Detection of *Haemophilus influenzae* type B DNA in a murine pneumonia model by *in situ* PCR

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This study estimated the value of *in situ* PCR (ISPCR) in the detection of *Haemophilus influenzae* type b (Hib) DNA in paraffin-embedded lung tissues of a murine pneumonia model. ICR mice were infected with Hib solution intranasally. In study group A ($n=20$), physiological changes and the number of deaths were recorded for 7 consecutive days after infection. In study group B ($n=10$), blood samples and lung tissues were obtained from the infected mice on the brink of death. In both groups, portions of the lung tissue were cultured for Hib, while other portions were submitted for histopathological studies. Conventional PCR, PCR followed by Southern blotting and ISPCR were performed to detect Hib in paraffin-embedded lung tissues. In control group A, six mice were inoculated intranasally with the same concentration of heat-inactivated Hib solution. In control group B, six healthy mice served as a blank control. Both control groups were managed using the same methods as those used in the study groups. The white blood cell count of the mice in the study group increased ($F=3.295$, $P<0.01$), with a high neutrophil count ($F=0.127$, $P<0.05$). In the histopathological study, various stages of pneumonia were found in the lung tissues of the infected mice examined by microscope; 80 % of the mice had moderate or severe pneumonia. Cultures of lung tissues in the study groups were all positive for Hib, while no bacteria were found in the control groups. Hib was detected in only 4 of 30 samples (13.3 %) of the study groups using conventional PCR, but in all 30 samples (100 %) using both Southern blotting and ISPCR. All three methods did not detect Hib in the control groups. Because of its sensitivity and specificity and its ability to locate the micro-organism, ISPCR can be considered suitable for the detection of Hib in paraffin-embedded lung tissues.

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INTRODUCTION

In China, community-acquired pneumonia (CAP) remains one of the major causes of child morbidity and mortality, with *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) responsible for the majority of cases. However, because most patients with CAP are likely to be treated with antibiotics before admission to hospital, isolation rates of the aetiological agent are very low. Furthermore, the role of these two pathogens in childhood CAP, especially in severe or fatal CAP, has not been fully clarified. Such information is important in the development of strategies for vaccine prophylaxis and targeted antibiotic therapy. Unfortunately, sensitive and specific

methods for determining the aetiology of pneumonia are still lacking (McIntosh, 2002; Nascimento-Carvalho, 2001).

Routine approaches for the diagnosis of Hib in the aetiology of pneumonia are blood culture and serology (Drummond *et al.*, 2000). Blood culture, although a highly specific method, very often fails to provide a reliable diagnosis, especially in patients previously treated with antibiotics. Meanwhile, a serological diagnosis of pneumonia usually takes a few days, as convalescent sera are generally required. Hence, this method cannot be used when rapid results are desirable. Other approaches, such as lung puncture and pleural fluid aspiration, are not commonly employed, particularly in developing countries. Currently, molecular methods are being used to determine the aetiology of pneumonia, but their value depends on the appropriateness of the site from which specimens are

Abbreviations: CAP, community-acquired pneumonia; Hib, *Haemophilus influenzae* type b; ISPCR, *in situ* PCR.

collected. Specific diagnostic techniques to identify the micro-organism 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* PCR (ISPCR) is particularly suitable: first, it allows localization of the bacteria in the lungs, and second, it requires very small samples.

The present study aims to evaluate the sensitivity and specificity of ISPCR in the aetiological detection of pneumonia in paraffin-embedded lung tissues in parallel with conventional PCR and Southern blotting. To achieve this objective, a murine model of Hib pneumonia was developed.

METHODS

Bacteria and media. A pharyngeal isolate of Hib was obtained from a hospitalized patient with pneumonia in Beijing Children's Hospital (BCH) in 1997. The isolate was identified as *H. influenzae* based on the requirement for both X and V factors for growth. The isolate was determined as type b through slide agglutination with specific sera (generously provided by John Robbins of the National Institutes of Health, USA). Non-type b *H. influenzae* strains and strains of *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pneumoniae* from the microbiological collection of BCH and non-type b *H. influenzae* strain ATCC 49247 obtained from John Robbins were also used in this study. These strains were cultured on appropriate media and incubated at 35 °C in a 5 % CO₂ atmosphere and later stored at -70 °C.

