Detection of Mycobacterial Infection in Paraffin-Embedded Pathologic Tissues by DNA Polymerase Chain Reaction: Comparison with Conventional Histochemical Stain

I-Ping Chiang[1], Lai-Fong Kok[1], Chi-Long Chen[1,2,3]

[1]Department of Pathology, China Medical University Hospital, Taichung; [2]Department of Pathology, Taipei Municipal Wanfang Hospital, [3]Department of Pathology, Taipei Medical University, Taipei, Taiwan.

Objectives. Tuberculosis caused by *Mycobacterium tuberculosis* is one of the most common infectious diseases worldwide. Pathologic confirmation by demonstrating mycobacterial bacillus in tissue section using histochemical stain or culture is the traditional diagnostic method. However, the diagnostic rate by both histochemical stain and culture is low. Polymerase chain reaction (PCR) is able to detect trace amounts of nucleic acid in paraffin sections. We detected mycobacterial DNA in pathological samples by PCR and compared the results with those of histochemical stain and culture.

Methods. Formalin-fixed paraffin-embedded tissue sections of 16 acid-fast positive and 17 acid-fast negative tissue samples showing granulomatous inflammation were retrieved retrospectively for PCR study.

Results. PCR detected mycobacterial DNA in 14 out of 16 acid-fast positive and 14 out of 17 acid-fast negative samples. The sensitivity and specificity for detecting *Mycobacterium* by PCR were much higher than acid-fast stain and culture.

Conclusions. PCR method was very useful in detecting mycobacterial infection in pathologic sections. We believe that the establishment of the PCR method in the department of pathology will improve the quality of clinical and pathologic diagnosis of tuberculosis. **(Mid Taiwan J Med 2005;10:25-31)**

Key words

Mycobacterium, PCR, tuberculosis

INTRODUCTION

Tuberculosis caused by *Mycobacterium tuberculosis* is one of the most common infectious human diseases worldwide [1-3]. The prevalence rate of tuberculosis in the general population has been increasing progressively for the past 10 years [1-3], particularly in patients with secondary immunodeficiency and acquired immunodeficiency syndrome (AIDS) [4]. Early and correct diagnosis of tuberculosis by mycobacterial culture or pathological examination is essential in prevention and treatment of tuberculosis in clinical practice.

Typically, *Mycobacterium* causes granulomatous inflammation of tissue, which presents as caseation necrosis, aggregates of epithelioid cells, and frequently multinucleated Langhans' giant cells. Tuberculosis is confirmed pathologically by the presence of mycobacterial bacillus in tissue sections using histochemical stain, the Ziehl-

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Address reprint requests to : Chi-Long Chen, Department of Pathology, Taipei Medical University, 250 WuXing Street, Taipei 110, Taiwan.

Table 1. The sequences of primers for polymerase chain reaction (PCR) used in this study

For *Mycobacterium*	
First run	5'-ACC AAC GAT GGT GTG TCC AT-3'
	5'-CTT GTC GAA CCG CAT ACC CT-3'
Nested	5'-GAG ATC GAG CTG GAG GAT CC-3'
	5'-AGC TGC AGC CCA AAG GTG TT-3'
For β-actin	5'-ATC ATG TTT GAG ACC TTC AA-3'
	5'-CAT CTC TTG CTC GAA GTC CA-3'

Neelsen stain and bacterial culture of the specimen. Direct detection of the bacteria by acid-fast stain on paraffin tissue section is a rapid method, but it is not very sensitive. It has been reported that at least 10^5 bacilli per milliliter are required for detecting the bacilli by acid-fast stain; furthermore, the detection rate of tuberculosis by histochemical stain has been shown to range from 22% and 78% according to different laboratories [5-7]. Culture is essential for definite diagnosis of microbial infection; however, the necessary incubation period takes from 6 to 8 weeks before any result can be obtained. Moreover, the sensitivity of culture is also relatively low [6,8].

