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ARTICLE

Pneumolysin polymerase chain reaction for diagnosis of pneumococcal pneumonia and empyema in children

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Abstract *Streptococcus pneumoniae* is the most important cause of childhood pneumonia and empyema, yet the diagnosis of pneumococcal infections by conventional methods is challenging. In this study, the clinical value of the pneumolysin-targeted real-time polymerase chain reaction (PCR) method for the diagnosis of pneumococcal pneumonia and empyema was evaluated with 33 whole blood samples and 12 pleural fluid samples. The analytical sensitivity of the PCR assay was 4 fg of pneumococcal DNA, corresponding to two genome equivalents of pneumococcal DNA per reaction. The PCR assay correctly detected all clinical isolates of *S. pneumoniae* tested, whereas all nonpneumococcal bacterial organisms tested were negative by PCR. In a clinical trial, *S. pneumoniae* was detected by PCR in the pleural fluid of 75% of children with empyema, increasing the detection rate of pneumococcus almost tenfold that of pleural fluid culture. However, in whole blood samples, PCR detected *S. pneumoniae* in only one child with pneumonia and one child with pneumococcal empyema and failed to detect *S. pneumoniae* in three children with blood cultures positive for *S. pneumoniae*. The present data indicate that pneumolysin-targeted real-time PCR of pleural fluid is a valuable method for the etiologic diagnosis of pneumococcal empyema in children. The ease and rapidity of the LightCycler technology (Roche Diagnostics, Mannheim, Germany) make real-time PCR an applicable tool for routine diagnostics. In the evaluation of blood samples, blood culture remains the superior method for the diagnosis of bacteremic pneumococcal disease.

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Introduction

Streptococcus pneumoniae is the most common causative bacterial pathogen of community-acquired pneumonia in children [1–4]. Pneumococcus is also the most common etiologic agent of childhood empyema, which has occurred with increasing incidence in Europe and in the USA over the last decade [5, 6]. Currently, the diagnosis of pneumococcal pneumonia and empyema using conventional methods is a challenging task. A definitive diagnosis requires the isolation of *S. pneumoniae* from normally sterile body sites such as blood or pleural fluid. However, blood cultures are positive in less than 10% of children with pneumonia [1, 2, 7], and pleural fluid cultures yield positive results in only 6–33% of children with empyema [5, 6, 8]. Other diagnostic tests, such as the detection of pneumococcal capsular antigens in urine, lack adequate diagnostic specificity in children [9, 10], and serological methods are not valid methods for routine clinical practice [11, 12].

Pneumolysin-based polymerase chain reaction (PCR) has shown promise in the detection of *S. pneumoniae* in clinical samples [13, 14]. In this study, the accuracy of pneumolysin-targeted real-time PCR for the detection of *S.*

pneumoniae was evaluated in whole blood and pleural fluid samples from children with pneumonia and children with pneumonia and empyema.

Patients and methods

Patients

The study enrolled children with radiologically verified pneumonia admitted to the Department of Pediatrics, Turku University Hospital, between April 2004 and January 2005, and children with pneumonia and empyema admitted to the Department of Pediatrics, Turku University Hospital, or to the Department of Pediatrics, Oulu University Hospital, between November 2003 and October 2005. In addition, children with a clinical picture suggestive of pneumococcal bacteremia without pneumonia were included in the study. Children with a clinically nonpneumococcal infection were used as controls. The study was approved by the Ethics Committee of the Hospital District of South-West Finland.

Blood and pleural fluid samples

Whole blood samples for PCR analysis were collected from children with pneumonia or suspected pneumococcal bacteremia without pneumonia and from control patients at presentation prior to the initiation of antibiotic therapy. The whole blood samples (0.5–3 ml) were collected in tubes containing EDTA in connection with the collection of peripheral blood samples for routine bacterial culture. Pleural fluid samples (0.5–3 ml) for PCR analysis and routine bacterial culture were collected from children with empyema in connection with diagnostic and therapeutic pleurocentesis within a median of 70 h after initiation of antibiotic treatment. In addition, three whole blood samples for PCR analysis were collected from children with empyema. The whole blood samples were collected within 0, 8, and 52 h after initiation of antibiotic treatment.

