

Evaluation of DNA extraction by boiling method for PCR-based detection of *Pneumocystis jirovecii* in sputum samples from immunocompromised patients

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Problem/background : *Pneumocystis jirovecii* can be detected by staining methods but the sensitivity is low. Polymerase chain reaction (PCR) technique is more sensitive and accurate but it requires high quality of DNA template. Although DNA prepared by boiling method may not generate high quality of DNA, it has been used for extraction of DNA from several organisms such as malaria, several bacteria and fungi for PCR detection with favorable results. Because boiling method is not time-consuming and inexpensive, we evaluated whether or not DNA of *P. jirovecii* could be prepared from sputum samples and amplifiable by diagnostic PCR. Results were also compared with those obtained from a commercial kit and Giemsa staining method.

Objective : To develop a rapid boiling method for detection of *P. jirovecii*.

Design : A descriptive study.

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Setting : *Department of Parasitology, Faculty of Medicine, Chulalongkorn University*

Materials and Methods : *An appropriate boiling time was determined for DNA preparation from 28 sputum samples from immunocompromised patients collected from April to October 2006 at King Chulalongkorn Memorial Hospital. DNA of these samples was also prepared using a commercial kit. All samples were stained with Giemsa for comparison.*

Results : *Boiling of sputum samples for 60 minutes gave positive PCR results in 67.86% (n = 19) of samples (n = 28) whereas boiling for either 30 or 90 minutes yielded positive results in 39.29% (n = 11). On the other hand, positive PCR results were obtained from all 28 samples (100%) that were prepared by using a commercial kit. Although DNA prepared with a commercial kit was superior to boiling method, it is more time-consuming and more expensive. Meanwhile, Giemsa stain could detected *P. jirovecii* in sputum of 5 patients (18%).*

Conclusion : *The sensitivity of PCR using DNA isolation by boiling method is far superior to Giemsa stain. The lower sensitivity of PCR using DNA prepared by boiling method than that by a commercial kit indicates that further improvement of the former method will be required before it can be used for general diagnostic laboratory.*

Keywords : *Boling method, DNA, PCR, Pneumocystis jirovecii.*

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ผลการเตรียม DNA โดยวิธีต้มเพื่อการตรวจหา*นิวโมซิสติสจิโรเวคซี* โดยวิธีปฏิกิริยาลูกโซ่
โพลีเมอร์เรส จากตัวอย่างเสมหะผู้ป่วยที่มีภาวะภูมิคุ้มกันบกพร่อง. *จุฬาลงกรณ์เวชสาร* 2555
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- เหตุผลของการทำวิจัย** : เชื้อ *Pneumocystis jirovecii* สามารถวินิจฉัยด้วยวิธีการย้อมสี แต่ความไวของวิธีนี้มีค่าต่ำ วิธีการตรวจโดยวิธีปฏิกิริยาลูกโซ่โพลีเมอร์เรส มีความไวและความถูกต้องสูงแต่มีความจำเป็นต้องใช้ดีเอ็นเอต้นแบบที่มีคุณภาพสูง ถึงแม้ว่าการเตรียมดีเอ็นเอด้วยวิธีการต้มจะไม่ได้ดีเอ็นเอที่มีคุณภาพสูง แต่วิธีการดังกล่าวได้ถูกนำมาใช้กับการเตรียมดีเอ็นเอในจุลชีพหลายชนิดอย่างเช่น มาลาเรีย แบคทีเรียหลายชนิด และรา เพื่อการตรวจโดยวิธีปฏิกิริยาลูกโซ่โพลีเมอร์เรส ซึ่งก็ให้ผลการศึกษาเป็นที่น่าพอใจ เนื่องจากการเตรียมดีเอ็นเอด้วยวิธีการต้มซึ่งเป็นวิธีการที่ประหยัดทั้งเวลาและค่าใช้จ่าย ดังนั้นคณะผู้วิจัยจึงได้ทดสอบว่าดีเอ็นเอของเชื้อ *P. jirovecii* สามารถเตรียมได้จากตัวอย่างเสมหะด้วยการต้มเพื่อใช้ตรวจโดยวิธีปฏิกิริยาลูกโซ่โพลีเมอร์เรสสำหรับการวินิจฉัยแยกเชื้อชนิดนี้ได้หรือไม่ นอกจากนี้ยังได้เปรียบเทียบผลการศึกษากับการเตรียมดีเอ็นเอด้วยชุดสกัดสำเร็จรูปและวิธีการย้อมสียิมซ่า
- วัตถุประสงค์** : เพื่อพัฒนาวิธีการเตรียมดีเอ็นเอแบบเร็วด้วยวิธีการต้มเพื่อใช้ในการวินิจฉัยเชื้อ *P. jirovecii*
- รูปแบบการวิจัย** : การศึกษาเชิงพรรณนา
- สถานที่ทำการศึกษา** : ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
- ตัวอย่างและวิธีการศึกษา** : เวลาที่เหมาะสมสำหรับการเตรียมดีเอ็นเอด้วยวิธีการต้มได้ถูกคิดค้นขึ้นเพื่อการเตรียมดีเอ็นเอจากตัวอย่างเสมหะจำนวน 28 ตัวอย่างจากผู้ป่วย ที่มีภาวะภูมิคุ้มกันบกพร่อง ซึ่งถูกเก็บรวบรวมในระหว่างเดือน เมษายน ถึง ตุลาคม ปี ค.ศ. 2006 จากโรงพยาบาลจุฬาลงกรณ์ ดีเอ็นเอของตัวอย่างเหล่านี้ยังได้ผ่านการเตรียมจากการใช้ชุดสกัดสำเร็จรูป และตัวอย่างเสมหะทั้งหมดถูกย้อมด้วยสียิมซ่า เพื่อการเปรียบเทียบผลการศึกษาทั้งหมด

