行政院國家科學委員會專題研究計畫成果報告

以聚合酶鏈鎖反應偵測結核病病人血中的結核菌 DNA

PCR Amplification of DNA of *Mycobacterium tuberculosis* from Peripheral Blood of Patients with Tuberculosis

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主持人：李麗娜　　國立台灣大學醫學院檢驗醫學科

' 八十六年度及以前的一般國科會專題計畫(不含產學合作研究計畫)亦可選擇適用，惟較特殊的計畫如國科會規劃案等，請先洽得國科會各學術處同意。

一、中文摘要

結核病的診斷在過去數十年間鮮有改變。最常使用的方法包括臨床檢體抹片，培養，胸部 X 光攝影，抗結核抗體檢查等。這些檢查有的雖迅速但敏感度不高(如抹片)，有的特異性不高(如抗結核抗體)。至於結核菌培養雖然敏感度、特異性佳，但需時 6-8 星期。近年來，由痰檢體中以聚合酶鏈鎖反應(PCR)增量結核菌 DNA 並加以偵測的方法，受到高度的重視。其優點是高敏感度、特異性以及迅速。但其缺點包括：(1)有些病人無法咳出痰，有些病人(如結核性腹膜炎、結核性腦膜炎、粟粒性結核患者)，其檢體的取得困難；(2)檢體(尤其是痰)的取得過程(如：經由生理食鹽水噴霧後取得)，或處理過程可能造成工作人員的感染；以及(3)檢體(如痰、胸水)中可能含有 PCR 抑制物。因此，吾人希望能用另一種較易取得，又較不具感染性的檢體來作結核菌 DNA 之 PCR。吾人所選擇的檢體，是病人血液中的單核球。因為早在 10 年前就有人報告，結核菌患者血中的單核球會被活化，產生多量的 interleukin-1，故推測在致病過程中，結核菌可能會進入血液中的單核球。本研究抽取結核菌患者的血液(每次 4 ml)，加入 Ficoll-Hypaque 離心後，取其中的單核細胞層，萃取 DNA，經過兩次 PCR 後，以 ethidium bromide 染色觀察，再以 DNA probe 作雜交。結果顯示，由 26 名肺結核病人取得的 30 支血液中，有 15 支在 PCR 後產生約 250 bp 長度之 DNA 片段，與陽性對照組所產生者相似。但經過 DNA probe 雜交後並未產生陽性結果，顯示這些 DNA 片段並非來自結核菌。此外在 9 位肺外結核(肋膜炎 5，淋巴結炎 3，腦膜炎 1)病人所抽取之 12 支血中，亦有 6 支在 PCR 後產生 250 bp 左右之 DNA 片段。但在與 DNA probe 雜交後並未產生任何陽性反應，顯示這些 DNA 片段亦非來自結核菌 DNA。檢討實驗未成功之原因可能包括：(1)PCR 抑制物；(2)血液量太少；(3)有些病人在抽血時已開始接受抗結核治療；(4)實驗本身之困難度高。目前作者仍繼續在嘗試改進，希望能得到預期之結果。

關鍵詞：結核菌，聚合酶鏈鎖反應，血液

Abstract

Traditional methods for the diagnosis of tuberculosis (TB) lack the sensitivity and rapidity. Amplification of mycobacterial DNA in sputum by polymerase chain reaction (PCR) has become a promising diagnostic tool. However, some patients are unable to provide sputum or other suitable specimens for smear/ culture/PCR, and the processing of infectious sputum may be hazardous. It has been shown that monocytes are activated in patients with pulmonary TB, suggesting that mycobacteria may enter bloodstream to trigger effector cells. Because blood is easy to obtain, we began to study the potential of peripheral blood PCR in the diagnosis of pulmonary and extrapulmonary TB. We drawn 4 ml of blood from patients with suspected pulmonary or extrapulmonary TB. Mononuclear cell layer was separated by Ficoll-Hypaque density centrifugation. Nested PCR was done and the products were examined by ethidium bromide staining and Southern hybridization. The results showed that in 30 peripheral blood samples taken from 26 patients with pulmonary TB, 15

showed a product similar to that produced by the positive control, but Southern blotting did not show positive hybridization, indicating negative results. In 12 peripheral blood samples taken from 9 patients with extrapulmonary TB, 6 showed a product like that produced by the positive control. But Southern blotting failed to show positive hybridization. Possible causes of the failure of PCR assay included: PCR inhibitors, small volume of blood samples, timing of the blood sampling and the inherent difficulty of the assay. We are now still trying to correct possible sources of failures.