Bacterial strains

Genomic DNA from *S. pneumoniae* ATCC 6314D (American Type Culture Collection, Manassas, VA, USA) and 33 clinical isolates of *S. pneumoniae* was used to determine the in vitro sensitivity of the PCR assay. The clinical isolates of *S. pneumoniae* were isolated from children with invasive pneumococcal infection and identified using standard microbiological methods as described previously [15]. The specificity of the PCR assay was determined with 20 different clinical isolates of streptococci other than *S. pneumoniae* and with 17 other bacterial organisms. The clinical isolates used in this study were obtained from the National Public Health Institute, Turku, Finland.

DNA extraction

Pleural fluid samples (0.5–3 ml) were concentrated by centrifugation (13,000×*g*, 5 min), and 200 μl of the concentrate was used for DNA extraction. Before DNA extraction, additional incubation at 37°C for 15 min with 15 μl of lysozyme (10 mg/ml) was carried out for prober lysis of the pneumococcal cell wall. DNA from clinical samples and from clinical isolates of *S. pneumoniae* and other bacterial organisms was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions. The samples (200 μl) were processed using the protocol for whole blood. DNA from all clinical samples was extracted within a median of 21 h of sample collection.

PCR

A 206-bp fragment of the pneumolysin-encoding gene of *S. pneumoniae* was used as a target in the PCR assay. The oligonucleotide sequences for primers and fluorescent-labeled hybridization probes used in this study have been described in detail earlier [14]. PCR was performed using the LightCycler Instrument (Roche Diagnostics), in which PCR amplification and analysis occur simultaneously. Assays were carried out in LightCycler capillaries in a 20-μl reaction volume using the LightCycler FastStart DNA Master Hybridization Kit (Roche Diagnostics) as described earlier [14]. In brief, the reaction mixture contained 2 μl of LightCycler FastStart DNA Master Hybridization probe reaction mixture, 4 mM $MgCl_2$, 1 μM of each primer, 0.2 μM of each probe, and 2 μl of extracted DNA template. The PCR was run as follows: initial denaturation at 95°C for 10 min followed by 50 cycles of amplification, each consisting of 10 s of denaturation at 95°C, 15 s of annealing at 57°C, and 9 s of elongation at 72°C. The melting curve was analyzed at 95°C for 20 s, 40°C for 20 s, and 85°C for 0 s. Standard precautions were taken to avoid contamination [16], and pneumococcal DNA from a clinical isolate and sterile distilled water were used as positive and negative controls in each run. Inhibition of PCR was assessed by spiking 40 fg of *S. pneumoniae* DNA in the reaction tube before retesting of the PCR-negative samples.

PCR was used to study the presence of macrolide resistance determinants *mef* (A/E), *erm* (B), and *erm* (TR) in the pleural fluid samples. The PCR method has been described previously by Rantala et al. [15].

Results

Analytical sensitivity and specificity of PCR

The sensitivity of the PCR assay was evaluated with tenfold dilutions of genomic DNA from *S. pneumoniae* ATCC 6314D. The lower detection limit was found to be 4 fg (two genome equivalents) of pneumococcal DNA per reaction. To determine the sensitivity of PCR for detecting whole pneumococci, whole blood was spiked with *S. pneumoniae* bacteria, and PCR sensitivity was found to be 0.02 colony-forming units per reaction. The PCR assay correctly detected all 33 clinical isolates of *S. pneumoniae* tested, resulting in an in vitro sensitivity of 100%, whereas all 37 nonpneumococcal bacterial organisms tested, including three organisms closely resembling *S. pneumoniae*, i.e. *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguis*, were negative by PCR, giving an in vitro specificity of 100%.

Patient characteristics

In total, 30 whole blood samples from children with pneumonia and three whole blood samples and 12 pleural fluid samples from children with pneumonia and empyema were analyzed by *S. pneumoniae* PCR. Of the pneumonia patients, 21 had a clinical picture suggestive of pneumococcal pneumonia. The criteria for suspected pneumococcal pneumonia were alveolar consolidation on chest radiograph and at least two of the following findings: fever of ≥39°C, leukocyte count $\geq 15 \times 10^9$/l, serum C-reactive protein ≥80 mg/l, and response to penicillin within 48 h. The criteria for empyema were radiological evidence of empyema and purulent pleural fluid and/or positive pleural fluid bacteriology. The median age of children with pneumonia was 3.9 years (interquartile range, 2.4–7.0 years). The median age of children with empyema was 5.6 years (interquartile range, 2.6–8.7 years). Seventeen percent of children with pneumonia and 33% of children with empyema had received antibiotic treatment before admission. The median duration of fever in children with pneumonia or empyema before admission to the Department of Pediatrics was 2 days and 5 days, respectively.

