

Invasive Pneumococcal Disease in Children: The Contribution of Real-Time PCR on Blood

Research Article

Volume 2 Issue 2 - 2015

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Received: March 10, 2015 | Published: April 09, 2015

Abstract

Objectives: This study aimed to determine the benefits of a real-time PCR test specific for *S. pneumoniae* performed directly on blood samples in children with suspected invasive pneumococcal disease (IPD).

Methods: We performed real-time PCR of the *ply* and *lytA* genes on blood samples from children with a suspicion of IPD, taken early after admission.

Results: We prospectively enrolled 76 children suspected of IPD over a 6-month period. Out of the 76 patients, 5 IPD were confirmed with two positive blood cultures and three positive pleural cultures for *S. pneumoniae*. Using specific real-time PCR on blood samples, eight pneumococcal infections were positive allowing the identification of five more cases of IPD than standard methods alone, including three of the four cases of pneumonia with pleurisy. The overall sensitivity of real-time PCR tests on blood samples was 80 % and the specificity was 98%.

Conclusion: Our study demonstrates the value of real-time PCR especially in case of pleurisy.

Keywords: *Streptococcus pneumoniae*; Invasive pneumococcal disease; Real-time PCR; *lytA*; Pediatric

Abbreviations: IPD: Invasive Pneumococcal Diseases; CRP: C-Reactive Protein; PCT: Procalcitonin

Introduction

Streptococcus pneumoniae is responsible for about 1 million childhood deaths per year worldwide [1]. Invasive pneumococcal diseases (IPD) are infections that occur in normally sterile sites such as the meninges, blood or pleura. The incidence of pneumococcal bacteremia varies with age but is higher at the extremes of life; for children aged up to four years it is between 13.5 and 20 per 100,000 with a mortality rate of up to 24% [2,3]. Nevertheless, many of these infections can be occult [1].

The diagnosis of IPD is usually obtained by cultures from blood, pleural fluid, cerebro-spinal fluid, or from other normally sterile sites. Culture methods can be disappointing and they often require at least 24 to 48 h to be detected as positive. Furthermore, empirical antibiotic treatments also underlie the difficulties in pneumococcal isolation. Detection of pneumococcal antigen in the urine or pleural fluid is another diagnostic method [4]. The value of this test is therefore particularly controversial in children [5,6].

More recently, molecular methods, including real-time PCR, have been developed to improve the evaluation of the incidence of IPD. Real-time PCR is theoretically useful for the diagnosis of IPD. However, these techniques are not in routine use for diagnosing pneumococcal infections and must be evaluated to ascertain their value among the tools used for clinical diagnoses especially when PCR is performed from blood samples [7]. PCR-based methods

usually target the *ply* gene encoding pneumolysin and the *lytA* gene encoding autolysin.

The aim of the present study was to evaluate the benefits of specific *S. pneumoniae* real-time PCR targeting the *ply* and *lytA* genes directly applied to blood samples from suspected IPD patients in comparison with standard microbiological methods.

Materials and Methods

Patients

The study included children aged from 0 to 18 years and who were suspected of suffering from pneumococcal infections, such as IPD-like meningitis, bacteriemia, pneumonia with pleurisy or who were hospitalized for pneumonia or isolated fever in children aged under 2 years. Cases of non-hospitalized pneumonia and otitis were excluded. During the study, invasive pneumococcal disease was defined as one of the above-mentioned diseases with the presence of *S. pneumoniae* in blood, CSF or pleural fluids confirmed by culture. Recent clinical history, previous vaccinations, and any underlying disease were recorded.

Study design

The study was an observational, prospective study designed to evaluate real-time PCR for the diagnosis of pneumococcal disease in a pediatric population. The pediatric study population was hospitalized in Tours university hospital or in the pediatric units at Orleans hospital. The study was performed over a 6-months period. A letter providing information about the study was given

to the parents. Verbal consent was obtained from the parents, although only specimens required for routine investigations were collected. Data on the children aged 0 to 18 years were provided. Pneumococcal vaccination, antibiotics during the last seven days, clinical symptoms, treatment and disease progress were recorded.

Biological specimens and clinical classification

Biological samples were obtained as soon as possible after hospital admission and were tested for biochemical parameters, and among them C-reactive protein (CRP), procalcitonin (PCT) and complete blood count. For bacterial purpose, blood cultures were performed for all patients with at least a BD Bactec Peds Plus Aerobic/F® vial and a BD Bactec Lytic Anaerobic/F® vials (BD Diagnostics, Germany). Pleural fluids were sampled in case of pleurisy for culture and molecular testing by real-time PCR. Nasopharyngeal aspirates were performed to screen for respiratory viruses.

