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Pneumolysin PCR-Based Diagnosis of Invasive Pneumococcal Infection in Children

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Blood-based pneumolysin PCR was compared to blood culture and detection of pneumolysin immune complexes, as well as to detection of antibodies to pneumolysin and to C polysaccharide, in the diagnosis of pneumococcal infection in 75 febrile children. Invasive pneumococcal infection was suspected on clinical grounds in 67 of the febrile children, and viral infection was suspected on clinical grounds in 8 of the febrile children. In addition, 15 healthy persons were examined to test the specificity of the PCR assay. Plasma, serum, and leukocyte fractions were analyzed by PCR. The combination of all test results led to the diagnosis of pneumococcal infection in 25 patients. Pneumolysin PCR was positive in 44% of these children, an increase occurred in the pneumolysin antibodies in 39% and in the C polysaccharide antibodies in 30% of the patients; pneumolysin immune complexes were found in convalescent serum in 30%, pneumolysin immune complexes occurred in acute-phase serum samples in 16%, and a positive blood culture was found in 20% of the patients. None of the healthy controls had positive results by PCR. The results suggest that the diagnosis of *Streptococcus pneumoniae* infection from blood samples necessitates the use of several different assays. Pneumolysin PCR was the most sensitive assay, but its clinical value is reduced by the fact that three blood fractions are needed.

Streptococcus pneumoniae is the predominant causative agent of childhood invasive bacterial infection in countries where infections caused by *Haemophilus influenzae* type b are eliminated by vaccinations (12, 24). The main clinical syndromes associated with invasive pneumococcal infection are occult bacteremia, pneumonia, meningitis, peritonitis, periorbital cellulitis, and septic arthritis (6, 7). One study suggests that if a child with occult pneumococcal bacteremia is not treated with antibiotics, there is a 6% risk for meningitis (2).

The differentiation of invasive pneumococcal infection from other febrile illnesses is difficult in the early phase of the disease. Children aged 3 to 36 months with fever of ≥39°C and a leukocyte (WBC) count of $\geq 15 \times 10^9$/liter should be suspected to have invasive bacterial infection (1, 9). These signs are, however, also common in children with viral infections (23).

A definitive diagnosis of invasive pneumococcal infection requires the isolation of *S. pneumoniae* from normally sterile sites such as the blood, lungs, pleural fluid, cerebrospinal fluid, or synovial fluid. Recently, antibody assays for *S. pneumoniae*, as well as measurement of circulating immune complexes, have proved useful in the study of the role of *S. pneumoniae* in the etiology of acute lower-respiratory-tract infections in young children (16, 20).

We compared pneumolysin PCR, blood culture, and detection of pneumolysin immune complexes, as well as of antibodies to pneumolysin and to C polysaccharide, for the diagnosis of invasive pneumococcal infection in febrile children.

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MATERIALS AND METHODS

Patients. Febrile children admitted during a 5-month period (beginning August 1996) to the Department of Pediatrics, Turku University Hospital, were enrolled in the study. The inclusion criteria were: a serum C-reactive protein (CRP) value of ≥100 mg/liter, a WBC count of $\geq 15 \times 10^9$/liter, or alveolar pneumonia. Sixty-nine patients fulfilled the criteria, and the final number of patients with suspected invasive pneumococcal infection was 67 after the exclusion of two patients with urinary tract infection. In addition, blood samples from eight febrile children with a virus-type infection (well-appearing children with a body temperature of <39.0°C, a CRP value of <80 mg/liter, and a WBC of $<15 \times 10^9$/liter) were included for comparison, and blood from 15 healthy persons was examined to test the specificity of the PCR assay.