Experimental Hib infection in mice. The Hib inoculum was obtained after an overnight incubation on chocolate agar at 35 °C in 5 % CO₂ and subsequent suspension in 0.9 % saline at a concentration of 10⁹ c.f.u. ml⁻¹. This concentration was determined using a nephelometer and confirmed by performing tenfold serial dilution (Scaglione *et al.*, 2003). Meanwhile, 4-week-old specific-pathogen-free (SPF) male ICR mice (20 g weight) were purchased from the Academy of Military Medical Science, China. The animals were anaesthetized by intraperitoneal administration of pentobarbital at a dosage of 50–70 mg (kg body weight)⁻¹ and then infected by intranasal inoculation with 0.05 ml of one of a Hib suspension at 10⁹ c.f.u. ml⁻¹ (study group A of 20 mice, study group B of 10 mice), a heat-inactivated Hib suspension at 10⁹ c.f.u. ml⁻¹ (control group A of 6 mice) or sterilized saline (control group B of 6 mice) (Miyazaki *et al.*, 2001; Xu *et al.*, 2008). After inoculation, the animals were observed continuously for 7 days. In study group A, the physiological index of the mice was recorded daily. In study group B, blood samples and lung tissues were obtained from the infected mice on the brink of death. The lungs were excised aseptically in the event that the animals died during the 7-day observation period. On day 7 following inoculation, all the mice from both the study and control groups were euthanized and their lungs were excised aseptically. Portions of all lung tissues were homogenized in 2 ml saline and 0.1 ml aliquots of the homogenates were cultured for Hib on chocolate media and incubated at 35 °C in 5 % CO₂ overnight. Other portions of lung tissue were fixed in 10 % neutral formalin and embedded in paraffin.

The criteria used for histological definition of severe pneumonia included the presence of consolidation with pulmonary infiltrates and intense polymorphonuclear leukocyte and/or lymphocyte accumulations in bronchioles and adjacent alveoli seen in several adjacent low-power microscope fields, with or without tissue necrosis (Corley *et al.*, 1997).

Hib DNA isolation. Bacterial DNA was extracted from paraffin-embedded lung tissue as follows. Tissue sections each measuring 10 µm in thickness were dewaxed using xylene and anhydrous ethanol and then digested with 200 µl buffer (100 mM Tris/HCl, pH 8, 50 mM EDTA, pH 8, 0.5 % NP-40) containing 0.5 mg proteinase K ml⁻¹ (SERVA). DNA was then isolated by phenol/chloroform extraction.

Conventional PCR. Two primers, HibFor (5'-CCTCGCAATGCAG-TTTATGGTCC-3') and HibRev (5'-AAGCGGGAATTTGATACCT-GATGC-3'), for the gene involved in Hib capsule synthesis were designed using the OLIGO program. Their specificity for Hib was confirmed using the BLAST software (available at <http://www.ncbi.nlm.nih.gov/BLAST>). The primers were purchased from Sangon Corp. (Shanghai, PR China). PCR was performed in 25 µl optimized reaction mixture containing 1.5 mM MgCl₂, 2 U *Taq* DNA polymerase and 200 µM dNTPs. The amplification program consisted of initial denaturation at 94 °C for 3 min followed by 37 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 40 s, with a final extension step at 72 °C for 5 min. DNA samples isolated from paraffin-embedded mouse lung tissue infected by Hib and normal lung tissue were used as positive and negative controls, respectively. Chromosomal DNA isolated from the Hib strain was also used as a positive control. PCR products were visualized using conventional electrophoresis in 1.5 % agarose gel containing ethidium bromide (0.5 µg ml⁻¹).