ผลการศึกษา : การต้มตัวอย่างเสมหะเป็นเวลา 60 นาที สามารถให้ผลบวกจากการทำพีซีอาร์ร้อยละ 67.86 (19 ตัวอย่างจากทั้งหมด 28 ตัวอย่าง) ส่วนการต้มด้วยเวลา 30 และ 90 นาที ให้ผลบวกเท่ากันที่ร้อยละ 39.29 (11 ตัวอย่าง) ในทางตรงข้ามการเตรียมดีเอ็นเอด้วยชุดสกัดให้ผลบวกพีซีอาร์ทั้งหมด 28 ตัวอย่างคิดเป็นร้อยละ 100 แม้ว่าวิธีการเตรียมดีเอ็นเอด้วยชุดสกัดนั้นให้ผลบวกโดยวิธีปฏิกิริยาลูกโซ่โพลีเมอร์เรสสูงกว่าวิธีการต้ม แต่เป็นวิธีการที่ใช้เวลา และค่าใช้จ่ายสูง ส่วนผลการย้อมสียิมซาสามารถวินิจฉัยเชื้อ *P. jirovecii* จากตัวอย่างเสมหะได้เพียง 5 ตัวอย่างหรือร้อยละ 18

สรุป : ความไวของการตรวจโดยวิธีปฏิกิริยาลูกโซ่โพลีเมอร์เรสจากการใช้ดีเอ็นเอที่เตรียมด้วยวิธีการต้มมีค่าสูงกว่าการตรวจด้วยวิธีการย้อมสี แต่ยังให้ผลความไวต่ำกว่าการใช้ชุดสกัด แสดงให้เห็นถึงควรมีการปรับปรุงวิธีการเตรียมดีเอ็นเอที่พัฒนาขึ้นในการศึกษาครั้งต่อไป เพื่อเพิ่มประสิทธิภาพของความไวในการตรวจก่อนที่จะนำไปใช้ในห้องปฏิบัติการโดยทั่วไป

คำสำคัญ : วิธีการต้ม, ดีเอ็นเอ, พีซีอาร์ นิวโมซิสติสซิโรเวคซี.

