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Pleural effusion with negative culture: a challenge for pneumococcal diagnosis in children☆

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ABSTRACT

Background: Pneumococcal parapneumonic effusion seems to be increasing in children in the postvaccine era and is frequently associated with negative culture. Due to the low yield of culture, culture-independent tools are evaluated.
Methods: Culture-negative pleural fluid specimens from 38 children with parapneumonic effusion were examined for pneumococcal *lytA* by quantitative polymerase chain reaction (qPCR) and soluble antigen (C-polysaccharide) using an immunochromatographic test (BinaxNow *Streptococcus pneumoniae*).
Results: In 81% (30/37) and 63% (24/38) of the specimens, a positive result was obtained by qPCR and antigen detection, respectively. Most mismatches were observed in specimens with low quantities of pneumococcal DNA and a negative antigen test.
Conclusions: Our results suggest an imperfect relationship between the 2 described methods. The immunochromatographic assay is a simple diagnostic tool, which can be used when resources are limited, and even after antibiotic use, but negative results may require confirmation through a more sensitive test, such as qPCR.

1. Introduction

Streptococcus pneumoniae is the most frequent cause of community-acquired pneumonia and is often associated with other infections such as sepsis and meningitis, as well as less severe conditions such as sinusitis, conjunctivitis, and otitis media (de Quadros, 2009; O'Brien et al., 2009). Because conjugated vaccines have been available, the burden of pneumococcal diseases has decreased, but an increase in the rates of complicated pneumonia has nevertheless been reported in some regions (Gupta and Crowley, 2006; Hendrickson et al., 2008). In Brazil, a pneumococcal 10-valent conjugated vaccine has been widely used since 2010, and its effects are being evaluated.

Parapneumonic effusion (PPE) and empyema are known complications of pneumococcal pneumonia. Effusion occurs in at least 40% of complicated pneumonias, with 60% resulting in the formation of empyema (Schultz et al., 2004). In some cases, surgical procedures, such as thoracentesis, chest tube drainage, and decortication, might be indicated as therapy in association with antibiotics (Light, 1995). Studies from some regions have reported an increase in the incidence of empyema in children in the postvaccine era, and most of these cases were associated with negative cultures (Byington et al., 2006; Hendrickson et al., 2008).

Pleural infection is complex, and bacterial causes differ between adult and pediatric patients, geographical regions, and coverage of immunization programs (Lisboa et al., 2011). Isolation of the causative agent is often difficult; detection from pleural fluid (PF) culture ranges from 18% to 33% and use of empiric rather than specific therapy is widely employed (Kunyoshi et al., 2006; Langley et al., 2008; Menezes-Martins et al., 2005; Picazo et al., 2013). In the last decade, several studies have suggested the use of molecular assays, such as nucleic acid amplification to determine the etiology of PPE, with higher sensitivity (Blaschke et al., 2011; Carvalho et al., 2007; Lahti et al., 2006; Menezes-Martins et al., 2005; Obando et al., 2008). However, nucleic acid assays are not readily available at many institutions. Simple and rapid tests for the detection of the urinary antigens of *S. pneumoniae* may be useful to confirm pneumococcal infections and indicate early and specific therapy. The BinaxNow *S. pneumoniae* test is an immunochromatographic test used to detect pneumococcal soluble antigen (C-polysaccharides) in the urine or cerebral spinal fluid, producing results within 15 minutes. However, the urinary test produces false-positive results when applied to children because of their dense

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colonization with pneumococci in the nasopharynx (Dowell et al., 2001; Hamer et al., 2002; Navarro et al., 2004), and use of this diagnostic tool has not been extensively investigated in other clinical specimens, such as PF.

The aim of this study was to evaluate the use of a quantitative polymerase chain reaction (qPCR)-based method and an antigen detection system for the recognition of *S. pneumoniae* in culture-negative pleural effusions from children presenting complicated pneumonia and PPE.

2. Materials and methods

2.1. Patient inclusion and exclusion criteria

We conducted a prospective study from June 2010 to June 2013, evaluating children with PPE and negative microbiology culture of the PF for *S. pneumoniae*, <18 years old, who attended at the pneumology unit of 2 pediatric hospitals in Porto Alegre, Brazil. Children with clinical features of pneumonia (history of cough or breathlessness, inability to feed, fever, tachypnea and/or tachycardia), radiological findings according to the "Standardized interpretation of pediatric chest radiographs for the diagnosis of pneumonia in epidemiological studies" (Cherian et al., 2005), and undergoing thoracocentesis were included in this study, and their medical records were reviewed. Patients with pleural effusion not requiring thoracocentesis or surgical decortications, such as direct chest tube insertion were excluded. Parents gave consent for their children's participation, and the research study was approved by the ethics committee.

2.2. PF sample

Convenience samples of PF obtained during thoracocentesis were sent for routine microbiology culture. During the surgical procedure, at least 2 mL of specimens were collected and frozen at −70°C for further analysis. For each child only 1 sample was included.