In addition, 19 whole blood samples from children with a clinical picture of suspected pneumococcal bacteremia without pneumonia were analyzed using *S. pneumoniae* PCR. The criteria for suspected pneumococcemia were as follows: fever ≥39°C, leukocyte count $\geq 15 \times 10^9$/l, serum C-reactive protein ≥80 mg/l, and penicillin response within 48 h (three of the four criteria were met in all children). The median age of children with suspected pneumococcemia was 2.1 years (interquartile range, 1.1–3.0 years), and the median duration of fever before admission was 1 day. No child had received antibiotic treatment before referral to the Department of Pediatrics.

Whole blood samples from 19 children with a clinical nonpneumococcal infection were used as control specimens. The clinical diagnoses of control patients were pyelonephritis, tonsillitis, or gastroenteritis. The median age of control patients was 1.6 years (interquartile range, 0.9–4.6 years). Of the control patients, 11% had received antibiotic treatment before referral to the Department of Pediatrics, and the median duration of fever before referral was 3 days.

S. pneumoniae PCR of whole blood samples from children with pneumonia

Among the 30 children with radiologically verified pneumonia, blood culture and real-time PCR from whole blood identified *S. pneumoniae* in three children (Table 1). Blood culture was positive in two children and PCR from whole blood was positive in one child, all three having a clinical picture suggestive of pneumococcal pneumonia. Culture-positive blood samples were not PCR positive and vice versa. DNA from whole blood samples of the two children with positive blood cultures but negative PCR results was extracted within 22 h and 21 h. No PCR inhibitors were detected in these samples. When frozen pneumococcal bacterial isolates from these two children were tested by the PCR assay, the results were positive.

S. pneumoniae PCR of pleural fluid and whole blood samples from children with empyema

Among the 12 children with empyema, pleural fluid culture and real-time PCR from pleural fluid identified *S. pneumoniae* in nine children (Table 1). In addition, one child with a positive PCR result also had *S. pneumoniae* isolated from a bronchoalveolar lavage fluid culture. Of the three children with empyema who had whole blood samples analyzed by PCR, one had a positive result by PCR. Although blood culture in this child was negative, *S. pneumoniae* was isolated from pleural fluid by both culture and PCR. Prior antibiotic therapy was probably the reason for the negative result from blood culture in this child.

S. pneumoniae PCR of whole blood in children with clinically suggestive pneumococcal bacteremia without pneumonia and in control patients

S. pneumoniae was isolated from blood culture in 1 of the 19 children with a clinical picture suggestive of pneumococcemia (Table 1). The PCR result in this child was negative, as were all other whole blood samples tested. DNA from the whole blood sample of this child was

Table 1 Clinical diagnosis and positive laboratory findings in blood and pleural fluid specimens from patients with pneumonia, empyema, and suspected pneumococcal bacteremia

Patient no.	Clinical diagnosis	Leukocyte count (10^9/l)	CRP value (mg/l)	Blood culture[a]	Blood PCR[a]	Pleural fluid culture[b]	Pleural fluid PCR[b]	Macrolide resistance genes
1	Pneumonia	22.3	79	Pos.	Neg.	ND	ND	ND
2	Pneumonia	34.5	311	Pos.	Neg.	ND	ND	ND
3	Pneumonia	15.4	64	Neg.	Pos.	ND	ND	ND
4	Empyema	10.1	155	Pos.	ND	Neg.	Pos.	Neg.
5	Empyema	52.0	274	Neg.	Pos.	Pos.	Pos.	Neg.
6	Empyema	8.3	281	Neg.	ND	Neg.	Pos.	*mef* (A/E)
7	Empyema	19.6	158	Neg.	ND	Neg.	Pos.	Neg.
8	Empyema	20.6	452	Neg.	ND	Neg.	Pos.	Neg.
9	Empyema	17.9	253	Neg.	Neg.	Neg.	Pos.	*mef* (A/E)
10	Empyema	20.1	176	Neg.	Neg.	Neg.	Pos.	Neg.
11	Empyema	12.7	317	ND	ND	Neg.	Pos.	Neg.
12	Empyema	17.2	251	Neg.	ND	Neg.	Pos.	Neg.
13	Pneumococcemia	22.5	5	Pos.	Neg.	ND	ND	ND