Pneumocystis jirovecii is an opportunistic ascomycetous fungal pathogen with a wide geographical niche. *P. jirovecii* pneumonia (PCP) is responsible for a significant morbidity and mortality among immunocompromised patients especially those who had underlying human immunodeficiency virus (HIV) infection with low CD4+ lymphocytes. During the first ten years after the outbreak of human immunodeficiency virus (HIV), more than 100,000 PCP cases were reported in the United States.⁽¹⁾ Although *P. jirovecii* is cosmopolitan in distribution, the prevalence of PCP seems to be highly variable depending on geographic location and population sampling.⁽²⁾ Presumptive diagnosis of *P. jirovecii* infection relies on clinical symptoms characterized by insidious or subacute onset of respiratory symptoms with non-productive cough, diffused interstitial infiltration on chest radiograph and underlying immunocompromised conditions. Because *P. jirovecii* cannot be cultured in vitro, definite diagnosis relies on demonstration of organisms from respiratory specimens such as sputum and bronchoalveolar lavage by staining methods. Early detection is crucial to enhance patient care whereas definitely negative laboratory results for *P. jirovecii* will lead to an avoidance of unnecessary treatment and reduce the risk of drug hypersensitivity and complication from multiple medication. Current laboratory diagnostic method for *P. jirovecii* in general practice relies on staining of sputum or other respiratory samples by various methods such as Giemsa, toluidine blue-O and Gomori's methenamine silver stains. Both trophic and cystic forms of *P. jirovecii* can be visualized by Giemsa stain albeit at lower sensitivity than the latter two staining protocols. Nevertheless, it is not

uncommon that *P. jirovecii* presents in samples fewer than microscopy detection threshold, resulting in false negative results.⁽³⁾

The most sensitive technique for diagnosing *P. jirovecii* is detection of specific DNA by polymerase chain reaction (PCR). However, the operating cost of PCR remains too expensive comparing with staining methods, especially when using commercial DNA extraction kit. On the other hand, DNA preparation by a simple boiling method is an inexpensive procedure that does not require expensive reagents and is less time-consuming than the commercially available kits. DNA preparation by simple boiling method has been used with satisfactory results for PCR-based diagnosis of various kinds of organisms such as malaria, bacteria, yeasts and fungi. For example, rapid boiling method has been applied for malaria using small amount of blood and provided enough DNA material for further analysis.⁽⁴⁾ The detection and genotyping of a common bacteria (*Chlamydia trachomatis*) causing sexually transmitted disease could be analyzed efficiently using DNA from boiling method under an optimal PCR amplification.⁽⁵⁾ Comparison of three different methods for diagnosing *Mycobacterium avium* in clinical samples that included real-time PCR using commercial DNA extraction kit, real-time PCR using DNA prepared from boiling method and direct cultivation using specific media revealed positive rates of 100 %, 90 % and 60 %, respectively.⁽⁶⁾ Therefore, application of DNA preparation for PCR diagnosis by boiling method seems promising for other organisms.

To date, all studies on molecular epidemiology and surveillance of drug resistance of *P. jirovecii* deployed DNA preparation from either phenol-

chloroform technique or commercial DNA extraction kits.⁽⁷⁻¹⁰⁾ Therefore, it is intriguing to evaluate whether or not a rapid DNA preparation by boiling method for *P. jirovecii* is suitable for diagnostic purpose. In this study, we aimed to look at the efficiency of PCR detection for *P. jirovecii* by using DNA prepared by boiling of sputum samples obtained from immunocompromised patients comparing with DNA preparation from a commercial kit and routine Giemsa staining method.

Materials and Methods

Clinical samples

Sputum samples from 28 PCP-suspected patients who attended the Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, King Chulalongkorn Memorial Hospital in Bangkok were collected after informed consent from April to October 2006. Inclusion criteria were patients more than 18 years old who had atypical pneumonic symptoms with bilateral infiltration on chest roentgenogram and sero-positive for HIV with CD4+ lymphocytes less than 500 cells/ μ L. Exclusion criteria were those who could not provide 1 ml of sputum sample and who did not provide informed consent to this study. Approximately 1 ml of sputum samples was collected in 15 ml conical-bottom sterile plastic tube. An equal volume of 0.3% dithiothreitol (DTT) was added to each sample to disrupt mucus materials. The samples were mixed well by vortexing and divided into 5 aliquots. After centrifugation, all supernatant was discarded. The remaining pellets were used for subsequent evaluation.