Peripheral blood samples. Blood samples were obtained during routine diagnostic evaluation. In 89% of cases, the samples for PCR and the samples for detection of antibodies and immune complexes were taken within 24 h after admission. From each patient, 3 ml of blood was collected for the serum sample, and 2 to 9 ml (mean, 6 ml) of blood was collected in tubes containing EDTA. One milliliter of the EDTA blood was used for separation of the plasma, and the rest was diluted with Hank's buffered saline with sodium bicarbonate at a ratio of 1:1. The WBC fraction was separated from the diluted blood by density centrifugation (Ficoll; [Pharmacia Biotech, Uppsala, Sweden] and Histopaque 1119 [Sigma Diagnostics, St. Louis, Mo.]). The layers of mononuclear cells and granulocytes were aspirated and then washed with phosphate-buffered saline (400 g for 10 min) in a total volume of 40 ml.

Purification of DNA from WBC, plasma, and serum. The serum samples were stored at −20°C before isolation of DNA. DNA was isolated from plasma and WBCs within 1 h in 41% of cases and within 24 h in 75% of cases. The WBC fraction was centrifuged for 10 s, and the pellet was suspended with 200 μl of gamma-irradiated water. The WBC fraction was incubated for 10 min at 94°C before proteinase K (2 μl, 10 mg/ml; Boehringer Mannheim, Mannheim, Germany) treatment. After incubation for 1 h or overnight at 56°C, the same protocol was used for 200 μl of plasma, serum, and WBC. First, 300 μl of sodium dodecyl sulfate (SDS) containing 0.1 M NaOH, 2 M NaCl, and 0.5% SDS was added to the suspension, which was then incubated for 15 min at 95°C. Then, 200 μl of 0.1 M Tris-HCl (pH 8.0) was added. DNA was extracted with phenol, precipitated with ethanol, and dissolved in 25 μl of Tris-EDTA.

Pneumolysin PCR. The PCR amplifications were done with a programmable thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer, Norwalk, Conn.) in a 50-μl volume with pneumolysin primers (25). The outer primers Ia (5′-ATTTCTGTAACAGCTACCAACGA-3′) and Ib (5′-GAATTCCCTGTCTTTTCAAAGTC-3′) amplified a 348-bp region of the pneumolysin gene, and the inner primers IIa (5′-CCCACTTCTTCTTGCGGTTGA-3′) and IIb (5′-TGAGCCGTTATTTTTTCATACTG-3′) amplified a 208-bp region. The reaction mixture contained 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton

TABLE 1. Age, CRP values, WBC counts, and clinical diagnoses for patients with a positive pneumolysin PCR with peripheral blood specimens[a]

Patient	Age (yrs)	CRP value (mg/liter)	WBC count (10^9/liter)	Temp (°C)	Clinical diagnosis	PCR-positive blood fraction (agarose/Southern)[b]
1	3.0	182	20.9	37.5	Fever without infection focus	P/P, W
2	2.6	158	16.1	39.7	Pneumonia	W/W
3	1.4	205	13.3	40.8	Pneumonia	P/P
4	1.5	159	18.5	40.4	Meningitis with blood culture-confirmed *S. pneumoniae* septicemia	W/W
5	4.0	146	22.6	38.9	Acute tonsillitis	W/W
6	1.5	157	21.9	37.5	Pneumonia	P/P
7	2.5	121	26.4	40.3	Pneumococcemia	S/S
8	3.8	105	26.3	39.8	Fever without infection focus	S/S
9	2.2	127	20.8	39.2	Pneumonia	S/S
10	2.9	143	23.1	40.4	Pneumonia	Negative/W
11	1.1	32	17.3	38.0	Pneumonia	Negative/P
Mean	2.4	140	20.7	39.3		

[a] CRP values, WBC counts, and temperatures are the highest values. P, plasma; W, WBC fraction; S, serum.
[b] Results are presented as follows: by agarose gel electrophoresis/by Southern hybridization.