To evaluate the benefits of real-time PCR on blood samples, patients were classified into four categories according to their probability of pneumococcal infections. These were: confirmed, probable, possible, and unlikely. A pneumococcal infection was confirmed if *S. pneumoniae* was isolated by culture of blood, CSF, or pleural fluid. Probable pneumococcal infection was considered if more than four of the following clinical features were present: fever >39° C, rapid clinical onset of symptoms, lobar pneumonia, WBC>15000/3mm, C-reactive protein> 40mg/L, or procalcitonin >0.5ng/L. Pneumococcal infection was considered as possible if less than four of the previous conditions were fulfilled without other differential diagnoses. If another infectious agent was identified and when no criteria indicated classification into the three previous categories, pneumococcal infection was considered to be unlikely. Clinical categories were reassessed after real-time PCR analyses.

Real-time PCR

Later on, real-time PCR analysis was performed and the treating physician was blinded to the results. DNA was extracted from whole blood using the Invisorb® kit (Invitek, Germany) in accordance with the manufacturer's instructions.

Two genes *ply* and *lytA* were targeted using primers *plyF/plyR* and *lytACDCf/lytACDCr* respectively. The primers for *ply* and *lytA* were previously described by Corless et al. and by Carvalho et al. respectively [8,9]. Amplifications were performed in a Smart Cycler® (Instrumentation Laboratory) with the final reaction mix containing 5 µL of DNA, 12.5 µL of Premix Ex Taq (TaKaRa®, Foster city, USA), 4.4 µL of water, 2.5 µL of SYBR® Green and 0.3 µL of each primer. The *S. pneumoniae* positive DNA control was from the reference strain R6 and was also included with each set of amplifications [10]. Thermal cycling conditions for *ply* and *lytA* were as follows: 1 cycle of 10s at 95°C followed by 40 cycles of 5s at 95°C and 20s at 60°C. Amplification of beta-globin gene was also performed to detect false-negative reactions. Discrepant results between the two targets were verified in another independent assay. If the discrepancy was confirmed, the amplified product was sequenced for confirmation using a BigDye® terminator v 3.1 cycle sequencing kit (Applied Biosystems). Sequencing analysis 5.1.1® software (Applied Biosystems) was used to analyse the DNA sequence. BLAST® software was used to compare the DNA sequence with sequences published in the National Center for

Biotechnology Information database.

Statistical analysis

The groups with positive or negative real-time PCR results were compared using the Chi2 test and p-values less than 0.05 were considered as statistically significant. The groups were analyzed with Kruskal-Wallis test. All continuous values were expressed mean+/-SD, with ranges given in parenthesis.

Results

Child population

Seventy-six children (38 male, 38 female) ranging from 17 days to 12 years were included into the study over a 6 month period. Thirty-six children (47.3%) were under two years of age. Six children out of 76, presented with risk factors of invasive pneumococcal disease; two had drepanocytosis, one had chronic adrenal gland insufficiency, and three had asthma treated with cortico-steroids. Seven children had a viral infection in the previous 15 days before the infection. Therefore, 17.1% of children (13/76) had a risk factor for IPD. Concerning vaccinations, 37 children had received Prevenar® and 3 had received Pneumo23® (52.6%). During the study, 20/76 children (26.3%) received antibiotics during the last seven days, including beta-lactams, amoxicillin (9/76, 11.8%), and amoxicillin-clavulanate (4/76, 5.6%). Other antibiotics that were used included phenoxymethylpenicillin in two cases of drepanocytosis and cefpodoxime in 3 children (2.7%). Only 2 children received macrolids (clarithromycin and josamycin).

Child population according to probability of pneumococcal disease

In the study population, blood cultures were performed for all children, pleural fluid was sampled in 12 children (15.8%) and respiratory samples were taken from 15 children (19.7%). Five children had confirmed pneumococcal infection based on pneumococcal culture of normally sterile sites. Among the patients with pneumonia, 3 had positive cultures of pleural fluid, although blood cultures were negative. Two blood cultures were positive in one case of lobar pneumonia and one case of pneumonia with pleurisy. In the latter, culture of pleural fluid was negative. The sensitivity of blood cultures among confirmed infections was therefore 2 out of 5.

Thirty-eight infections were considered as probable (50% of the studied population) before molecular analysis. Twenty-four of these (63% of probable infections) were cases of pneumonia. Two of the pneumonia cases were associated with a pleural effusion of less than 2 cm. Five of the pneumonia cases were associated with pleurisy. Twenty-five children (32.9%) in the study population initially presented with possible pneumococcal disease. The final diagnoses of these patients were nine cases of pneumonia (36%), two pharyngitis (2.6%), five cases of RSV bronchiolitis (6.6%), one case of metapneumovirus bronchiolitis (1.3%), five cases of infection considered as viral (6.6%), two cases of asthma (2.6%), and one case of macrophage-activation syndrome (1.3%).

