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BRIEF REPORT

Streptococcus pneumoniae as a frequent cause of severe community-acquired pneumonia among children in Beijing

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Community-acquired pneumonia (CAP) is a major cause of childhood morbidity worldwide and is a significant cause of child death in developing countries [1, 2]. The gram-positive pathogen *Streptococcus pneumoniae* (*S. pneumoniae*) is one of the major causative agents of bacterial CAP. In many countries, the incidence of pneumococcal pneumonia has been reduced by the development and widespread use of pneumococcal vaccine [3]. In China, however, the causative agents of child CAP, especially in severe cases, have not been routinely determined. Such information is important in the development of strategies for vaccine prophylaxis and antibiotics therapy. Specific diagnostic techniques identifying the microorganism at the site of infection (lung tissue) are certainly helpful in determining the causal relationship, particularly in fatal cases. In line with this, *in situ* polymerase chain reaction (ISPCR) is particularly suitable because, first, it provides information relative to the localization of the bacteria in the lungs, and, second, it requires only very small samples. The present study was performed to analyze the role of *S. pneumoniae* as the causative agent of fatal outcome of severe pneumonia in Chinese children. In order to achieve this objective, conventional PCR, Southern blotting, and ISPCR were applied to detect the presence of *S. pneumoniae* in paraffin-embedded autopsy lung tissue samples from historic cases of fatal childhood CAP.

A total of 202 paraffin-embedded lung autopsy tissues of children aged 1 month to 5 years old (≤2 years n=172, >2 years n=30) who died of CAP were selected at random from the Beijing Children's Hospital (BCH). Of these 202 samples, 116 were collected between 1953 and 1969, while 86 samples were collected between 1980 and 2002. Clinically documented data on CAP were available for 174 cases.

Bacterial DNA isolation and conventional PCR were performed as previously described [4, 5]. In brief, tissue sections of 10 μm in thickness were digested with 0.5 mg/ml Proteinase K (Serva, Germany), and DNA was isolated by phenol/chloroform extraction. Two primers (5′-CTACG CATTTCACCGCTACAC-3′ and 5′-AAGGTGCACTTG CATCACTACC-3′) were used for the amplification of the 16SrRNA gene of *S. pneumoniae* [6]. The conventional PCR consisted of initial denaturation at 94 for 3 min followed by 38 cycles of denaturation at 94 for 30 s, annealing at 60 for 1 min, extension at 72 for 40 s, and another extension at 72 for 5 min during the last step. PCR products were detected by conventional electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 μm/ml).

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Procedures involved in DNA labeling by digoxigenin, Southern blotting, and immunological detection were performed according to the protocol provided by the manufacturer (Roche Corp., Germany).

In situ PCR was performed as previously described [7, 8]. After being de-waxed, tissue sections of 5 μm in thickness fixed on organosilane-coated glass slides were permeabilized with 20 μg/ml Proteinase K. ISPCR was performed in a 25-μl reaction mixture, among which 5% of dTTP in the reaction mixture was replaced by digoxigenin-11-dUTP (Roche Corp.). The ISPCR reaction conditions were the same as that described for conventional PCR, except the number of ISPCR cycles was modified to 30. *In situ*-generated PCR products that included digoxigenin-11-dUTP were analyzed under a microscope by immunohistochemistry. The paraffin-embedded mice lung tissues infected by *S. pneumoniae* fixed on the glass slides were used as positive controls. Normal lung tissue, PCR mixtures that lacked *Taq* DNA polymerase or primers, were used as negative controls.

Statistical analysis was performed using SPSS for Windows 10.0. All average values were expressed as mean ± standard deviation (SD). The statistical methods used in this study include the independent samples *t*-test (two-sided) and Chi-Square test, with $P<0.05$ considered as significant for all comparisons.