A tool for early detection and rapid identification of the mycobacterial bacillus is valuable in patients suspected of having tuberculosis. Since the development of molecular techniques and polymerase chain reaction (PCR) in 1986 [9], detection of nucleic acid of pathogenic microorganism using paraffin-embedded pathologic tissues has been possible [10-15]. In this study, we used PCR method to detect mycobacterial DNA in formalin-fixed paraffin-embedded pathologic sections retrieved from our pathology file; the rate of detection by PCR was then compared with the rate of detection by conventional histochemical stain, and mycobacterial culture.

MATERIALS AND METHODS

Thirty-three formalin-fixed paraffin-embedded tissue specimens from 33 patients with granulomatous inflammation obtained from July 2001 to June 2002 were retrieved from the files of the Department of Pathology, China Medical University Hospital for extraction of nucleic acid followed by polymerase chain reaction (PCR) experiments. The tissues included 10 lymph node samples; soft tissue: 6; lung, 5; skin, 2; and pleura, 3. The other tissues included small intestine, testis, prostate, bursa, synovium, fallopian tube, and pericardium. The Ziehl-Neelsen acid-fast stain had been performed on all 33 tissue samples. Of them, results of mycobacterium culture were available in 24 patients, 13 in the acid-fast positive group and 11 in the acid-fast negative group. The clinical data and clinical presentations were collected from charts of all patients.

For extraction of DNA from these samples, three continuous 8-μm thick sections were cut from each paraffin-embedded tissue block and placed into a clean autoclaved Eppendorf tube. To avoid cross contamination of the specimens, the microtome blade was changed and the microtome was cleaned with xylene and alcohol after each block was cut. Tissues were then deparaffinized by xylene and centrifuged. The supernatant was removed and the pellet was re-suspended in 300 μL of PCR lysis buffer containing proteinase K (200 μg/mL, Boeringer-Mainheimn, Germany), 50 mM Tris-HCl, 0.5% Tween 20, carrier glycogen 100 μg/mL, and 1.5 mM $CaCl_2$. The mixture was then incubated for 1 to 2 days at 60˚C, followed by the addition of 200 μL of chelex-100 (BIO-RAD) and boiling for 10 min. The samples were then centrifuged at 13,000 rpm for 10 min. DNA was extracted with a DNA extraction kit (Boerhinger-Mainheimn, Germany) according to the manufacturer's instructions.

The primers used for PCR amplification are shown in Table 1 [16]. Positive control samples of *M. tuberculosis* consisted of extracted DNA from cultured bacilli (kindly provided by Dr. Cheng-Hsiang Hsiao, Department of Pathology, National Taiwan University Hospital). The negative control in each reaction consisted of ionized water. The β-actin gene served as the internal control for monitoring successful DNA extraction; the gene was amplified to yield a 316-bp product. Nested PCR amplified the conserved region among *Mycobacterium* species to increase the detection rate of mycobacterial DNA. For

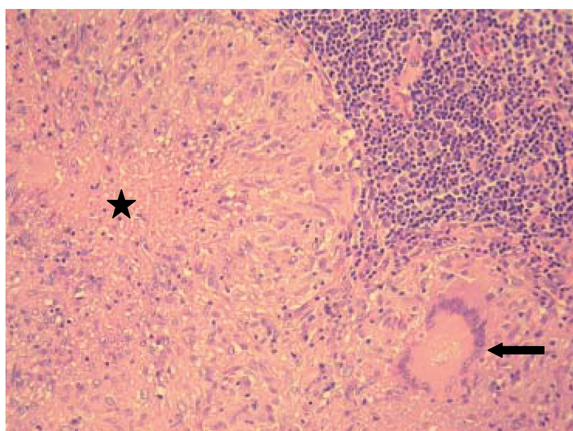

Fig. 1. Typical histological findings of granulomatous inflammation in lymph node with mycobacterial infection. The granulomatous lesion was composed of aggregated epithelioid cells, lymphocytes, Langhans' multinucleated giant cells (arrow), and central caseous necrosis (asterisk). (H&E, original magnification 200 ×)

