Detection and Identification of *Mycobacterium* Species by Polymerase Chain Reaction (PCR) from Paraffin-Embedded Tissue Compare to AFB Staining in Pathological Sections

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Background: *Polymerase chain reaction (PCR) is a recent, rapid and reliable method in the detection of causative organism. The authors tried to determine the possibility of using PCR technique as an alternative way to detect mycobacterial DNA from paraffin-embedded tissue to avoid repeated biopsy from the patient.*
Material and Method: *Paraffin-embedded tissue blocks, the corresponding histopathologic slides, and cultural results were retrospectively searched for according to the patient's records, the granuloma clinic, Department of Dermatology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand from 1994-2000. One hundred and thirty-one tissue blocks and slides were found but only 120 cultural results were retrieved. Histologic sections were reviewed for AFB findings and PCR was done using 16S rRNA sequences to detect M. tuberculosis by one-tube nested technique and multiplex PCR for M. marinum and M. fortuitum complex.*
Results: *The causative organisms were identified by AFB staining in pathologic sections 31.29%, by PCR 35.87%, and by culture 30.00% of tested samples. The sensitivity of PCR when compared to AFB result was 29.26%, specificity 61.11% but when compared to cultural results, the sensitivity of PCR was 66.67% and AFB sensitivity was 41.66% with specificity 76.19% and 72.61% respectively.*
Conclusion: *The low sensitivity of the PCR method may be due to formalin fixation, deparaffinization process, DNA extraction method, the use of 16S rRNA-based primers and the length of the expected product, and the tissue type that may have Taq polymerase inhibitor. Therefore, PCR should be used to augment the information of the conventional method in the diagnosis of mycobacterial infection.*

Keywords: *Mycobacterial skin infections, Polymerase Chain Reaction, Acid fast bacilli*

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The diagnosis of mycobacterial skin infections poses a big problem in clinical practice. The incidence of mycobacterial infections, tuberculous and non tuberculous, has increased dramatically both in industrialized and developing countries[1]. Clinical manifestations are not specific enough for diagnosis alone without laboratory support. Cultural method remains the gold standard, but it takes up to six weeks to obtain a result and the microscopic detection of acid fast bacilli requires large numbers of mycobacteria in the sample. In particular cases with unexpected histopathological findings, there is no fresh specimen left for culture, so that diagnosis depends on a morphological examination and microscopic detection of acid fast bacilli. Polymerase chain reaction (PCR) for detection of mycobacteria in formalin-fixed, paraffin-embedded tissue is a recent technique that could provide a more rapid, reliable and species specific method which may be a guide for appropriate management.

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In the present study, the authors tried to detect mycobacterial DNA by the PCR method from routinely processed paraffin-embedded tissue and compared the PCR results with microscopic detection of acid fast bacilli in a pathologic section.

Material and Method

The records of patients who attended the granuloma clinic, Department of Dermatology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand from 1994-2000 were analyzed. The clinical data (age and sex of the patient, site, number and morphology of skin lesions), pathological number, and cultural results were retrieved from the patients' records. The patients who attended the clinic had various forms of asymptomatic and slowly progressive skin lesions as well as cervical lymphadenitis with draining sinus (scrofuloderma-like). Skin biopsy was performed at the affected site for histopathological and microbiological study. The tissue for pathological study was fixed in formalin and embedded in paraffin as routine standard procedure. These paraffin-embedded tissue blocks and the corresponding histopathologic sections were searched.

The histopathologic sections were reviewed blindly for acid fast bacilli (AFB) detection by 2 independent observers. In cases with discordant findings, the sections were reexamined by the two observers together in order to come to an agreement.

Sensitivity, specificity and accuracy of PCR and staining method in detection of mycobacterial infection were calculated using cultural result as a gold standard. Then sensitivity, specificity and accuracy of PCR were calculated again by using AFB result as a gold standard.

Preparation of Paraffin block

Possible contaminated DNA at the surface of the tissue block was decontaminated by the following procedures. The chosen paraffin blocks were cleaned by whipping with 70% alcohol. The side of the embedded tissue was put up in the safety cabinet 60 cm away from the UV-lamp and exposed to UV for 90 min. After UV decontamination of the surface, each paraffin block was kept in each small plastic sealed bag until they were sliced by microtome with 3 mm thick for 30 slices. These specimens were then deparaffinized and used as DNA sources.