In paraffin-embedded lung tissue samples of the 202 children who died of pneumonia, conventional PCR, Southern blotting, and ISPCR revealed the presence of *S. pneumoniae* in 7/202 (3.5%), 107/202 (53.0%), and 106/202 (52.5%) samples, respectively ($\chi^2=3.703$, $P>0.05$). Using ISPCR, *S. pneumoniae* was found to be distributed in the lung tissue, especially in areas of hyperemia or hemorrhage (Fig. 1). A combined total of 57.4% of the samples (116/202) were found to be *S. pneumoniae*-positive by Southern blotting and/or ISPCR. *S. pneumoniae* was identified in 64 of 116 samples (55.2%) collected in 1953 to 1969 and 42 of 86 samples (48.8%) collected in 1980 to 2002 ($\chi^2=1.470$, $P>0.05$). In CAP cases younger than 2 years of age, 90 of 172 (52.3%) were positive for *S. pneumoniae*, and in cases 2 years of age or older, 16 of 30 (53.3%) were positive ($\chi^2=3.772$, $P>0.05$).

Clinical data were available for 106 of 116 CAP cases of pneumococcal pneumonia determined by PCR, Southern blotting, and ISPCR. Out of 106 patients, 77 (72.6%) had fever for three days from the day of admission, and most of them (74 patients) had temperatures equal to or higher than 38.5°C. Hypothermia was observed in five patients (4.7%); 78 patients (73.6%) had tachypnea with nasal flaring and a high respiratory rate (50 to 128 breaths per min), while 47 had prominent intercostal and subcostal retractions. Most of the patients with CAP showed leukocytosis (from 10×10^9/L to 81×10^9/L), with an increased number of neutrophils

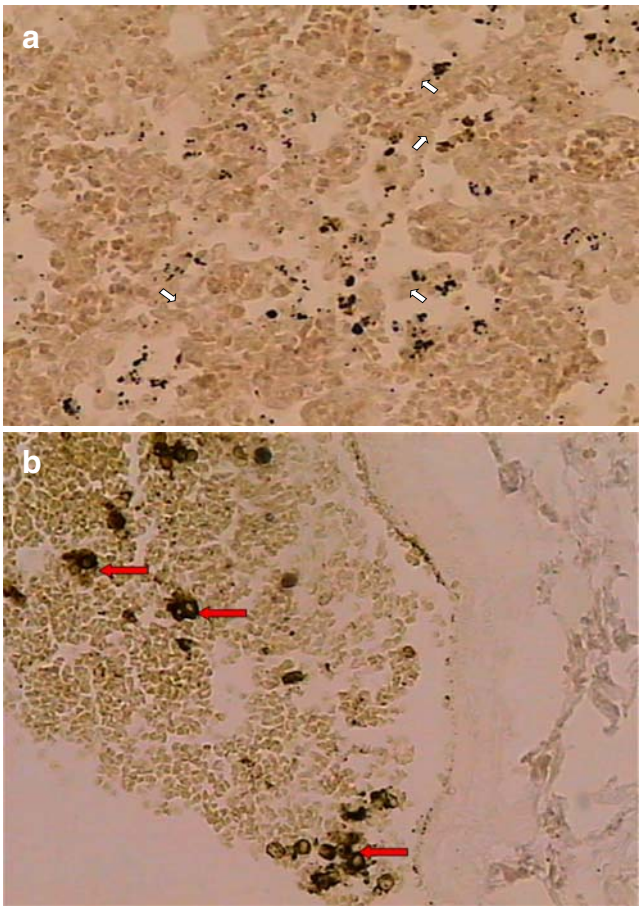

Fig. 1 **a** *In situ* polymerase chain reaction (ISPCR) detection of *Streptococcus pneumoniae* in child paraffin-embedded lung tissues. *S. pneumoniae* localized in alveoli with pulmonary infiltrate and among lung tissue cells; without counterstain; magnification×20. *S. pneumoniae* cells are indicated *by white arrows*. **b** Some *S. pneumoniae* were localized among the red blood cells in dilated and congested vessels; without counterstain, magnification×40. *S. pneumoniae* cells are indicated by *red arrows*

(from 50 to 90%). All of the subjects received antibiotics (from 1 day to 5 days) prior to death.

Among the samples identified in this study as *S. pneumoniae*-positive, only 20 patients had microbiologic ($n=19$) nasopharyngeal culture and/or blood culture, and the identity of one isolate ($n=1$) was confirmed by capsule swelling test using Omni sera against *S. pneumoniae* on admission. Although the rate of microbiologic positivity to pneumonia by blood culture ($n=7$) was found to be 57.1% (4/7), none of the samples were found to be *S. pneumoniae*-positive. Only the sample tested by Omni sera was *S. pneumoniae*-positive.