X-100, 200 μM deoxyribonucleotides, 50 pmol of primers, 1.0 U of DNA polymerase (Dynazyme; Finnzymes, Espoo, Finland), a drop of mineral oil, and various amounts of DNA (1:1 and 1:10 dilutions) extracted from WBC fraction, plasma, and serum specimens (5 μl). The amplifications were repeated 40 times as follows: 30 s at 94°C for denaturation, 30 s at 56°C for annealing, and 30 s at 72°C for extension. Nested amplifications were carried out as for the first-round PCR. A pneumococcal DNA preparation (ATCC 49619) was used as a positive control. At every amplification, a negative control was included. The PCR products were stored at 4°C prior to analysis by agarose gel electrophoresis.

A 10-μl volume of the PCR product was separated by using 1.5% agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. DNA was transferred to a nylon membrane (Hybond-N+; Amersham) with 0.4 M NaOH by Southern blotting. The membrane was prehybridized for 15 min at 42°C in a solution containing 15% formamide, 1% SDS, 20% dextran sulfate, 58 g of NaCl per liter, and 1% blocking reagents. Hybridization was carried out for 3 h at 42°C with a radioactively labeled ([^{32}P]ATP) oligonucleotide probe (5′-TTGGAGAAAGCTATCGCTACTTGC-3′). The membrane was washed three times: first with 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) at room temperature for 5 min, then at 42°C with 2× SSC–1% SDS for 30 min, and finally at room temperature with 0.1× SSC for 30 min. Autoradiography was carried out overnight at −70°C.

Blood culture. Blood samples for cultures were obtained prior to the initiation of antimicrobial therapy. The blood culture bottles (Dupont Isolator system; Merck, Darmstadt, Germany) were inoculated with blood obtained by peripheral venipuncture from 66 children upon admission; 59 of the 67 patients in the study group and 7 of the 8 children in the comparison group were tested.

Determination of antibodies to pneumolysin and to C polysaccharide and pneumolysin immune complexes. Paired serum specimens were obtained from 54 patients: 50 of the 67 patients in the study group and 4 of the 8 patients in the comparison group. The acute-phase samples were drawn from all patients at the first visit, and the convalescent-phase samples were collected 34 ± 20 (mean ± standard deviation [SD]) days later. These samples were used for the determination of pneumolysin antibodies, C polysaccharide antibodies, and pneumolysin immune complexes. A twofold or greater rise in antibody titer to pneumolysin or C polysaccharide in paired sera or the presence of pneumolysin-specific immune complexes in any serum sample were the serological criteria used to determine the presence of pneumococcal infection (13, 16, 18).

RESULTS

In vitro sensitivity of PCR. The sensitivity of the PCR was evaluated by using pneumococcal DNA as a target at 10-fold dilutions. Both in the first amplification round and in the nested PCR, sensitivity was 90 fg as detected by visualization of PCR products of the expected 208-bp length by agarose gel electrophoresis. Southern hybridization of the PCR products caused a 10-fold increase in the sensitivity of the PCR reactions. When whole pneumococci were used as a target in serial dilutions, the sensitivity of the assay was 10 CFU and 1 CFU after Southern hybridization of the PCR product.

Patient characteristics. The clinical diagnosis and numbers of pediatric patients fulfilling the study inclusion criteria (n = 67) were as follows: pneumonia, 39; fever without infection focus, 16; acute respiratory tract infection, 5; pneumococcemia, 2; meningitis with blood culture confirmed *S. pneumoniae* septicemia, 1; periorbital cellulitis with blood culture confirmed *S. pneumoniae* septicemia, 1; periorbital cellulitis, 1; *Bacteroides fragilis* septicemia, 1; and acute tonsillitis, 1. All patients were treated with antibiotics. The patients in the comparison group (n = 8) received the following diagnoses: interstitial pneumonia, 4 patients; acute respiratory tract infection, 2 patients; parotitis, 1 patient; and aseptic meningitis, 1 patient.