ND not done
[a] Blood culture and PCR of whole blood in 30 patients with pneumonia and in 19 patients with suspected pneumococcal bacteremia without pneumonia; blood culture and PCR of whole blood in 11 and 3 empyema patients, respectively
[b] Culture and PCR of pleural fluid in 12 empyema patients

extracted within 21 h, and no PCR inhibitors were detected in the sample. When a frozen pneumococcal bacterial isolate from this child was analyzed using PCR, the result was positive.

All whole blood samples from control patients were negative by blood culture and by PCR.

Discussion

Recent studies suggest that up to 37–44% of childhood pneumonia cases are caused by *S. pneumoniae* [2, 13], but the etiology often remains unestablished in clinical practice. We carried out this study to determine whether pneumolysin PCR assay from whole blood and pleural fluid improves the clinical diagnosis of childhood pneumonia and empyema. The LightCycler Instrument was the chosen PCR method because of its rapidity and ease of use, making it applicable to routine diagnostics. The pneumolysin-targeted real-time PCR used in this study showed optimal in vitro sensitivity (100%) and specificity (100%) when tested with isolated strains. In a clinical trial, we found that *S. pneumoniae* was detected by PCR in the pleural fluid of 75% of children with empyema, increasing the detection rate of pneumococcus almost tenfold that of pleural fluid culture. However, PCR detected *S. pneumoniae* in whole blood samples in only one child with pneumonia and one child with pneumococcal empyema and failed to detect *S. pneumoniae* in three children with a positive blood culture result for *S. pneumoniae*.

Although empyema is a relatively rare complication of childhood pneumonia, it is always important to determine its etiology. First, because the incidence of empyema in children is increasing [5, 6], it is important to identify the pathogen(s) responsible for the increase. Second, a definitive diagnosis permits adequate antibiotic treatment, reducing inappropriate use of broad-spectrum antibiotics. The main finding of this study was that *S. pneumoniae* was detected in the pleural fluid of most children with empyema. Compared to pleural fluid culture, the PCR assay increased the *S. pneumoniae* detection rate from 8 to 75%. Our finding is in agreement with the results of Eastham et al. [6], who found evidence of *S. pneumoniae* infection by pneumolysin PCR in over 70% of culture-negative children with empyema. Using broad-range 16S rDNA PCR, Saglani et al. [17] also detected *S. pneumoniae* in 41% of children with empyema compared to 3% detected by culture. Whether these positive PCR findings represent true positive findings is difficult to determine, because collection of pleural fluid samples from healthy children for specificity studies is not possible. However, considering that all 37 nonpneumococcal bacterial organisms as well as all 19 whole blood samples from children with clinically nonpneumococcal infection tested were negative by PCR, specificity was clearly no problem in this study. In addition, the LightCycler technology minimizes carryover contamination from one sample to another during a PCR run, because the amplification and detection of PCR products are carried out in a closed system. The most probable reason for the failure of culture to detect *S. pneumoniae* in PCR-positive samples was the prior antibiotic treatment

that all study children with empyema had received before collection of pleural fluid samples.

Our data suggest that *S. pneumoniae* might be responsible for the increase in the incidence of childhood empyema. Similar results are reported by Byington et al. [5], who compared the incidence and microbiological etiology of childhood empyema during 1993 through 1999 in the USA. However, because our findings are based on the application of a method more sensitive than that previously used, more data are needed to make a definite conclusion. An interesting question is whether the increase in the incidence of childhood empyema is related to the increase in the antibiotic resistance of *S. pneumoniae*. While pleural fluid cultures are mainly negative in patients with empyema, the antibiotic resistance profile is difficult to define using conventional microbiological methods. By PCR, the determination of penicillin and macrolide susceptibility in patients with culture-negative but PCR-positive results is possible using penicillin-binding protein genes and macrolide resistance genes as a PCR target [18–20]. In studies from the USA and the UK, most *S. pneumoniae* isolates causing empyema were found to be penicillin sensitive [5, 6]. Because the macrolide resistance of *S. pneumoniae* is increasing and because macrolide-resistant pneumococcus can cause treatment failures [21, 22], macrolide resistance was studied in our empyema patients by multiplex PCR targeted to macrolide resistance genes *mef* (A/E), *erm* (B), and *erm* (TR). Macrolide-resistant *S. pneumoniae* was detected in 2 of the 12 (17%) study children with empyema. The macrolide resistance rate seen in our study is similar to that (23%) observed in the general pediatric population of our area in 2002 (personal communication, Merja Rantala).