DNA labelling, Southern blotting and detection. DNA probe-labelling by digoxigenin, Southern blotting and immunological detection of the Hib DNA band were performed according to the protocol provided by the manufacturer (Roche). In brief, PCR products were subjected to agarose gel electrophoresis and transferred onto a nylon membrane overnight. The nylon membrane was then incubated at 80 °C for 2 h. The membrane was prehybridized in solution containing 5× SSC, blocking reagent (1 %) and SDS (0.02 %) at 65 °C for 1 h and hybridized with the gene probe in the same solution at 65 °C overnight. The membrane was washed twice (5 min each time) at room temperature in a solution containing 2× SSC and 0.1 % SDS and twice (15 min each time) at 65 °C in a solution containing 0.1× SSC and 0.1 % SDS. The membrane was later washed in a buffer containing 100 mM Tris/HCl (pH 7.5) and 150 mM NaCl (buffer 1) for 3 min, incubated for 1 h at room temperature in buffer 2, prepared by dissolving 1 % blocking reagent in buffer 1, and incubated in fresh buffer 2 (containing 1:5000-diluted, alkaline phosphatase-conjugated anti-digoxigenin Fab fragments) for 1.5 h. Afterwards, the membrane was washed three times (5 min each time) in buffer 1 and washed for 3 min in buffer 3, containing 100 mM Tris/HCl (pH 9.5), 100 mM NaCl and 50 mM MgCl₂. Finally, the membrane was stained with 10 ml buffer 3 containing 200 µl NBT/BCIP solution in a darkroom until staining was complete. The reaction was stopped by rinsing in distilled water.

Comparison of sensitivity of conventional PCR and Southern blotting. In order to analyse the sensitivity of PCR and Southern blotting, tenfold serial dilutions of the Hib DNA template were performed.

ISPCR. *In situ* PCR was performed as described previously (Praveena *et al.*, 2007; Ma *et al.*, 2007; Zhaori *et al.*, 1996). Tissue sections measuring 5 µm in thickness were fixed on organosilane-coated glass slides and sequentially dewaxed in xylene and ethanol (70, 95 and 100 %). Samples were subsequently rehydrated in double-distilled water and permeabilized with 20 µg proteinase K ml⁻¹ in TE (100 mM Tris/HCl, pH 8, 10 mM EDTA, pH 8) for 30 min at 37 °C. ISPCR was then performed in a 25 µl reaction mixture containing 2.5 mM MgCl₂, 5 U *Taq* DNA polymerase, 250 µM dNTPs and 10 pM primers HibFor and HibRev. A total 5 % of the dTTP in the

reaction mixture was replaced by digoxigenin-11-dUTP (Roche). ISPCR conditions were the same as those described for conventional PCR except that the number of cycles was reduced to 30. *In situ*-generated PCR products that included digoxigenin-11-dUTP were visualized directly by immunohistochemistry according to a method described previously (Komminoth *et al.*, 1992). Following this, slides were hydrated for 2 min in buffer 1 and then blocked for 30 min in buffer 2. Next, the samples were incubated for 1 h at room temperature with 1:5000-diluted, alkaline phosphatase-conjugated anti-digoxigenin Fab fragments in buffer 2. The slides were washed three times (5 min each time) in buffer 1 and then for 3 min in buffer 3. Finally, the slides were stained with NBT/BCIP as described above. The slides were then analysed by microscopy. Infected paraffin-embedded mouse lung tissues fixed on glass slides were used as positive controls. Normal lung tissue was used as a negative control.

Statistical analysis. Statistical analysis was performed using SPSS for Windows 10.0. Mean values are expressed as means $\pm$ SD. The statistical methods used in this study included independent-samples *t* test and chi-square test, with $P < 0.05$ considered significant for all comparisons. In comparing the sensitivity, specificity and positive and negative predictive values of the diagnostic methods (conventional PCR, Southern blotting and ISPCR), the Kappa test was used. Culture of specimens served as the gold standard.