Deparaffinization of tissue section

Paraffin was removed by extraction with xylene. A 1.5 ml Eppendorf tube containing 30 pieces of 3 mm thick microtomic section from each block was vortexed after being filled with 1,200 ml xylene. The tube was centrifuged at 12,000 rpm (Hermle ZK 380, Germany) for 5 min to collect the cell pellet. After discarding xylene, the cell pellet was consecutively extracted with xylene plus absolute ethanol, 90%, 80% and 70% ethanol respectively with repetition of 5 min vortexed and centrifuged for 5 min in each step as previously described.

DNA extraction from cell pellet

The cell pellet obtained from each tissue block was used for DNA extraction using QIAamp® DNA Mini Kit (QIA GEN lot. No 51304, Germany) and proceeded according to the manufacture's instructions. Briefly the cell pellet was incubated with 180 ml ATL and 20 ml proteinase K (10mg/ ml) at 56 C overnight. Two hundred microliters of AL was added to the tube. It was vortexed and incubated at 70 C for 10 min, then 200 ml absolute ethanol was added. The tube was vortexed and centrifuged at 8,000 rpm for 1 imn to collect splash. All the suspension was pipetted into a QIAamp spin column and spinned down at 8,000 rpm for 1 min, and then the collection tube was replaced with a new one. Five hundred microliters of buffer AW1 were filled in the column and centrifuged again at 8,000 rpm 1 min and the flow through liquid was discarded. The steps were repeated once with 500 ml buffer AW2 and centrifuged at 13,000 rpm for 3 min. DNA from QIAamp spin column was eluted with 50 ml buffer AE, incubated at 70 C 5 min and spinned down at 8,000 rpm for 1 min. The eluted DNA solution was used as the DNA template in one-tube nested and multiplex PCR.

One-tube nested and multiplex PCR

Amplification of nucleic acid was done by 2 steps. The first step was the one-tube nested method using 16S rRNA sequence as the target to detect a 555 bp DNA fragment for common *Mycobacterium* species and 306 bp DNA fragment for *M. tuberculosis* complex. The specimen that had both 555bp and 306 bp fragment or had only 306 bp fragment was diagnosed as *M. tuberculosis* infection. The specimen with only 555 bp fragment was detected proceeded to the second step, which was multiplex PCR to detect 298 bp product for *M. marinum* and 216 bp product for *M. fortuitum-chelonae* complex. The primers 16SOL-16SOR and 16SIL-16SIR were used in one-tube nested PCR for detection of *Mycobacterium tuberculosis* complex,

whereas 16SOL-16SOR, ACML-ACMR and SML1 primers were used in one-tube multiplex PCR for detection of *M. marinum* and *M. fortuitum* complex. Both PCR systems could reveal specific amplified product from 100 fg each of purified DNA. The sequences of the primers and the temperature profiles for amplification were previously described by Gengvinij, et al, and Chaiprasert et al (Application for Patents on June 29, 2001 and No. to Department of Intellectual Property). Amplification was accomplished in DNA Thermal Cycler Perkin 480. The amplified products were detected with 4% Nusieve-agrose gel electrophoresis in Mupid II minigel apparatus (Japan) at 100V for 300 min, staining with ethidiumbromide (1 mg/ml), visualized on UV-transilluminator and photographed with polaroid camera.

Result

One hundred and ten cases, 58 males and 52 females, of suspected mycobacterial infections were found. All of these suspected cases had one or multiple chronic, asymptomatic, slow progressive skin lesions without systemic symptoms, or had cervical lymphadenitis. Leprosy cases were excluded. Deep fungal infections confirmed by histopathologic and culture results were also excluded. The age of the patients ranged from 2 to 73 years of age (mean 34.6 years of age). One hundred and thirty-one paraffin-embedded tissue blocks were retrieved from these 111 cases because 16 cases were biopsied more than once. PCR and AFB staining were done in all cases but only 120 specimens were simultaneously cultured at the same time that the specimens were processed for pathological study.