Eight children (10.5%) had an unlikely *S. pneumoniae* infection. In these, another bacterium or another cause was identified. Among them, one child had pneumonia with pleurisy and positive IgA for *Mycoplasma pneumoniae*. Other diagnoses were

lymphoma, pneumonia due to *Haemophilus influenzae*, two cases of pyelonephritis due to *E. coli*, a case of pneumonia with pleurisy due to *S. aureus*, and a case of *Streptococcus pyogenes* bacteriemia.

Results of real-time PCR analysis in comparison to the degree of certitude of pneumococcal disease

During the study, real-time PCR targeting the *ply* and *lytA* genes was performed on blood samples from 76 children. Results are presented in Table 1. Among the five confirmed infections, the sensitivity of real-time PCR was 60% (3/5). However, two of the positive blood cultures gave a negative PCR. The three children

with a positive PCR test had pneumonia with pleurisy (Table 2). Among the 38 probable infections, five children had positive real-time PCR tests. Of these, four had pneumonia with pleurisy, and one had pneumonia with a pleural effusion of less than 2 cm (Table 2). Amplification of the *lytA* gene agreed with the results for the *ply* gene except for one case of pneumopathy. Twenty-five of the possible pneumococcal infections were negative on real-time PCR tests for the *ply* and *lytA* genes. Among the unlikely infections, 7/8 had a negative real-time PCR test. The one positive PCR test for the *ply* gene was seen in a child with a positive bacteriemia due to *S. pyogenes*.

Table 1: Bacteriological and real-time PCR test results on blood samples according to the degree of probability of pneumococcal disease defined by non-molecular methods.

Probability of pneumococcal disease (N° of cases)	Positive blood culture (Nb tested)	Positive pleural culture (Nb tested)	Positive blood PCR targeting <i>ply</i> gene	Positive blood PCR targeting <i>lytA</i> gene
Confirmed (5)	2 (5)	3 (4)	3	3
Probable (38)	- (38)	- (5)	6	5
Possible (25)	- (25)	-	-	-
Unlikely (8)	- (8)	- (3)	1	-
Total (76)	2 (70)	3 (12)	10	8

Table 2: Clinical and biological features of the patients with confirmed invasive pneumococcal infections (cases 1 to 5) and with probable pneumococcal infection (cases 6 to 10) presenting with a positive real-time PCR test result for *Streptococcus pneumoniae* in blood.

Cases	1	2	3	4	5	6	7	8	9	10
Age	4 years 9 months	4 years 3 months	7 years	11 years	10 years 6 months	2 years 10 months	2 years 5 months	4 years 10 months	5 years 5 months	5 years 8 months
Clinical symptoms	pneumonia with pleurisy	pneumonia with pleurisy	pneumonia with pleurisy	pneumonia	pneumonia and secondary pleurisy	pneumonia with pleurisy	pneumonia with pleurisy	pneumonia and reactional pleural effusion	pneumonia with pleurisy	pneumonia with pleurisy
WBC/mm ³ (neutrophils polynuclear)	21500/mm ³ (18060/mm ³)	21200/mm ³ (16670/mm ³)	16400/mm ³ (14550/mm ³)	26960/mm ³ (21790/mm ³)	19000/mm ³ (17290/mm ³)	11300/mm ³ (8810/mm ³)	36200/mm ³ (23890/mm ³)	16500/mm ³ (13200/mm ³)	13200/mm ³ (10940/mm ³)	4500/mm ³ (2480/mm ³)
C-reactive protein (mg/L)	331.6	225	238	277	328	309.6	257.6	395.9	256	293
Procalcitonin (ng/L)	7.4	4.2	9.9	156.7	7	21.2	5.6	21.8	10	42.5
Blood cultures	Sterile	Sterile	Sterile	<i>S. pneumoniae</i>	<i>S. pneumoniae</i>	Sterile	Sterile	Sterile	Sterile	Sterile
Pleural fluid culture	<i>S. pneumoniae</i>	<i>S. pneumoniae</i>	<i>S. pneumoniae</i>	Not done	Not done	Sterile	Sterile	Not done	Sterile	Sterile
Blood real-time PCR	Positive	Positive	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Positive
Antibiotics before PCR	Yes Amoxicillin + clavulanic acid for 2 days	Yes Amoxicillin for 3 days	No	No	No	No	Yes (amoxicillin for 3 days before hospitalization)	No	No	No

These findings demonstrate that real-time PCR tests for *S. pneumoniae* in blood samples combined with blood-culture tests improves the diagnosis of IPD with a sensitivity of 76.9% and a specificity of 95.6%. In children with confirmed pneumococcal disease or positive PCR tests, the median level of CRP was 276.3 mg/L (82-395.9) and the median level of PCT was 8.65 ng/mL (0.14-156.7). CRP and PCT levels were significantly higher in the groups with confirmed infection or positive real-time PCR tests than in the other group ($p < 0.0001$ and $p < 0.007$, respectively).