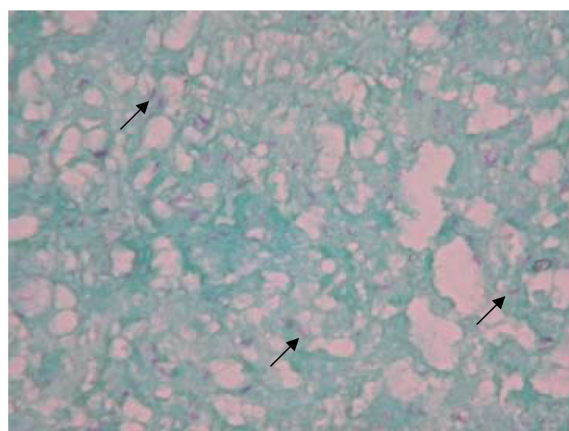

Fig. 2. The Ziehl-Neelsen acid fast stain method showed slender acid-fast positive bacilli (arrows) in granulomatous areas of granulomatous inflammation. (Ziehl-Neelsen method with light green counterstain, original magnification 400 ×)

nested amplification, 2 μL of first-round PCR product was mixed with 23 μL of PCR mixture.

For the first round of PCR, primary denaturation was performed at 95˚C for 5 min followed by 30 cycles of amplification with denaturation at 94˚C for 1 min, annealing at 60˚C for 1 min, and extension at 72˚C for 1 min. A final cycle of extension at 72˚C for 10 min was stopped by cooling the reactant at 4˚C. For the second round of PCR, the conditions were the same as for the first round, except that the amplification cycles were increased to 35 times. All of the reactions were performed in a Biometra thermocycler (Biometra, Germany).

Finally, the PCR products were fractionated on 3% agarose gel in 0.5X TAE buffer, stained with ethedium bromide, and visualized and photographed under UV light. For *Mycobacterium*, the products of nested PCR were 383 base pairs.

RESULTS

The study included 33 samples from19 males and 14 females ranging in age from 5 to 75 years. The clinical presentations included enlarged painless nodular lesions in the neck, inguinal, soft tissue, and skin for variable periods. All patients with lung diseases presented with chronic cough accompanied by pulmonary opaque lesions in image studies. All patients with pleural lesions presented with pleural effusion. One of the patients had late stage acquired immunodeficiency syndrome (AIDS).

Pathologic changes in most of the biopsy specimens revealed typical granulomatous inflammation with or without caseation necrosis. Multinucleated Langhans' giant cells were found in the granulomatous area of most tissue samples (Fig. 1). Tuberculosis was diagnosed in 16 patients because the Ziehl-Neelsen acid-fast stain revealed acid-fast-positive slender bacilli in varying amounts (16/33, 48.5%) (Fig. 2). Granulomatous inflammation was diagnosed in 17 patients because no acid-fast-positive bacilli were found in the sections by the Ziehl-Neelsen method. There was no difference in morphology of tissues with granulomatous inflammation between acid-fast positive and acid-fast negative staining.

The samples were divided into acid-fast positive (n = 16) and acid-fast negative (n = 17) groups. Culture for *Mycobacterium* was performed on most samples from both groups; 4 of the 13 (4/13, 30.8%) in the acid-fast positive group were positive for *Mycobacterium* while only 2 of the 11 (2/11, 18.2%) in the acid-fast negative were positive. The overall positive culture rate for *Mycobacterium* was 25% (6/24).