In the present study, three molecular techniques (conventional PCR, Southern blotting, and ISPCR), which are presumed to have higher specificities and sensitivities for *S. pneumoniae* detection in comparison with traditional methods, were used. All of these methods were developed from the experimental murine *S. pneumoniae* pneumonia

model and were subsequently used for the detection of *S. pneumoniae* in 202 paraffin-embedded lung tissue autopsy samples from children who died of CAP.

ISPCR is a novel molecular technique that can provide an extremely sensitive detection of the pathogen and its distribution in tissue section, as well as the pathological changes in the tissue. An ISPCR can be divided into four phases: section pretreatment, PCR amplification, hybridization, and detection. In each of these phases, there are multiple potential variables that can affect sensitivity, specificity, and background staining. In the current study, several of these factors' effects on sensitivity and background staining had been examined for each phase. Optimal results were obtained when the tissues were cut at 5-μm thickness and then permeabilized with 20 μg/ml Proteinase K for 30 min at 37°C. ISPCR actually amplifies specific DNA fragments directly within isolated cells in tissue sections and does not require DNA isolation. In addition, signal amplification in the detection of amplified DNA can further enhance the sensitivity of the method, as opposed to conventional PCR, where extensive extraction and purification procedures can end up in the loss of bacterial DNA. However, ISPCR is costly and time-consuming; it also requires skilled personnel and the use of specialized equipment.

S. pneumoniae was found to be localized in lung tissues, mainly among the lung cells, in the pulmonary alveolus, and in/or around the dilated and congested vessels. It was not found inside the lung cells.

Southern blotting and/or ISPCR revealed the presence of *S. pneumoniae* in 116 lung samples; this is significantly higher than that detected by conventional PCR (seven *S. pneumoniae*-positive samples). Thus, these sensitive methods demonstrate that the clinical importance of *S. pneumoniae* in fatal childhood CAP is significantly higher than that previously reported [9, 10].

In 95.4% of *S. pneumoniae*-positive patients, the presence of bacterial infections was demonstrated by the increase in the number of leukocytes and neutrophils in the blood. More than 69% of *S. pneumoniae*-positive patients had symptoms of severe pneumonia, as defined by the presence of high fever (≥38.5°C) within 72 h of admission, breathlessness with flaring of the alae nasi, and three depression signs [11].

In the present study, the analysis of two age groups (younger than 2 years old and older than 2 years old) revealed a similar occurrence of *S. pneumoniae* in the lung tissues, demonstrating the clinical importance of *S. pneumoniae* among Chinese children of different ages.

The traditional diagnostic methods of pneumonia result in significant differences in the identification of *S. pneumoniae* in clinical samples. Indeed, among the 20 pneumococcal pneumonia cases diagnosed by Southern blotting and/or ISPCR, only one case was *S. pneumoniae*-positive, as previously determined by pharyngeal swab culture test, blood culture test, and/or Omni sera. This observation can be attributed to the indiscriminate use of antibiotics in China. Most patients with CAP are likely to have been treated with antibiotics before visiting the hospital; this can affect the etiological diagnosis of CAP using traditional culture methods. On the contrary, molecular techniques can detect the presence of *S. pneumoniae* DNA in killed or damaged bacterial cells, as it occurs after antibiotic treatment. In most children with CAP, the identification of the causative organism is not critical [12], but patients with severe symptoms of CAP should undergo urgent diagnostic testing. In identifying *S. pneumoniae*, ISPCR and Southern blotting can be applied not only on paraffin-embedded tissues but also on lung puncture tissue or sputum from the lower respiratory tract. Thus, the methods introduced in the present study, being highly sensitive and specific, may be useful for the identification of *S. pneumoniae* in such high-risk cases. However, further studies are needed to detect pneumonia-causing pathogens in a sample of pulmonary irrigating solution rather than in biopsies.

An alternative way for the community to resist *S. pneumoniae*-caused pneumonia is the administration of pneumococcal vaccine [3]. To date, pneumococcal vaccine has not been used in China due to the limited data on the causative agents of CAP, especially severe CAP. The results of this study show that at least half of the deadly cases of CAP may be caused by *S. pneumoniae*, and, therefore, a vaccination program against pneumococcal pneumonia in China may be very important in protecting children from severe diseases and death.

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