The overall result is shown in Table 1. Evidence of mycobacterial infection was confirmed by AFB finding 31.29%, by culture 30%, and by PCR 35.87% of cases. Among these cases (Table 2), the evidence of mycobacterial infection could be confirmed by AFB alone in 16.03% (21 of 131), by culture alone in 9.16% (12 of 131), and by PCR alone 19.84% (26 of 131). Evidence of mycobacterial infection was not detected by using all these 3 methods in 32.82% (43 of 131). In the group that had culture results and mycobacterial DNA was amplified by PCR method (row 1 and row 5 of Table 2), the sensitivity of PCR result could be checked. PCR and cultural results were perfectly matched in 4 cases (*M. marinum* 1 case, *M. fortuitum* 3 cases). Ten cases that PCR results were positive for *M. tuberculosis* complex and *M. marinum* had culture identified as *M. tuberculosis* in 2 cases, *M. marinum* 4 cases, *M. fortuitum* 2 cases, and *M. chelonae* 2 cases. These were acceptable results because the selected primers that could amplify 306 bp product, could cross amplify some strains of *M. fortuitum* complex and *M. marinum* as in the previous report (Gengvinij, *et al*)[9]. One sample had only 555 bp product amplified, and the culture grew *M. szulgai*. This was also an acceptable result since 555 bp product could be amplified from some strains of *M. szulgai*. Only 1 unexpected result in which PCR was positive for *M. fortuitum* complex but the culture grew *M. tuberculosis*.

Because PCR was the test method in the present report and culture was not done in all cases, the authors compared the PCR results with AFB results (Table 3). The authors also compared AFB and PCR results using culture results as the gold standard in Table 4 and 5.

Table 1. Overall results of mycobacterial infections detected by 3 methods

	AFB (%)	PCR* (%)	Culture* (%)
Positive	41 (31.29)	47 (35.87)	36 (30.00)
Negative	90 (68.71)	84 (64.13)	84 (70.00)
Total	131 (100.00)	131 (100.00)	120 (100.00)

* for all tested species, AFB = acid fast bacilli detected in pathologic section, PCR = polymerase chain reaction

Table 2. Comparative result between the 3 methods

			Number (%)
AFB +	PCR +	Culture +	7 (5.35)
AFB +	PCR +	Culture -/ND	5 (3.82)
AFB +	PCR -	Culture +	8 (6.11)
AFB +	PCR -	Culture -/ND	21 (16.03)
AFB -	PCR +	Culture +	9 (6.87)
AFB -	PCR +	Culture -/ND	26 (19.84)
AFB -	PCR -	Culture +	12 (9.16)
AFB -	PCR -	Culture -/ND	43 (32.82)
	Total		131 (100.00)

AFB = acid fast bacilli detected in pathologic
PCR = polymerase chain reaction, ND = not done
Row 4 mycobacteria detected by AFB alone
Row 6 mycobacteria detected by PCR alone
Row 7 mycobacteria detected by culture alone

Discussion

The detection rate of mycobacteria from clinical specimens, by all 3 methods (AFB staining, PCR, and culture) in the present study yielded around 30% which is in the same range as a previous report[2]. The low detection rate was still a problem in the present study. AFB findings and cultural method require skilled

Table 3. Results of PCR amplification compared to AFB

PCR*	AFB		Total (%)
	Positive (%)	Negative (%)	
Positive	12 (29.27)	35 (38.89)	47 (35.88)
Negative	29 (70.73)	55 (61.11)	84 (64.12)
Total	41 (100.00)	90 (100.00)	131 (100.00)

Sensitivity 29.26% (12 of 41), specificity 61.11% (55 of 90), accuracy 51.14%, positive predictive value 25.53% (12 of 47), negative predictive value 65.42% (55 of 84)
* for all tested species

Table 4. Results of PCR amplification and cultural results

PCR*	Culture		Not cultured (%)	Total (%)
	Positive (%)	Negative (%)		
Positive	24 (66.67)	20 (23.81)	3 (27.27)	47 (35.88)
Negative	12 (33.33)	64 (76.19)	8 (72.73)	84 (64.12)
Total	36 (100.00)	84 (100.00)	11 (100.00)	131 (100.00)

sensitivity 66.67% (24 of 36), specificity 76.19% (64 of 84), accuracy 73.33%, positive predictive value 54.54% (24 of 44), negative predictive value 84.21% (64 of 76)
* for all tested species