Results of real-time PCR in cases of presumed pneumococcal pneumonia with pleurisy

Of the nine patients with presumed pneumococcal pneumonia with pleurisy, seven had positive real-time PCR tests. The sensitivity of bacteriological identification was therefore 77.8% for real-time PCR versus 44.4% for cultures of blood or pleural fluids (four out of nine patients positive). For real-time PCR, the positive predictive value was 78.8% versus 44.4% for blood/pleural fluid culture. Similarly the negative predictive value for real-time PCR was 75% versus 42.3% for blood/pleural fluid culture.

Discussion

Real-time PCR on blood samples offers an opportunity to readdress the problem of diagnosing infections due to *S. pneumoniae*. The main objective of our study was to determine the clinical value of real-time PCR on blood samples in routine practice. According to the classification based on probability of pneumococcal disease, real-time PCR appears to be useful in confirmed infections, with a sensitivity of 60% versus 40% for blood cultures. These results are apparently inconsistent because in confirmed infections, two of the patients with positive blood cultures had negative PCR tests and in three of the patients with positive PCR tests, blood cultures were negative. In these cases, the quantity of DNA in blood is therefore often below the threshold of detection. In children aged <2 years, the incidence of bacteremia has been reported to be 1.9% [11]. In 2008, Azzari et al. [2] showed a sensitivity of 81.8% with PCR compared with only 18.2% for blood culture.

In cases of positive PCR tests with negative cultures, antibiotics or inhibitors may be responsible for inhibiting the culture. In five cases of probable infection according to clinical and biological parameters, the positive PCR confirmed the suspicion of pneumococcal disease. If we retained positive cultures of samples from a sterile site and positive PCR tests in cases of probable infection, 10 pneumococcal diseases would be diagnosed. Our study has shown that real-time PCR detects pneumococcus in blood samples, and combined with blood cultures it improves greatly the rate of diagnosis with a sensitivity of 80% and a specificity of 97.1% (the false positive amplification of *ply* in the *Streptococcus pyogenes* bacteremia was excluded from these calculations). Furthermore, the prevalence of confirmed pneumococcal diseases in the population studied increased from 6.6% to 13.2% with the PCR test. No PCR tests were positive in cases of possible infection. In case of pneumonia, PCR for pneumococcus was not very contributory, as it was positive in only one case of pneumonia with a pleural effusion of less than 2 cm. A higher sensitivity of 44% was demonstrated with PCR on samples from pleural effusion, blood, nasopharynx and throat. The interest of semi-quantitative PCR has been highlighted by Greiner

et al. [12]. In our study, a certain number of pneumonia cases were either unaccompanied by bacteremia or were not bacterial. Nevertheless, our study demonstrates the value of real-time PCR on blood samples in diagnosing the etiology of pneumonia with pleurisy. In this scenario, the positive predictive value was 78.8% and the negative predictive value was 75%. Pleural fluid was obtained in 11 of the 12 cases of pneumonia with pleurisy and nine of these were presumed to be due to pneumococcus. Antibiotherapy was started before pleural puncture in eleven cases. Blood real-time PCR tests were positive in 7 out of 9 cases (77.8%) of suspected pneumococcal pneumonia with pleurisy. Analysis of PCR tests for pneumococcus has only previously been performed on pleural fluid. Menezes-Martin et al. [13] have shown that PCR for pneumococcus in pleural fluid is more sensitive than pleural culture. In our study, only three pleural-fluid cultures allowed the culture of *S. pneumoniae*. Antibiotics given before pleural puncture can explain this low sensitivity. The interest of our findings is the high sensitivity of real-time PCR on blood in cases of pneumonia with pleurisy. However, bacteriological culture of pleural fluid allows sensitivity to antibiotics to be determined and eventually to identification of the serotype causing the disease.

The advantage of *ply* and *lytA* gene amplification on blood is the ease by which this tissue can be obtained. Moreover, results can be given within 2h whereas culture requires 24 to 48h. Real-time PCR test for *S. pneumoniae* on blood samples has improved the diagnosis of probable pneumococcal infection, particularly for pneumonia with pleurisy. The choice of the target gene is important because a false-positive result was observed in the sample of a child with a *Streptococcus pyogenes* bacteremia with *ply* gene but not with *lytA*. Indeed, *ply* appears to be less specific than *lytA* [8,14]. Other studies have also targeted genes which may be more specific than *lytA* for pneumococcus such as, *spy*, *spn9802* and *spn9828* [15].

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