The age of the children was 4.3 ± 3.7 years in the study group and 4.8 ± 3.1 years in the comparison group (mean ± SD). The highest CRP values were 130 ± 68 and 27 ± 24 mg/liter, the highest WBC count values were 22.2×10^9 to $\pm 8.0 \times$ and 7.6×10^9 to $\pm 3.2 \times 10^9$/liter, and the highest body temperatures were 39.0 ± 1.1 and 38.6 ± 1.2°C (mean ± SD), respectively.

PCR-positive peripheral blood specimens. There were 12 PCR-positive peripheral blood samples. Nine were found by visualization of the PCR products by agarose gel electrophoresis: three from the WBC fraction, 3 from the plasma samples, and three from the serum samples (Table 1). All positive results were confirmed by Southern hybridization. In addition, three samples were positive after Southern hybridization of the PCR product. Two of these were positive WBC fractions, and one was a positive plasma sample. In one patient, the infection was diagnosed from the plasma sample by agarose gel electrophoresis and, additionally, from the WBC fraction by Southern hybridization. Thus, 11 patients were found to have positive samples by PCR. None of the patient samples from the comparison group and none of the samples of the healthy controls were found to be positive by PCR.

Blood culture-positive specimens. *S. pneumoniae* was isolated from blood culture in four patients. The densities of bacteria in the four cultures were <1, 1, 2, and 200 bacteria/ml. The first case was diagnosed by pneumolysin PCR, as well as by an increase in pneumolysin and C polysaccharide antibodies. The second case was not positive by any other method. The third case was determined to be positive by detection of pneumolysin immune complexes in the convalescent serum, and the

TABLE 2. Positive results by the pneumolysin PCR, blood culture, and pneumolysin immune complexes or diagnostic increases in pneumolysin or C polysaccharide titers and the clinical diagnosis of patients[a]

Patient	PCR	Blood culture	Ply IC		Ply Ab	Cps Ab	Clinical diagnosis
			Acute	Convalescent			
1	+	+			+	+	Pneumococcemia
2	+	ND		+	+		Pneumonia
3	+				+	+	Fever without infection focus
4	+	+					Meningitis*
5	+				+		Pneumonia
6	+			ND	ND	ND	Fever without infection focus
7	+	ND					Pneumonia
8	+						Pneumonia
9	+						Pneumonia
10	+						Pneumonia
11	+						Acute tonsillitis
12		+		+			Periorbital cellulitis*
13		+		ND	ND	ND	Pneumococcemia
14		ND	+	+			Pneumonia
15			+			+	Pneumonia
16			+	+			Pneumonia
17			+				Fever without infection focus
18		ND		+	+		Febrile seizures
19				+			Pneumonia
20				+			Pneumonia
21		ND			+	+	Pneumonia
22					+	+	Fever without infection focus
23					+	+	Fever without infection focus
24					+		Pneumonia
25						+	Pneumonia
N	11	4	4	7	9	7	

[a] Results are negative unless otherwise stated. PCR, polymerase chain reaction; Ply IC, pneumolysin immune complexes; Ply Ab, pneumolysin antibodies; Cps Ab, C polysaccharide antibodies; ND, not done; N, number of patients with positive results; *, with blood culture-confirmed *S. pneumoniae* septicemia.

fourth case was found to be positive by pneumolysin PCR. The two patients with negative PCR results had been on antibiotic treatment for 2 and 5 days before the blood samples for PCR were taken, and the two patients with positive PCR results had been on antibiotic treatment for 1 day.

Positive results in pneumolysin immune complexes or diagnostic increases in pneumolysin or C polysaccharide antibody titers. Pneumococcal infection was diagnosed from an increase in pneumolysin or C polysaccharide antibodies or by the presence of pneumolysin immune complexes in 19 patients. Four of these were also diagnosed by PCR, and two were diagnosed by blood culture (Table 2). None of the patients with pneumolysin immune complexes in their acute-phase serum samples had positive results by pneumolysin PCR or diagnostic increases in pneumolysin antibody titers. Four patients had pneumolysin immune complexes in their sera both in the acute and the convalescent phases. Two of these children were from the comparison group with interstitial pneumonia with a WBC count of $<15 \times 10^9$/liter and a CRP value of <80 mg/liter.