Table 5. Result of AFB findings in pathologic section and culture results

AFB	Culture		Not cultured (%)	Total (%)
	Positive (%)	Negative (%)		
Positive	15 (41.67)	23 (27.38)	3 (27.27)	41 (31.30)
Negative	21 (58.33)	61 (72.62)	8 (72.73)	90 (68.70)
Total	36 (100.00)	84 (100.00)	11 (100.00)	131 (100.00)

sensitivity 41.66% (15 of 36), specificity 72.61% (61 of 84), accuracy 63.3%, positive predictive value 39.47% (15 of 38), negative predictive value 74.39% (61 of 82)

personnel and a large number of organisms in the sample[1]. It has been described in the literature that the effectiveness of PCR with formalin-fixed, paraffin-embedded tissue is impaired by multiple interacting factors, including the type of fixative[2-5], the fixation time[3], the DNA extraction procedure, the length of PCR target, the concentration of target DNA amplified, and PCR protocol[6]. Formalin-fixation gave a slightly less satisfactory result compared to ethanol or Histo-choice (new fixative)[4], and formalin might degrade DNA that could cause PCR inhibition[7,8]. Prolonged fixation time is another factor that has been reported as causing DNA structural changes[8].

The PCR system the authors used in the present study had a detection limit of 100 fg (equivalent to 20 mycobacterial cells)[9] which was in the same range of detection limit as the culture method but PCR can also detect non-viable mycobacteria that cannot be grown by culture[1]. This aspect makes the PCR method more useful in the diagnosis of partially treated cases in which the number of viable organisms is low.

According to type of tissue tested, it has been reported that, in some tissue types such as the lung and spleen, DNA could be amplified in every case; an amplification sensitivity of 75% being obtained in lymph node samples. Mycobacterial DNA in liver samples could not be amplified at all[6]. The negative result of PCR may also be due to the presence of inhibitors detected in the tissue itself. Chan *et al* have reported that inhibitors were detected more frequently in extrapulmonary than in pulmonary tissue[10]. Solid specimens such as skin and lymph nodes also cause difficulties in DNA extraction and discordant results in different methods may be caused by non-homogenous distribution of AFB in the specimen[11].

Many genes and sequences are used as target DNA for amplification. They are repetitive and non-repetitive DNAs. IS *6110* is one of the frequently used repetitive sequences while 16S rRNA is a non-repetitive sequence[9,12]. IS *6110* is a good target for amplification because it presents in high copy number but some strains of *M. tuberculosis* do not contain IS *6110* sequence in their genomes[2,13]. The 16S rRNA is an essential gene in every organism. In the authors' previous study[9,12], one-tube nested PCR using sequences based on 16S rRNA gene and successfully detected 21 species and 41 strains of mycobacteria (555 bp product) and 306 bp products were found in 59 strains of M. tuberculosis complex. Cross amplification was observed *with M. marinum, M. ulcerans,* and some strains of *M. fortuitum* complex[9]. The same result occurred again in this study. Many recent studies [1,2,6,8,14-16] have reported very high sensitivity (80-100%) and specificity used IS *6110* sequence to detect shorter DNA product (123-245 bp)with a limitation unit of 10 fg (equivalent to 2 *M. tuberculosis* cell).

In conclusion, PCR should be used in combination with other diagnostic methods as well as clinical correlation to reach appropriate treatment for the patients.