2.3. Real-time qPCR

DNA was extracted from 0.2 mL of the specimens using the QiAamp DNA Mini Kit with modifications (QIAGEN; Valencia, CA, USA), and stored at −20°C. Briefly, PF was added to 100 μL of lysis buffer (Tris-EDTA buffer [Sigma-Aldrich; St. Louis, MO, USA], containing 0.04 g/mL lysozyme [Sigma-Aldrich] and 75 U/mL of mutanolysin [Sigma-Aldrich]) and incubated for 1 hour at 37°C; all subsequent steps were performed following the manufacturer's instructions. The quantity and quality of the DNA were determined by spectrophotometric analysis in the BioSpec-Nano (Shimadzu Scientific Instruments; Kyoto, Japan).

qPCR (SYBR-Green dye) was conducted according to Carvalho et al. (2007), with some modifications. The oligonucleotide primers designed for the *lytA* gene were the same as the original assay (*lytA*-CDC), performed in a final 12 μL reaction volume using Fast SYBRGreen Master Mix (Applied Biosystems; Foster City, CA, USA), with 2 μL of DNA sample and a final primer concentration of 200 nM. The DNA was amplified with the 7500 Real Time PCR System (Applied Biosystems) using the following cycling parameters: 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 95°C for 15 seconds and 60°C for 1 minute. During each analysis, a no-template control and a standard curve were developed using a template containing known concentrations of *lytA* target. Serial 6-fold dilutions in TE buffer (Tris-EDTA) of purified synthesized template (Sigma-Aldrich) were made to prepare a standard DNA curve ranging from 10^5 to 32 copies. To determine the low limit detection, we also have made a serial dilution ranging from 10^5 to 1 copy. Identification of the copies in the PF was determined by the equation, $C = Q \times V_{DNA}/V_{PCR} \times 1/V_{EXT}$, where C is the number of copies per μL of PF, Q is the number of copies calculated by the software, V_{DNA} is the volume of extract, V_{PCR} is the volume of DNA in the reaction, and V_{EXT} is the volume of PF used for the extraction. RNase P amplification was conducted as a positive amplification control to exclude samples with reaction inhibitors. TaqMan Copy Number Reference Assay RNase P (Applied Biosystems) was conducted following the manufacturer's instructions. The amplification data, number of *lytA* copies, and melting curves of the assays were analyzed by 7500 Software v2.0.6 (Applied Biosystems).

2.4. Antigen detection

Pneumococcal antigen detection by immunochromatographic testing was performed with BinaxNow*S. pneumoniae* (Alere; Scarborough, ME, USA) in PF specimens following the manufacturer's instructions for urine specimens.

2.5. Analysis of patient's records

Patient's medical history includes the previous use of antibiotics and results of viral tests (immunofluorescent staining for respiratory virus in nasopharyngeal swab), and microbiological cultures (from PF, and blood) were obtained from the patient's charts.

2.6. Statistical analysis

Categorical data were shown in frequencies, and the continuous data for DNA quantification were ranked from the lowest number to the highest, then divided in quartiles, since the distribution was not normal because of the amplitude. These quartiles were used to separate the DNA copy number in groups and then associate these with other factors. Pneumococcal DNA load data were natural logarithm transformed (Ln) to assume a normal distribution, because the results from qPCR analysis follow a logarithmic distribution. This transformation was used only for statistical analysis using *t*test comparing previous antibiotic use/nonuse and antigen positive/negative samples. Also, κ coefficient was used to measure the agreement rate between qPCR and the antigen test; a $P = 0.05$ was considered to be significant. All analyses were made using PASW Statistics version 18.0 (SPSS; Chicago, IL, USA).

3. Results

Thirty-eight children with PPE were enrolled in this study. The average age was 5 years (range 11 months to 16 years), 65% were male, and were hospitalized from emergency room and basic health units (66%), or regional hospitals (34%). By the time that the PFs were collected, 33 (86.8%) of the children had been treated with antibiotics prior PF collection (Table 1), and all had received at least 1 β-lactam agent with penicillin, the most frequent antibiotic used for 26/38 (68.4%) children.

In only 1 culture of PF, a positive result was obtained, for methicillin-resistant *Staphylococcus aureus* (MRSA), and blood cultures were negative for all of the children. Six patients presented results for respiratory virus, 3 positive results were detected, 2 for syncytial respiratory virus (LP27 and LP29), and 1 for influenza A (LP23).

All specimens were tested by qPCR for RNase P; one sample (LP32) did not amplify RNase P and was therefore excluded from the qPCR assays. The lowest limit detection for the *lytA* qPCR was 1 copy, with a template melting temperature of 77.15 ± 0.35°C. The standard curves presented slopes from −3.18 to −3.34, with a R^2 value >0.99, and efficiencies ranged from 99% to 106% (Supplementary Fig. 1).

Of the 37 PFs, 30 (81%) were positive for *S. pneumoniae* by qPCR. Quantification of *lytA* showed a median of 64 copies/μL (range 2 to 153,000). Table 1 presents the results of the *lytA* qPCR and quantification based on the quartile distribution (<14, 14–64, 65–409, >409 DNA copies/μL). The proportion of children who had not been treated with antibiotics prior PF collection was larger in the group with a high copy number (fourth quartile, >409 copies/μL), 42.8% (3/7); however, in the groups with a low copy number (first and second quartiles, <14 and 14–64 copies/μL), the proportions were 12.5% (1/8). Of the children