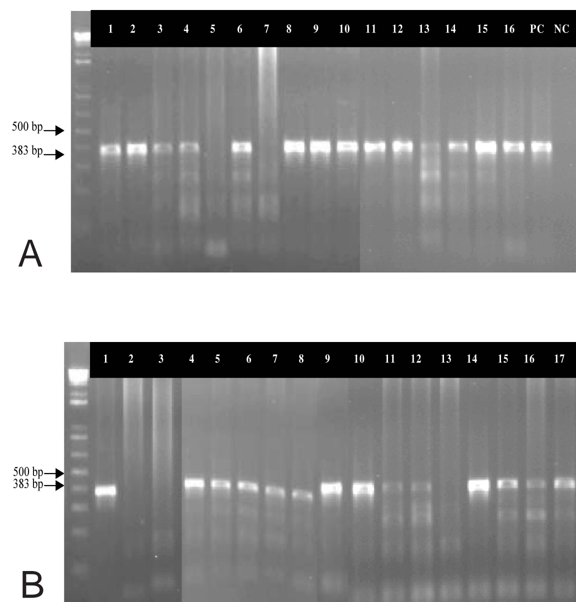

Fig. 3. PCR for mycobacterial DNA showed products of 383 bp indicating presence of *Mycobacterium*. A: Fourteen of 16 acid-fast positive cases were positive for mycobacterial DNA. B: Fourteen of 17 acid-fast negative cases were positive for mycobacterial DNA. No mycobacterial DNA was detected by PCR in cases 5 and 7 in the acid-fast positive group and cases 2, 3, and 13 in the acid-fast negative group. PC = positive control; NC = negative control.

PCR revealed that 14 of 16 samples (14/16, 87.5%) in the acid-fast positive group (Fig. 3A) and 14 of 17 samples (14/17, 82.4%) in the acid-fast negative group had PCR products of 383 bp (Fig. 3B). The overall PCR-positive rate was 84.8% (28/33). The specificity of PCR in this study was 83.3% (15/18). The clinical data and results of acid-fast stain, culture and PCR for *Mycobacterium* are presented in Table 2.

DISCUSSION

The definitive diagnostic methods for confirming tuberculosis are acid-fast stain and bacterial culture. The diagnostic rate of tuberculosis by histochemical stain ranges from 22% to 78% [5-7]. The necessary incubation period takes from 6 to 8 weeks before any result of mycobacterial culture can be obtained. Since the introduction of polymerase chain reaction (PCR) in detecting trace amounts of nucleic acid [9], detection of trace amounts of mycobacterial DNA in formalin-fixed paraffin-embedded tissue sections by PCR has been reported in several studies [10-15].

In this study, we established the PCR method for detecting mycobacterial DNA in paraffin-embedded tissue sections in acid-fast positive and acid-fast negative tissues using published DNA sequences of *Mycobacterium* by PCR and compared the results with those of culture and histochemical stain. As shown in Table 2, our results showed that PCR had a very high rate of detecting mycobacterial DNA in paraffin-embedded tissue sections with granulomatous inflammation, which was in good agreement with previous reports [14-19]. The sensitivity of PCR in detecting mycobacterial infection in this study was 84.8% (28/33) and the specificity was 83.3% which were consistent with previous reports [5,6,10-15,17-19]. The sensitivity of the PCR method in detecting *Mycobacterium* was much higher than the sensitivity of acid-fast stain (84.8% *vs* 48.5%) or culture (84.8% *vs* 25%).

PCR detected mycobacterial DNA in 82.4% (14/17) of acid-fast negative samples. There are two possible reasons which may explain this phenomenon. First, acid-fast stain is a method with relatively low sensitivity to detect mycobacterial bacillus in paraffin sections. Second, PCR is a very sensitive and powerful detection method, requiring very trace amounts of DNA in tissue; in contrast, acid fast stain requires at least 105 bacilli per milliliter to accurately detect *Mycobacterium* [5-7]. Also, fragmented mycobacterial DNA can only be detected by PCR but cannot be detected by acid-fast stain.

In this study, PCR failed to detect mycobacterial nucleic acid in two samples which were acid-fast positive; one of them was positive for mycobacterial culture. The reason for this discrepancy might be because no mycobacterial DNA or intact mycobacterial DNA fragments were left in the studied sections since only three thin paraffin sections were used in each PCR experiment. In addition, fixatives used in routine tissue processing of paraffin embedded tissue sections may affect the PCR results [20].