RESULTS AND DISCUSSION

Specificity of Hib identification

In this study, specificity of primers HibFor and HibRev for Hib identification was tested using DNA isolated from different bacterial species (Hib, non-type b *H. influenzae*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *E. coli* and *Streptococcus pneumoniae*). All Hib DNAs used as templates produced PCR fragments with the expected size of 774 bp. Non-type b *H. influenzae* strains did not produce any amplification bands. These results demonstrate the specificity of the primers HibFor and HibRev for Hib identification.

Hib detection in an experimental pneumonia model

Mice were infected by intranasal inoculation with a Hib suspension, a convenient and economical way to establish a murine pneumonia model of Hib which, at the same time, imitates the natural route of Hib respiratory tract infection. Following infection, the mice in the study group were noted to develop a clumsy gait, shortness of breath and weight loss. The total mortality rate in the study group was 15% (3/20), most of which died within the first 3 days. The white blood cell count of the mice increased (7.699 ± 1.726) ($F = 3.295$, $P < 0.01$) and the neutrophil count increased specifically (10.685 ± 2.781) ($F = 0.127$, $P < 0.05$). Lung tissues of the study group mice were likewise noted to have a dull red colour, were not glossy and had petechiae. In contrast, the lung tissues of control mice were pink, glossy and had clear striations. It was also noted in the study group that the mean body mass of the mice decreased from 22.7 to 21.0 g and the mean mass of

the lung tissue was about 327.7 mg; the lung tissue:body mass ratio was 15.6×10^{-3} (mean). However, in the control group, the mean body mass of the mice increased to 32.1 g and the mean lung tissue mass was 275 mg; the lung tissue:body mass ratio was 8.6×10^{-3} (mean value). These results indicated that the mass of lung tissue in infected mice increased as a result of the infection.

Subsequently, lung tissue sections were homogenized and bacteria were cultured on chocolate agar. Hib was cultured from all infected mice, while no Hib was detected in the control groups.

Lung tissue sections of all Hib-infected mice were analysed by microscopy. Most showed different stages of inflammation; 80% (24 of 30) showed moderate or severe pneumonia (Fig. 1) with typical pathological findings, including consolidation with pulmonary infiltrates and the presence of intense polymorphonuclear leukocytes in bronchioles and adjacent alveoli.

In the present study, in addition to culture, three molecular techniques (conventional PCR, Southern blotting and ISPCR) presumed to have higher specificities and sensitivities compared with traditional methods were employed to analyse the presence of Hib in lung tissues.

Sensitivity of conventional PCR and Southern blotting

By using a tenfold serial dilution of Hib DNA template, the last dilution from which a visible amplification band could be detected following conventional PCR was the 10^{-3} dilution, while a band could be detected following Southern blotting from the 10^{-5} dilution (data not shown). This indicates that Southern blotting is more sensitive than conventional PCR in detecting Hib in paraffin-embedded lung tissues.

PCR identified Hib only in 4 out of 30 lung-tissue homogenates of infected mice (13.3% sensitivity and 100% specificity; Table 1). On the other hand, both Southern blotting and ISPCR detected Hib in all 30 infected mice (100% sensitivity and specificity). In the control groups, Hib was not detected using any of these three methods. The lowest sensitivity of the conventional PCR correlated with a previous result on the detection of bacterial pathogens in paraffin-embedded tissue samples (Johansen *et al.*, 2004). In contrast, using a combination of PCR and Southern blotting, we detected Hib in all 30 infected mice (Fig. 2). It is also worth mentioning that our study employed primers HibFor and HibRev, which amplify a 774 bp fragment. This may be too long for a diagnostic PCR; such long fragments may result in a low amplification efficiency, a possible explanation for the low sensitivity of the PCR used in this study. Primers HibFor and HibRev were selected since, in ISPCR, if the amplification product is too short (< 400 bp), it can spread outside the bacterial cell and yield false *in situ*

Fig. 1. Paraffin-embedded lung tissues of mice in the Hib murine pneumonia model and control group. (a) Histopathological changes in infected tissue include pulmonary infiltrate and intense polymorphonuclear leukocytes and/or lymphocytes accumulation in bronchioles and adjacent alveoli. Haematoxylin-eosin; magnification $\times 20$. (b) Normal lung tissue of mice in the control group. Haematoxylin-eosin; magnification $\times 20$.

results (Nuovo *et al.*, 1991; Retzel *et al.*, 1994; Tani *et al.*, 1998).