Morphological diagnosis

The pellets from an aliquot was smeared on a clean glass slide, dried and fixed with absolute methanol by dipping in a Coplin jar before staining with 1:20 vol/vol Giemsa staining solution (MERCK, Germany) for 30 minutes.⁽¹¹⁾ After washing with clean water and dried, slides were examined for cystic and trophic stages of *P. jirovecii* under light microscope by an experienced microscopist who was blinded from the results of other test methods.

DNA extraction

Commercial DNA extraction kit

DNA was extracted from the pellet of each sample using QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Briefly, 180 μ L of buffer ATL was added to the pellet, mixed with 20 μ L of proteinase K and incubated at 56°C in a shaking heat block for 5 hours. Buffer AL solution (200 μ L) was then added and incubated at 70°C for 10 minutes. After mixing thoroughly, 200 μ L of ethanol was added, transferred all solution into a column with collection tube and centrifuged for 1 minute at maximum speed. The column was placed in a new collection tube, 500 μ L of buffer AW1 was then added and centrifuged for 1 minute. The column was transferred to the new collection tube, 500 μ L of buffer AW2 was added and centrifuged for 3 minutes. Finally, the column was removed to a new 1.5 mL microcentrifuge tube, 50 μ L of AE buffer was applied directly into the column membrane and incubated at room temperature for 1 minute before centrifugation at 8,000 rpm for 1 minutes.⁽¹²⁾ The DNA sample was kept frozen at -40°C until use.

Boiling method

The remaining 3 aliquots of pellets were filled with 50 μ L distilled water and boiled at 100 $^{\circ}$ C in a water-bath for 30, 60 and 90 minutes in separate tubes.^(4,6) After spinning down using microcentrifuge, all supernatant was transferred to 0.5 μ L micro-tube. The samples were kept at -40 $^{\circ}$ C until use.

PCR Analysis

Nested PCR targeting mitochondrial large subunit ribosomal RNA (mtLSU rRNA) of *P. jirovecii* was performed by using specific oligonucleotide primers as previously described.⁽¹³⁾ Primers pAZ 102-H: 5'-GTGTACGTTGCA AAGTACTC-3' and pAZ 102-E: 5'-GATGGCTGTTTCCAAGCCCA-3' were used for primary PCR generating 347-bp fragment. The nested PCR primers generating 252-bp product were pAZ 102-X: 5'-GTGAAATACAAATCGGGCTAGG-3' and pAZ 102-Y: 5'-CACTTAATATTAATTGGGGAGC-3'.⁽¹³⁾ Total volume of PCR reaction was 20 μ L containing template DNA, 2.5 mM each deoxynucleoside triphosphate, 1.5 μ L of 10x PCR buffer, 0.3 μ M of each primer and 0.4 unit of Ex Taq DNA polymerase (Takara, Seta, Japan). PCR reaction was amplified using Applied Biosystem GeneAmp[®] PCR system 9700 thermocycler (PE Biosystems, Foster City, CA). The thermal cycling profile for primary PCR included a pre-denaturation at 94 $^{\circ}$ C for 2 min; 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s; primer-template annealing at 55 $^{\circ}$ C for 30 s; primer extension at 72 $^{\circ}$ C for 1 min, and post amplification extension at 72 $^{\circ}$ C for 5 min. Nested PCR was done using the same thermal cycling condition except that an annealing temperature was set to 50 $^{\circ}$ C. PCR products were analyzed by 2% agarose gel electrophoresis. Ladder

100 bp was used as a DNA marker to compare the size of PCR products. The positive control was known *P. jirovecii*-positive samples by microscope and negative control was distilled water.

Positive and negative controls

DNA of *P. jirovecii* from an isolate that had been previously determined the small subunit ribosomal RNA sequence was used as a positive control (Putaporn et al, unpublished). Sterile water was used as negative control. All PCR procedures were performed under strict physical separation of laboratory procedures by experienced researchers.