Keywords: Mycobacterium tuberculosis, PCR, blood

INTRODUCTION

Diagnostic methods of tuberculosis (TB) have changed very little for many years. Smear/culture of clinical specimens, chest radiographs and serologic tests are the most commonly used methods. These conventional methods have the disadvantages of low sensitivity (smear), low specificity (serology) or being very slow (culture). Recently, amplification of mycobacterial DNA from sputum by polymerase chain reaction (PCR) has become a promising method for rapid diagnosis of pulmonary TB (1-4). In 1996, Condos and colleagues (5,6) reported that they had used peripheral blood to undergo PCR assay in patients with pulmonary TB, and had found the overall sensitivity and specificity for the diagnosis of pulmonary TB was 95% and 89%, respectively. The potential role of the peripheral blood PCR assay in extrapulmoanry TB, however, was not accessed. Because patients with either pulmonary or extrapulmonary TB sometimes have difficulty in providing a proper specimen for smear/culture/PCR, we began to study the peripheral blood PCR assay in patients with suspected pulmonary, as well as in those with extra-pulmonary TB.

MATERIALS AND METHODS

(1) **Peripheral blood samples**:

Peripheral blood samples were taken from patients with suspected pulmonary or extra-pulmonary TB. For conventional diagnosis of TB, three clinical specimens were smeared and cultured.

From each patient 4 ml of blood was collected in tubes containing EDTA, diluted with 4 ml of phosphate buffered saline (PBS). The diluted blood was slowly layered over Ficoll-Hypaque in a ratio of 3:1 (blood:Ficoll). After density gradient centrifugation (700 g, 40 min), the mononuclear cell layer was aspirated, washed and centrifuged (700 g, 10 min). Cells were then suspended in 40 ul of tris-EDTA (TE) buffer, and 60 ul of 10% sodium dodecylsulphate (SDS) and 10 ul proteinase K (20 mg/ml) were added. Cells were incubated at 56°C for 1 hr and at 95°C for 10 min. Then phenol-chloroform extraction of DNA was done.

(2) **PCR**:

Primers homologous to the mycobacterial insertion sequence IS6110 were used. This sequence is specific for the *Mycobacterium tuberculosis* complex (including *M. tuberculosis, M. bovis, M. africanum* and *M. microfti*) and will not detect organisms of *M avium* complex or other atypical mycobacteria.

Nested primer pairs were used. The outer primer, designed for complementarity with the inverse terminal repeat region of the IS6110 insertion sequence consisted of oligonucleotide: 5' TGA ACC GCC CCG GCA TGT CCG GAG ACT 3' The inner primer pair included: sense primer: 5' CGT GAG GGC ATC GAG GTG GC 3', and antisense primer: 5' GCG TAG GCG TCG GTG ACA AA 3' The final PCR product is a 253 bp segment of the IS6110. For the second (nested) PCR, 2.5 ul of the initial PCR product was added as the template DNA. The PCR was carried out for both the outer and the inner primer pair under the following conditions for 30 cycles:

Denature	95°C for 1 min
Annealing	50°C for 1 min
Extension	72°C for 1 min

In both the initial and nested reactions, negative and positive controls were included. The negative control reactions consisted of no template. Positive control was genomic DNA extracted from a clinical isolate of *Mycobacterium tuberculosis* in the mycobacte-

riology laboratory of our hospital. PCR products were analysed by agarose gel (2%) electrophoresis and ethidium bromide staining, under ultraviolet transillumination.

(3) **Southern hybridization**:

After electrophoresis, PCR products were blotted onto Hybond-N nylon membranes, dried and hybridized with a non-radioactive, 253 bp DNA probe (see below for preparation) homologous to the expected PCR product, using the Enhanced Chemiluminescence (ECL) labeling and Detection kit (Amershan, UK). The membrane was washed, the substrate of peroxidase added, the chemiluminescence detected and autoradiographed according to manufacturer's instructions.

