

TABLE 3. ROC curve values for prediction of *Streptococcus pneumoniae* infection and bacteremia in patients with CAP

ROC curve parameter ^a	Marker of infection values		
	BDL	WBC	CRP
<i>S. pneumoniae</i> infection			
AUC (95% CI)	0.82 (0.68–0.92)	0.79 (0.63–0.90)	0.72 (0.56–0.85)
Optimal cutoff value	125	13.3	187
Sensitivity (%)	65	82	65
Specificity (%)	100	62	77
PPV (%)	100	42	43
NPV (%)	90	91	87
<i>S. pneumoniae</i> bacteremia			
AUC (95% CI)	0.86 (0.72–0.95)	0.80 (0.65–0.91)	0.81 (0.66–0.91)
Optimal cutoff value	225	16.8	187
Sensitivity (%)	75	67	83
Specificity (%)	97	87	77
PPV (%)	69	35	23
NPV (%)	97	96	98

^a The units of measurement for the optimal cutoff values: BDL, CFU equivalents/ml; WBC, number of cells $\times 10^9$ /liter; CRP, mg/liter. 95% CI, 95% confidence interval.

FIG. 1. Distribution of the BDL in relation to the type of pneumonia. The P value reflects the Kruskal-Wallis analysis of variance among groups. The dashed line indicates the lower detection limit, equiv, equivalents.

the CRP level (43 and 23%, respectively); the NPV of these parameters were in the same range (approximately 90 and 97%).

Association of the BDL with clinical and laboratory variables. The *S. pneumoniae* BDLs were higher in patients with *S. pneumoniae* bacteremia compared to those in patients with nonbacteremic pneumococcal pneumonia and with pneumonia of another origin (Fig. 1). Presentation with SIRS upon admission was associated with a higher BDL ($P = 0.04$). Positive correlations were observed for the BDL with the CRP value ($r_s = 0.55$; $P < 0.001$) and with the WBC ($r_s = 0.43$; $P < 0.01$). In addition, a positive correlation was noted between the BDL and the length of stay ($r_s = 0.30$; $P < 0.05$). No other associations were observed between the BDL and the variables listed in Table 2. In patients with *S. pneumoniae* pneumonia, the BDL was associated with the length of stay ($r_s = 0.49$; $P = 0.04$), mental status alteration ($P = 0.04$), and CRP level ($r_s = 0.66$; $P < 0.001$). Finally, there was a tendency for higher BDLs to occur in patients with a PSI class higher than 2 ($P = 0.08$).

DISCUSSION

In this study, we showed that the *S. pneumoniae* BDL in blood samples is a diagnostic marker of pneumococcal infection and relates to the severity of disease in adult patients with CAP. We performed a quantitative analysis of *S. pneumoniae* DNA in blood samples, while previous studies have focused on the diagnostic concordance of PCR (DNA positive or negative) with blood culture results (7, 14, 23, 24, 31). The introduction of the BDL as a marker of infection enables clinical prediction of *S. pneumoniae* infection and helps gain insight into the clinical interpretation of a positive DNAemia. In our study, 77% of patients with bacteremia had *S. pneumoniae* DNA detected in whole-blood samples. This is a higher level of

sensitivity than that reported previously in adult CAP patients (i.e., around 40%) and in patients with invasive pneumococcal disease (69%), but it is lower than the level in a cohort of patients with suspected meningitis and/or septicemia (92%) (7, 23, 24). In another study, *S. pneumoniae* DNA was detected in all three patients with bacteremia in a cohort of 28 patients with CAP and in 3/5 patients with a positive urinary antigen test and negative blood culture (14). In our study, *S. pneumoniae* DNA was detected in 67% of patients with microbiologically confirmed pneumococcal pneumonia, which is higher than the level reported by Lorente et al. (55%) (16). In that study, a higher rate of PCR-positive results was observed in patients with bacteremia than in patients with nonbacteremic pneumonia. We had a relatively high proportion of CAP patients with bacteremia, which may have introduced some bias. This was partially overcome by using a corrected disease prevalence value in the calculation of the PPV and NPV of the ROC curves. The specificity of *S. pneumoniae* DNA in blood was 100% for pneumococcal etiology. As such, a positive *S. pneumoniae* DNAemia value in blood samples excluded other causes of CAP in our setting.

The BDL in our study was in concordance with the range of BDLs described in another study of adult patients with CAP and was higher than those reported for Malawian children with pneumonia (5, 14). The BDL had a good discriminative value for the *S. pneumoniae* origin of infection and for the presence of bacteremia. The values for the area under the curve (AUC) of the ROC curves for the BDL (0.82 and 0.86 during infection and during bacteremia, respectively) were higher than those of the WBC and CRP level. This was not statistically different, which might be due to our small study population and possibly relatively high discriminative values of the CRP level and the WBC in our study: a recent study by Müller et al. describes AUC values of 0.68 and 0.71 for the WBC and the CRP level, respectively, for the prediction of bacteremia (33/42 of pneumococcal origin) in 373 patients with CAP (17). The PPV of the BDL for the prediction of *S. pneumoniae* infection (100%) in our study were much higher than those of the WBC (42%) and the CRP level (48%), while the NPV were equally high for all three parameters. As such, the BDL is likely to add to the CRP level and the WBC in confirmation of the pneumococcal origin of pneumonia. This prompts treatment for pneumococcal infection regardless of the CRP level and the WBC results if the BDL is above the defined cutoff value. In this respect, the BDL also improves upon blood culture, because the BDL can be available within a few hours compared to 1 to 2 days for blood culture.

We observed a relationship between the BDL and several clinical and laboratory markers of the severity of disease, as follows: SIRS, mental status alteration upon admission, and the level of CRP. Also, the BDL tended to be higher in patients with invasive disease and in those with a PSI class higher than 2. This confirms earlier findings where a higher *S. pneumoniae* BDL in children with meningitis was reportedly compared to those in children with pneumonia, and the BDL in nonsurvivors was compared to that in survivors (5). In addition, another study reported an association of the *Neisseria meningitidis* BDL with more-severe disease in children with meningitis (12). Altogether, these data suggest a role for the testing of BDL in identification of patients with high risk for

severe or complicated disease. Possibly, the *S. pneumoniae* BDL can be used to support the choice between hospital admission and outpatient treatment of CAP patients. As such, a large prospective study is highly warranted to define the clinical application and benefit of the *S. pneumoniae* BDL in the treatment of patients with CAP. Monitoring of the course of the BDL during treatment is another interesting potential application (19, 21). Like the CRP level, the course of the BDL might be indicative of its response to treatment or the development of complicated disease (22). Although the kinetics of microbial DNA in blood are largely unknown, monitoring of the BDL under treatment certainly warrants further study. Important determinants for the potential application and clinical utility of the BDL will be the performance, turnaround time of the assay, logistics, organization of the laboratory services, and the costs.

In conclusion, a positive BDL value supports the diagnosis of *S. pneumoniae* infection in patients with CAP and should prompt targeted treatment, while a negative BDL value does not exclude *S. pneumoniae* infection. The BDL provides a putative quantitative marker for the severity of disease.

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