Ethical aspect

The ethical approval of this study has been obtained by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University (IRB No. 176/51).

Statistical analysis

Percentage of positive tests per total number of samples was calculated. This is a pilot study that did not recruited PCP-negative individuals, precluding an evaluation of diagnostic performance of the test method.

Results

A total of 28 patients offered adequate (~1 ml) sputum samples. There were 10 males (35.71%) and 18 females (64.29%) with age range from 19 to 55 years (mean 34 years). Twenty-six patients had HIV infection and 25 (96.2%) of these had CD4+ lymphocyte \leq 200 cells/ μ L. The remaining 2 patients had lymphoma. Co-infections

with other infectious agents included *Mycobacterium tuberculosis*, viral hepatitis type B and type C. All patients were treated with cotrimoxazole (Table 1).

DNA extraction by boiling method for 30, 60 and 90 minutes yielded positive PCR tests in 11 (39.29%), 19 (67.86%) and 11 (39.29 %) samples, respectively (Table 2). All positive samples gave PCR

products of expected size. No false amplification was observed when water was used as control. However, all 28 samples were positive in nested PCR using DNA from the commercial kit. On the other hand, both trophic and cystic stages of *P. jirovecii* were demonstrated by Giemsa stain in 5 samples (17.86%) (Figure 1).

Table 1. Demographic and clinical profiles of patients with *Pneumocystis jirovecii* pneumonia (n = 28).

	Range	Mean	N	%
Age	19 - 55 years	34 years		
sex				
male			10	35.71
female			18	64.29
Underlying illness				
HIV			26	92.85
Lymphoma			2	7.15
Other infection				
Tuberculosis			2	7.15
Hepatitis B virus			2	7.15
Hepatitis C virus			2	7.15
Treatment				
Co-trimoxazole			28	100
CD4+ lymphocyte	1-442 cells/ μ l	54.9 cells/ μ l		
> 200 cells/ μ l			1	3.6
\leq 200 cells/ μ l			27	96.4

Table 2. Comparison of three boiling times for DNA preparation for PCR-based detection of *Pneumocystis jirovecii*.

PCR (n = 28)	Boiling time (minutes)		
	30	60	90
Positive	11 (39.29%)	19 (67.86%)	11 (39.29%)
Negative	17 (60.71%)	9 (32.14%)	17 (60.71%)

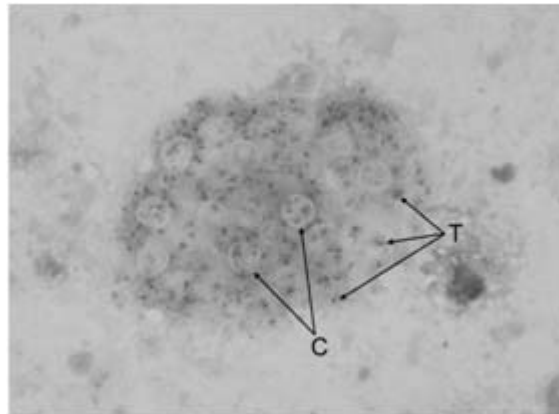


Figure 1. Agarose gel electrophoresis of the polymerase chain reaction products generated from primers pAZ 102 X and pAZ 102 Y using DNA extracted from sputum by boiling for 60 minutes (sample nos. 4 to 37). M= 100 bp maker, Neg = negative control, Pos = positive control.

When the results of PCR using DNA extracted with the commercial kit were considered as gold standard, the diagnostic sensitivity of Giemsa stain and PCR using DNA template prepared by boiling method were 18% and 67.9%, respectively

(Figure 2, Table 3). The positive ratio of PCR using DNA template prepared by boiling method and Giemsa stain was 3.8 whereas the ratio of positive PCR results from boiling method and commercial kit was 0.68.