DISCUSSION

This study shows that the diagnosis of invasive pneumococcal infection in children necessitates the use of a combination of several tests. Pneumococcal infection was diagnosed in 25 of the patients in this study group and in 2 patients in the comparison group. Of the 25, 44% could be diagnosed by pneumolysin PCR, 39% by an increase in pneumolysin antibodies, 30% by an increase in C polysaccharide antibodies, 30% by the presence of pneumolysin immune complexes in convalescent-phase serum samples, 20% by blood culture, and 16% by the presence of pneumolysin immune complexes in acute-phase serum samples.

Only a few studies have been published in which PCR was used to diagnose invasive pneumococcal infection. Rudolph et al. (21) studied 16 adults with culture-proven pneumococcal bacteremia by using nested PCR, with primers designed from pneumolysin and autolysin genes. In vitro sensitivity was 10 fg or 200 CFU for whole bacteria and 20 CFU for buffy-coat samples. The sensitivity of the assay when buffy-coat fraction samples from eight blood culture-positive patients were studied was 75% with pneumolysin and 63% with autolysin primers. When eight whole-blood specimens were tested, the sensitivity was 37.5% with either set of primers, and the specificity of the assay was 93%. In another study from Gambia, 25 adults with suspected pneumonia were examined by PCR also with primers detecting the autolysin gene sequence (10). In vitro sensitivity was 50 fg or 3 CFU. The samples were first cultured and the DNA for PCR analysis was extracted from blood culture bottles either 48 h after inoculation, if positive growth was recorded, or after 7 days, when the culture bottles were discarded. *S. pneumoniae* was isolated from blood cultures in 12 patients. In four patients, PCR was positive with supernatants from both paired culture bottles, whereas pneumococci were cultured from only one. Salo and coworkers (25) studied 20 serum samples from adult patients with blood culture-confirmed acute pneumococcal pneumonia. These authors used a nested PCR method with primers similar to those used in the present study designed from the pneumolysin gene. The in vitro sensitivity was 24 fg (10 bacterial equivalents). All 20 samples were positive by PCR. To assess clinical specificity, 100 serum specimens from healthy adults were tested and 94 were found to be negative (specificity, 94%). Zhang and coworkers (26) used whole-blood PCR to study 36 pediatric patients with suspected bacteremia. Their primers and probe were derived from the penicillin-binding protein 2B gene. The in vitro sensitivity was 100 fg or 1 CFU. Four of five blood culture-positive patients were diagnosed, as well as five additional cases from the 31 culture-negative samples. Dagan and coworkers (4) undertook a prospective study to evaluate the accuracy of pneumolysin PCR of serum for the detection of pneumococcal infections in children. The in vitro sensitivity of the pneumolysin PCR assay was 10 CFU, whereas the clinical sensitivity of blood and cerebrospinal fluid culture-positive samples from 13 patients was 100%. The positivity rates for patients with lobar or segmental pneumonia or acute otitis media and for healthy controls were 38, 44, and 17%, respectively. The results indicated that although pneumolysin PCR was sensitive, it was not very useful for the detection of deep-seated pneumococcal infections because a high rate of positivity was seen in the controls. It should be noted that the positive results by agarose gel electrophoresis in the present study were not confirmed by Southern hybridization. In our study, 69 serum, plasma, or WBC fraction samples from patients with a virus-type infection or from healthy controls were tested by PCR, and none of them were found to be positive, indicating a good specificity and a lack of contamination in our PCR procedure. Our problem was sensitivity rather than specificity, because we found only 12 PCR-positive samples among the 201 serum, plasma, or WBC samples tested.