Other mycobacterial species, such as *M. bovis, M. microti, M. africanum, M. avium, M.*

Table 2. Clinicopathological data, results of Mycobacterial culture, and results of mycobacterial polymerase chain reaction (PCR) in acid-fast positive and acid-fast negative cases in this study

	Age (yr)	Sex	Organ	Culture	PCR
Acid-fast (+)					
1	46	F	Lymph node	–	+
2	67	F	Intestine	–	+
3	50	M	Lymph node	ND	+
4	47	M	Lung	–	+
5	40	F	Lymph node	–	–
6	61	M	Testis	ND	+
7	45	F	Lung	+	–
8	40	F	Soft tissue	ND	+
9	32	M	Soft tissue	–	+
10	67	F	Bursa	–	+
11	44	F	Lung	–	+
12	59	M	Pleura	–	+
13	75	M	Prostate	–	+
14	30	M	Soft tissue	+	+
15	75	F	Synovium	+	+
16	38	M	Lymph node	+	+
Acid-fast (–)					
1	56	M	Lymph node	ND	+
2	19	F	Fallopian tube	–	–
3	46	M	Lung	–	–
4	64	F	Pleura	–	+
5	16	F	Lymph node	–	+
6	18	M	Soft tissue	ND	+
7	5	M	Soft tissue	–	+
8	74	M	Soft tissue	–	+
9	48	M	Pericardium	+	+
10	71	F	Lung	–	+
11	29	M	Lymph node	–	+
12	28	M	Skin	–	–
13	33	F	Lymph node	+	+
14	26	M	Skin	ND	+
15	68	M	Lymph node	ND	+
16	48	F	Lymph node	ND	+
17	41	M	Pleura	ND	+

ND = not done.

paratuberculosis, and other slow-growing mycobacteria, may cause granulomatous inflammation in humans [21]. The PCR primers used in this study covered the common sequences of different mycobacteria. Multiplex PCR using paired primers for specific mycobacterial species may improve sensitivity [22].

In conclusion, PCR was very sensitive for detecting trace amounts of mycobacterial DNA in formalin embedded paraffin samples. Since *M. tuberculosis* is still a major medical problem in Taiwan, we highly recommend that a molecular diagnostic laboratory be established in the department of pathology to improve the clinical and pathologic diagnosis of tuberculosis.

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以DNA聚合酶鏈反應法自病理石蠟組織切片偵測分枝桿菌感染：與傳統組織化學染色結果之比較

江宜平[1] 郭麗芳[1] 陳志榮[1,2,3]

中國醫藥大學附設醫院 病理部[1]

台北市立萬芳醫院 病理科[2]

台北醫學大學 病理科[3]

目的 結核分枝桿菌引起的結核病是世界上最重要的傳染病之一。由組織作分枝桿菌培養或自組織的病理切片作抗酸染色證實有分枝桿菌是傳統的確診方法，但是兩種傳統診斷方法的診斷率都很低，而且相當耗費時間與人力。自1986年核酸的聚合酶鏈反應法被開發以來，這種能快速且有效偵測微量核酸的方法就被廣泛的使用於特定核酸片段的偵測，包括各種微生物之核酸。

方法 本研究自16例抗酸染色陽性病例和17例抗酸染色陰性病例的福馬林固定石蠟包埋的病理組織切片中，以聚合酶鏈反應法偵測分枝桿菌DNA片段並與組織化學染色結果做比較。

結果 本研究中以聚合酶鏈反應法發現16例抗酸染色陽性病例和17例抗酸染色陰性病例中各有14例在石蠟組織切片中有分枝桿菌DNA片段。以聚合酶鏈反應法偵測分枝桿菌DNA片段的靈敏性和特殊性，顯然比傳統診斷結核菌感染的細菌培養和抗酸染色要高。

結論 以靈敏的聚合酶鏈反應法偵測微量DNA片段在偵測病理切片中的分枝桿菌是非常有效的方法，本文強烈建議在病理科室設立高品管的分子病理實驗室，將可以大幅增加結核菌感染的診斷率。（中台灣醫誌 **2005;10:25-31**）

關鍵詞

分枝桿菌，核酸聚合酶鏈反應，結核病

聯絡作者：陳志榮

地　　址：110台北市信義區吳興街250號

台北醫學大學　病理科

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