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การตรวจหาเชื้อกลุ่มมัยโคแบคทีเรียด้วยวิธี Polymerase Chain Reaction (PCR) จากตัวอย่างเนื้อเยื่อที่ฝังในขี้ผึ้งพาราฟินเปรียบเทียบกับผลการตรวจหาเชื้อโดยวิธีย้อมสีทนกรดจากสไลด์พยาธิวิทยา

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ในการวินิจฉัยการติดเชื้อกลุ่มมัยโคแบคทีเรีย จำเป็นต้องตรวจพบเชื้อก่อโรคโดยวิธีการเพาะเชื้อ หรือการย้อมสีทนกรด แต่ในบางครั้งวิธีการเพาะเชื้ออาจมีข้อจำกัดเนื่องจากมิได้เตรียมเนื้อเยื่อไว้ก่อน เพื่อที่จะหาวิธีช่วยในการวินิจฉัยและบอกชนิดของเชื้อ โดยผู้ป่วยไม่ต้องถูกตัดชิ้นเนื้อซ้ำเป็นครั้งที่สอง คณะผู้วิจัยได้ทำการตรวจหาดีเอ็นเอของเชื้อโดยวิธี Polymerase Chain Reaction (PCR) จากชิ้นเนื้อที่ฝังไว้ในขี้ผึ้งพาราฟิน ซึ่งเป็นการเตรียมเนื้อเยื่อเพื่อตรวจทางพยาธิวิทยา เปรียบเทียบผลการตรวจโดยวิธี PCR กับการตรวจพบเชื้อโดยวิธีย้อมสีทนกรดในสไลด์พยาธิวิทยา และได้ทำการศึกษาย้อนหลังเพื่อเปรียบเทียบความไวและความจำเพาะของวิธีการทั้งสองโดยใช้ผลการตรวจเพาะเชื้อของผู้ป่วยรายนั้นเป็นหลักด้วย

คณะผู้วิจัยทำการตรวจหาเชื้อโดยวิธีย้อมสีทนกรด (AFB) และตรวจหา ดีเอ็นเอด้วยวิธี PCR จำนวนทั้งสิ้น 131 ตัวอย่างในจำนวนนี้ได้รับการเพาะเชื้อร่วมด้วย 120 ตัวอย่าง ใช้วิธี one-tube nested PCR เพื่อตรวจหา Mycobacterium tuberculosis และใช้วิธี Multiplex PCR ในการตรวจหา M. marinum และ M. fortuitum-chelonae complex โดยใช้ 16S rRNA gene เป็นเป้าหมาย สามารถตรวจพบเชื้อด้วยวิธีย้อม AFB ร้อยละ 31.29 ด้วยวิธี PCR ร้อยละ 35.87 และวิธีเพาะเชื้อร้อยละ 30 ของตัวอย่างส่งตรวจทั้งหมด พบว่าความไวของวิธี PCR เมื่อเทียบกับการตรวจพบ AFB ได้เพียงร้อยละ 29.26 มีความจำเพาะร้อยละ 61.11 แต่หากใช้ผลการเพาะเชื้อเป็นมาตรฐานในการเปรียบเทียบความไวและความจำเพาะของวิธี PCR และการตรวจพบ AFB ในสไลด์พบว่าความไวคิดเป็นร้อยละ 66.67 และ 41.66 ตามลำดับ ความจำเพาะคิดเป็นร้อยละ 76.19 และ 72.61 ตามลำดับ การที่ความไวของวิธี PCR มีค่าต่ำอาจเนื่องมาจากปัจจัยต่อไปนี้คือ การใช้น้ำยา formalin ในการดองเนื้อเยื่อ, กรรมวิธีในการสกัดขี้ผึ้งพาราฟินออกจากเนื้อเยื่อ และวิธีการสกัดสายดีเอ็นเอออกจากเนื้อเยื่อ ทำให้ความเข้มข้นของสายดีเอ็นเอลดลง, การใช้ 16S rRNA เป็นเป้าหมายในการเพิ่มจำนวนสายดีเอ็นเอ และชนิดของเนื้อเยื่อซึ่งเนื้อเยื่อผิวหนังอาจมีตัวยับยั้งการทำงานของเอนไซม์ที่ทำหน้าที่สร้างสายดีเอ็นเอเพิ่มขึ้น (Taq polymerase inhibitor) ดังนั้นจึงสรุปว่าวิธีการตรวจหาเชื้อกลุ่มมัยโคแบคทีเรียด้วยวิธี PCR เป็นเพียงวิธีหนึ่งที่จะช่วยในการวินิจฉัย ร่วมกับการตรวจหาเชื้อด้วยวิธีอื่นเท่านั้น ไม่สามารถใช้เป็นหลักในการวินิจฉัยโรคได้