Several factors may limit the sensitivity of PCR with samples from the peripheral blood in a clinical setting. First, the density of *S. pneumoniae* in the bloodstream is often low. In the Turku University Hospital, *S. pneumoniae* was isolated from blood cultures of 22 children during 1994 to May 1997. Of these, 23% had <1 bacteria/ml, 27% had 1 to 9 bacteria/ml, 23% had 10 to 100 bacteria/ml, and 28% had >100 bacteria/ml. This indicates that the sensitivity of the PCR may not be high enough for all blood culture-positive cases. The second limitation of PCR with blood specimens is the inhibition of DNA polymerase by porphyrin compounds (11). This decreases the sensitivity of the PCR in clinical samples compared to in vitro conditions. Moreover, antibiotic treatment before sampling decreases the yield of positive findings by PCR. Dagan and coworkers did not detect pneumococcal DNA in the serum 48 h after the initiation of antibiotic treatment (4). In our study, the two blood culture-positive patients who were pneumolysin PCR negative had been on antibiotic treatment for 2 and 5 days before the samples for PCR were taken.

Blood culture is only seldom positive in children with pneumococcal pneumonia in developed countries (3, 8, 22). Therefore, various antibody assays and the detection of pneumococcal immune complexes have been used to study the etiology of pneumonia. The diagnostic methods have shown the following sensitivities in children: pneumococcal antigen in acute serum, 33 to 39%; antibodies to type-specific capsular polysaccharides, 32 to 37%; pneumolysin antibodies, 7 to 30%; C polysaccharide antibodies, 12 to 15%; and pneumococcal antigen in an acute-phase urine sample, 2 to 5% (14, 15). Korppi and Leinonen (16) found diagnostic levels of immune complexes in nearly one-half of the pneumococcal pneumonia cases diagnosed by antigen, free antibody, or circulating immune complexes. The assays, also used in the present study, have been validated in healthy children and in young adults with a common cold; a positive result or a diagnostic rise in titers between paired sera have been present in <1% by the above criteria also used in this study (17, 18, 19).

Our study may well underestimate the value of blood culture in the detection of invasive pneumococcal infection. Isaacman et al. (12) found that a single small-volume blood culture fails to identify a significant proportion of children with bacteremia. Differences occur between blood culture methods used to detect bacteremia and fungemia. The isolator system used in our hospital has been found to be less sensitive than the Bactec system for the detection of bacteremia (5, 12). Our study is also limited by the fact that the samples for pneumolysin PCR and the samples for the detection of antibodies and immune complexes were not regularly taken before antibiotic treatment, as were the samples for blood cultures. Therefore, the results of the different assays are not fully comparable. None of the samples from patients in the comparison group or from controls were positive by pneumolysin PCR. However, pneumolysin PCR was positive with the WBC fraction of a 4-year-old girl with unilateral tonsillitis. Although this patient may have had invasive pneumococcal infection, we cannot exclude the possibility that this PCR result could also be a false-positive finding, especially because none of the other pneumococcal tests confirmed the pneumococcal etiology.

The recent apparent increase of penicillin-resistant pneumococcal infections has emphasized the necessity of accurate clinical diagnosis and laboratory confirmation of pneumococcal infections. In this study, several diagnostic methods, including blood culture, detection of pneumolysin immune complexes and antibodies to pneumolysin and to C polysaccharide, and pneumolysin PCR, were compared in the diagnosis of invasive pneumococcal infection. We conclude that a combination of several methods is needed for the detection for invasive pneumococcal infection. Pneumolysin PCR was the most sensitive assay tested and increased the number of patients with a microbiological diagnosis of pneumococcal infection. However, for optimal sensitivity, several blood fractions should be tested and this is laborious and expensive. We conclude that pneumolysin PCR is currently not a feasible method for routine diagnostics of invasive pneumococcal infection in children.

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