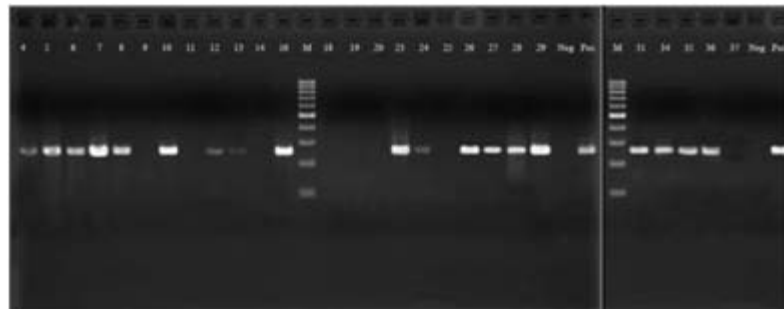


Figure 2. *Pneumocystis jirovecii* stained with Giemsa. C (cystic form) and T (trophic form).

Table 3. Comparison of *Pneumocystis jirovecii* detection from sputum by Giemsa stain and PCR using templates from boiling method and a commercial kit.

Method	Result	
	Positive	Negative
Giemsa stain	5 (17.86%)	23 (82.14%)
PCR (Template prepared by boiling)	19 (67.86%)	9 (32.14%)
PCR (Template prepared by a commercial kit)	28 (100%)	0 (0%)
Total	28 (100%)	28 (100%)

Discussion

Of the 28 positive samples for *P. jirovecii* in this study, the sensitivity of Giemsa stain in sputum specimens was 18%, a consistent results similar to a previous study.⁽³⁾ The optimum time required for boiling samples to yield maximum amount of DNA of *P. jirovecii* for PCR detection was 60 minutes that gave 68% sensitivity when results from PCR using DNA extracted with commercial kit was used as reference. Although Giemsa stain has been widely used in most diagnostic laboratories, high false negative results with low sensitivity were observed. The PCR method is more accurate to detect the organism but this method is still an expensive one especially the cost of DNA preparation.

Rapid DNA extraction by boiling is simple and inexpensive. Its successful results could be envisaged in several microorganisms such as extraction of malarial DNA by 10 minute boiling.⁽¹⁴⁾ DNA extraction by boiling method was also very efficient for diagnosing *Chlamydia trachomatis* infection because boiling method generated the highest positive results compared with phenol-chloroform extraction and proteinase K digestion.⁽¹⁵⁾ Furthermore, preparation of DNA of *Myclobacterium* spp. for real-time PCR by 5-minute boiling gave superior results (90% sensitivity) to that prepared with commercial kits.⁽⁶⁾ On the other hand, DNA preparation from *Aspergillus fumigatus* by freeze-thaw

method, freeze-boil method, bead-beating method and enzyme extraction using real-time PCR have shown that bead-beating method was the most successful, giving high quality and quantity DNA.⁽¹⁶⁾

To our knowledge, this is the first study that applies a rapid boiling method for preparing DNA of *P. jirovecii* for PCR diagnosis. Although the efficiency of DNA extraction by boiling seems to be lower than that prepared by a commercial DNA extraction kit, further improvement of the method such as using more volume of samples and fine adjustment of boiling time is mandatory before the method can be used for routine laboratory diagnosis. If boiling method could be improved to an acceptable level, a remarkable reduction in turn-around time and a dramatic reduction in expense per unit cost will make the method be of practical value for routine laboratory use (Table 4). Nevertheless, PCR diagnosis of *P. jirovecii* using DNA template prepared by the boiling method is unequivocally far superior to Giemsa stain in this study. Meanwhile, limitations in our study include small number of samples recruited in analysis and the lack of PCP-negative samples, precluding assessment of diagnostic specificity. Undoubtedly, further study using more samples from various groups of subjects along with an improvement in DNA extraction protocol during or after the boiling method will be required.

Table 4. Comparison of unit cost for DNA preparation.

Procedure	Unit Cost (Baht)	
	Commercial kit method	Boiling method
DNA preparation	190	10
PCR reagents	20	20
Net operating cost	210	30

Conclusion

The preparation of *P. jirovecii* DNA by the boiling method for PCR diagnosis is promising if it can be further improved because it could substantially reduce false negative tests by Giemsa stain and reduce turn-around time and expenses of using commercial kit for DNA extraction.

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