PCR of whole blood samples was not useful in this study because it failed to detect *S. pneumoniae* in whole blood samples from three children with positive blood culture results, and, in total, *S. pneumoniae* was detected by PCR in whole blood from only 2 of the 52 children with pneumonia, empyema, or suspected pneumococcemia. The reason for the low clinical sensitivity of the pneumolysin PCR in whole blood samples in our study is not clear. Previous studies investigating the applicability of PCR of blood samples to the diagnosis of pneumococcal pneumonia in children and adults have shown clinical sensitivity and specificity ranges of 29–100% and 83–100%, respectively, compared with blood culture results [13, 23–28]. The in vitro sensitivity of the two genome equivalents of pneumococcal DNA per reaction seen in this study is similar to earlier reported sensitivities. The pneumolysin primers, DNA extraction method, and LightCycler technology employed in this study have been successfully used in studies of PCR used for the diagnosis of other pneumococcal infections [14, 29]. Furthermore, the DNA extracted from whole blood samples can be considered good quality because no significant delay occurred in DNA extraction and no inhibitory agents were detected in the samples.

The probable explanation for the low clinical sensitivity of PCR of whole blood samples simply seems to be the low bacterial concentrations in the blood in our patients. Sullivan et al. [30] as well as Bell et al. [31] found that the magnitude of *S. pneumoniae* bacteremia correlates with the severity of the infection. Children with pneumococcemia or pneumococcal pneumonia have low levels of bacteremia, usually <10 cfu/ml, whereas children with meningitis often have >100 cfu/ml. The level of bacteremia in 22 children with blood-culture-proven pneumococcal infection was studied at the Turku University Hospital from 1994 to May 1997, and 50% of the children were reported as having <10 bacteria/ml [26]. As only a small part of the original sample can be exploited in the PCR analysis, it is possible that with so few bacteria in the blood, no bacteria are present in the final PCR reaction. In our study, children with pneumonia or suspected pneumococcemia were referred to the hospital at a very early phase of the illness, were generally in good condition, and recovered rapidly and uneventfully. The severity of the infection and the level of bacteremia have therefore probably been very low in our study children. In a study by Michelow et al. [13], who reported 100% sensitivity of pneumolysin-based PCR in children with a lower respiratory tract infection, all study children were high-risk hospitalized children, indicating that the magnitude of bacteremia in these children was probably higher. The characteristics of our study children with clinically suggestive pneumococcal pneumonia, i.e. age, duration of fever before admission, laboratory findings, and outcome, were similar to those of children with proven bacteremic pneumococcal pneumonia in Finland during 1985 through 1994 [32], indicating that our patient material was representative. The use of different blood fractions, i.e. buffy coat, serum, and plasma, instead of whole blood as samples for PCR would probably have improved the sensitivity of our PCR, as Toikka et al. [26] and Michelow et al. [13] have previously shown in their studies of PCR used for the diagnosis of pneumococcal infections. However, the use of several blood fractions is laborious and expensive and therefore is not feasible for clinical diagnostics.

In conclusion, our data indicate that pneumolysin-targeted real-time PCR of pleural fluid is a promising method for the etiologic diagnosis of pneumococcal empyema in children. In our study, PCR increased the detection rate of *S. pneumoniae* almost tenfold that of pleural fluid culture. With the DNA extraction method and the LightCycler technology used in this study, PCR results are available in 3 h, permitting administration of adequate antibiotic therapy in early illness. However, the clinical

sensitivity of PCR in whole blood samples from children with pneumonia was insufficient in our patients. Therefore, blood culture remains superior to PCR in the clinical diagnosis of bacteremic pneumonia in children. To improve the clinical sensitivity of PCR in blood samples, further research is needed for the development of a method that concentrates bacteria without concentrating the excessive human DNA present in original samples. In fact, the level of bacteremia in pneumonia patients may be too low and transient for blood samples to be considered optimal diagnostic samples in pneumonia.

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