***In situ* PCR**

An ISPCR can be divided into four phases: section pretreatment, PCR amplification, hybridization and detection. In each of these phases, there are potential variables that could affect sensitivity, specificity and background staining. In our study, the effects of several of these factors on sensitivity and background were examined for each

Table 1. Sensitivity and specificity of conventional PCR compared with culture

Sensitivity = $4/(4+26) = 13.3\%$. Specificity = $12/(12+0) = 100\%$. Positive predictive value = $4/(4+0) = 100\%$. Negative predictive value = $12/(26+12) = 31.6\%$.

Method	Culture (infected and controls)		
	Positive	Negative	Total
Conventional PCR			
Positive	4	0	4
Negative	26	12	38
Total	30	12	42

phase. The optimal results were obtained when tissues were cut at $5\ \mu\text{m}$ thickness and then permeabilized with $20\ \mu\text{g}$ proteinase K ml^{-1} for 30 min at $37\ ^\circ\text{C}$. ISPCR revealed results similar to those obtained by Southern blotting but with a further advantage; ISPCR amplifies specific DNA fragments directly within isolated cells in tissue sections and does not require DNA isolation. In addition, signal amplification in detection of amplified DNA can enhance the sensitivity of the method further, in contrast to conventional PCR, where extensive extraction and purification procedures could result in the loss of bacterial DNA.

Fig. 2. Results of conventional PCR and Southern blotting. (a) Four of 30 lung-tissue homogenates of infected mice showed the expected fragment following conventional PCR (lanes 4–6 and 8; lane 7, blank). (b) Hib was detected in all 30 infected mice by Southern blotting (lanes 1–6 show some examples; lane 7, blank).

Fig. 3. ISPCR detection of Hib in paraffin-embedded mouse lung tissues. (a) Hib localized in alveoli with pulmonary infiltrate and among lung tissue cells. No counterstain; magnification $\times 40$. Hib cells are indicated by arrows. (b) Hib localized among lung tissue cells in the thickening wall of alveoli; in the lower-left corner, some Hib cells are visible localized among red blood cells in dilated and congested vessels. No counterstain, magnification $\times 40$. Hib cells are indicated by arrows. (c) Normal lung tissue of mice in the control group. No counterstain; magnification $\times 20$.

ISPCR provides valuable information on the localization of Hib in tissues and its correlation with histopathological changes. In this study, Hib was found to be localized in lung tissues, particularly around the lung cells, in the pulmonary alveoli and in or around dilated and congested vessels, but not inside macrophages or lung cells (Fig. 3).

Unfortunately, ISPCR is costly and time-consuming and requires skilled personnel and specialized equipment. Further studies are needed to attempt the detection pneumonia-causing pathogens in a sample of pulmonary irrigating solution rather than in biopsies. Recently, conventional PCR, Southern blotting and ISPCR were used to examine paraffin-embedded lung tissue samples from 202 children who died of pneumonia. A total of 13.9% of the samples (28 out of 202) were found to be positive by both Southern blotting and ISPCR. No Hib was detected in similar specimens from the control group by any of the three methods (Hu *et al.*, 2008). This study therefore provides strong evidence that Hib is an important pathogen in childhood pneumonia and, taking into account the mortality rate, shows the importance of the WHO policy to implement Hib vaccination in Asian countries, especially in China.

In conclusion, all three methods (PCR, Southern blotting and ISPCR) used in this study are specific for Hib identification from lung tissues, but ISPCR is the most appropriate method, providing both 100% sensitivity and images of the pathological changes in the lung tissue as a consequence of Hib infection.

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