(4) **Preparation of the probe**:

PCR product of the positive control was purified using the method of rapid freezing and thawing (7). The purified DNA fragment was then labeled using the ECL labeling kit, according to manufacturer's instructions.

Results

Active pulmonary TB

Of the 30 peripheral blood samples obtained from 26 patients with culture-proven pulmonary TB, 15 showed a product of about 250 bp after nested PCR. However, when being Southern hybridized, none of them showed a positive signal, indicating that they were nonspecific amplification products not from *M. tuberculosis* complex.

Extrapulmonary TB

Of the 12 peripheral blood samples obtained from 9 patients with culture-proven extrapulmonary TB (5 tuberculous pleurisy, 3 tuberculous lymphadenitis, 1 tuberculous meningitis), 6 showed an amplification product of about 250 bp after nested PCR. But none of them showed positive hybridization with the probe after Southern blotting.These bands were nonspecific amplification products, too.

DISCUSSION

PCR assay for the detection of mycobacterial DNA in respiratory specimens such as sputum and tracheal aspirates is an established laboratory methods for the diagnosis of pulmonary TB, with a sensitivity ranging from 66.7% to 84.4%, and a specificity between 98% and 100% (1-4, 8,9). The collection of proper respiratory specimens, however, is sometimes hard if not impossible. Patients, especially children, may have difficulty in expectoration. To obtain proper clinical specimens for smear/culture/PCR is often more difficult in patients with extrapulmonary TB, such as those with tuberculous meningitis and increased intracranial pressure, those with tuberculous peritonitis without ascites, or those with miliary TB who refuse bone marrow biopsy/culture. The infectious nature of the sputum (when collected through induction by nebulized saline or when handled in the laboratory) makes the procedures potentially hazardous. The sputum specimens may contain PCR inhibitors which may be responsible for the sometimes suboptimal sensitivity of sputum PCR assay (1-4,8,9). For these reasons we started this study to investigate the possibility of using peripheral blood PCR assay for the diagnosis of pulmonary and extrapulmonary TB. Because blood is easy to obtain, and in patients with pulmonary TB the activation of peripheral blood monocytes, shown by the increased interleukin-1 production and enhanced expression of interleukin-2 receptors, suggests that mycobacteria enter bloodstream and trigger effector cells (10-12).

But we failed to detect mycobacterial DNA in the peripheral blood mononuclear cells from any patient with pulmonary or extrapulmonary TB. Although we visualized amplification products of about 250 bp in length, which were similar to the PCR products from positive controls, they did not hybridize with the probe on Southern blot analysis. Condos and colleagues found the overall sensitivity and specificity of the peripheral blood PCR assay for the diagnosis of pulmonary TB was 95% and 89%, respectively (5,6). The reasons of our failure to demonstrate a similar effectiveness may be: (1) The presence of PCR inhibitors in the sample. They could have come from residual Ficoll-Hypaque, SDS, proteinase K or phenol. (2) The volume of peripheral blood we drawn

may be too small. Although Condos recommended 4 ml, which was also the volume we drawn, the mononuclear cell layer we obtained after Ficoll-Hypaque density gradient centrifugation was always very thin. After further preparation including the use of proteinase K, SDS, phenol and chloroform, mycobacteria which had entered blood stream, if any, may have been lost. (3) Some of the peripheral blood samples were taken one or two days after the patient had been put on anti-TB chemotherapy, and the number of mycobacteria in bloodstream may have been reduced. (4) The technique may be a tricky one, because there have been no further reports demonstrating similar effectiveness of peripheral blood PCR assays after the initial two by Condos and his group. At the present time we are still trying to improve the methodology by: (1) more excessive washing to remove possible PCR inhibitors such Ficoll-Hypaque; (2) getting a larger volume (12 ml) of blood; (3) obtaining the blood sample before anti-TB chemotherapy was started.

CONCLUSION

We tested the potential usefulness of a peripheral blood PCR assay in the diagnosis of pulmonary and extrapulmonay TB, and found that it could not be used to diagnose either. Possible reasons for the failure were discussed.

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