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A simple and efficient DNA extraction from respiratory samples for PCR detection of *Pneumocystis jirovecii*

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Background : *Pneumonia caused by Pneumocystis jirovecii (PCP) is a leading cause of morbidity and mortality in immunocompromised individuals. Although microscopic detection of stained clinical samples and immunofluorescence assay (IFA) have been widely used for diagnosis of P. jirovecii infections, polymerase chain reaction (PCR)-based detection of P. jirovecii provides a higher diagnostic performance. DNA preparation by simple boiling method could be useful for PCR detection albeit the sensitivity remained remarkably low.*

Objective : *To refine P. jirovecii DNA preparation by boiling method in order to improve the diagnostic yield of subsequent PCR detection.*

Methods : *Two induced sputum and 4 bronchoalveolar lavage (BAL) samples from PCP patients were recruited for initial assessment of DNA extraction by boiling for 10, 20, 30, 60 and 90 minutes. Performance of each DNA preparation was determined by dilution of samples and tested by P. jirovecii-specific nested PCR. Evaluation of the best boiling time for DNA extraction was done with 51 paired sputum and BAL samples from PCP patients using data from a commercial kit as references.*

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Results : *Boiling of samples for 20 minutes gave the best nested PCR results. Application of DNA extraction of 51 paired sputum and BAL samples by boiling for 20 minutes offered 92.16% and 96.08% sensitivity, respectively; both yielded 100% specificity.*

Conclusion : *DNA of P. jirovecii could be efficiently extracted from BAL fluids and sputum samples by boiling for 20 minutes, almost comparable to that obtained from a commercial DNA extraction kit.*

Keywords : *Pneumocystis jirovecii, boiling method, DNA extraction, brochoalveolar lavage, sputum.*

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เหตุผลของการทำวิจัย : *โรคปอดอักเสบจากเชื้อนิวโมซีสติสจิโรเวคซีเป็นสาเหตุการการเจ็บป่วยและเสียชีวิตที่สำคัญในผู้ที่มีภาวะภูมิคุ้มกันบกพร่อง แม้ว่าการตรวจหาเชื้อภายใต้กล้องจุลทรรศน์จากตัวอย่างที่ย้อมสี และการตรวจโดยวิธีอิมมูโนฟลูออเรสเซนซ์มีการใช้อย่างแพร่หลายสำหรับการวินิจฉัยการติดเชื้อนี้ แต่การตรวจโดยวิธีปฏิกิริยาลูกโซ่โพลิเมอร์เรสหรือวิธีพีซีอาร์ สำหรับนิวโมซีสติสจิโรเวคซี พบว่าให้ผลการตรวจที่มีประสิทธิภาพมากกว่า ทั้งนี้การเตรียมดีเอ็นเอโดยวิธีต้มสามารถใช้ได้กับวิธีพีซีอาร์ แต่วิธีดังกล่าวยังให้ผลการตรวจที่มีความไวต่ำ*

วัตถุประสงค์ : *เพื่อพัฒนาวิธีการเตรียมดีเอ็นเอของเชื้อนิวโมซีสติสจิโรเวคซีโดยวิธีต้มเพื่อเพิ่มประสิทธิภาพการตรวจหาเชื้อดังกล่าวโดยวิธีพีซีอาร์*

วิธีการทำวิจัย : *ในเบื้องต้นได้ทำการประเมินผลการเตรียมดีเอ็นเอจากตัวอย่างเสมหะจำนวน 2 ตัวอย่างและตัวอย่างจากน้ำล้างปอดจำนวน 4 ตัวอย่าง โดยทำการต้มเป็นเวลา 10, 20, 30, 60 และ 90 นาทีทำการเปรียบเทียบผลการตรวจโดยวิธีพีซีอาร์จากตัวอย่างดีเอ็นเอที่เจือจางในระดับต่าง ๆ หลังจากนั้นทำการประเมินวิธีการเตรียมดีเอ็นเอโดยการต้มในระยะเวลาที่ให้ผลการตรวจที่ดีที่สุด โดยใช้ตัวอย่างจากผู้ป่วยโรคปอดอักเสบจากการติดเชื้อนิวโมซีสติสจิโรเวคซีจำนวน 51 รายที่มีตัวอย่างเสมหะและน้ำล้างปอด การเปรียบเทียบโดยใช้ผลที่ได้จากการเตรียมดีเอ็นเอโดยใช้ชุดตรวจสำเร็จรูป*

ผลการศึกษา : *การต้มเป็นเวลา 20 นาทีเป็นการเตรียมดีเอ็นเอที่ให้ผลการตรวจดีที่สุดโดยวิธีพีซีอาร์ เมื่อทำการประเมินวิธีการเตรียมดีเอ็นเอดังกล่าวกับตัวอย่างเสมหะ และน้ำล้างปอดจากผู้ป่วย 51 ราย พบว่าการตรวจโดยวิธรพีซีอาร์ให้ความไวร้อยละ 92.16 และ 96.08 ตามลำดับ ในขณะที่มีความจำเพาะร้อยละ 100 เช่นเดียวกัน*

สรุป : *ดีเอ็นเอของเชื้อนิวโมซีสติสจิโรเวคซีสามารถเตรียมได้จากตัวอย่างเสมหะและน้ำล้างปอดได้อย่างมีประสิทธิภาพ โดยวิธีการต้มเป็นเวลา 20 นาที ซึ่งให้ผลการตรวจที่ใกล้เคียงกับการใช้ชุดเตรียมดีเอ็นเอสำเร็จรูป*

คำสำคัญ : *นิวโมซีสติสจิโรเวคซี, การต้ม, การสกัดดีเอ็นเอ, น้ำล้างปอด, เสมหะ.*

Atypical pneumonia caused by *Pneumocystis jirovecii*, an ascomycetous opportunistic pathogen, is a leading cause of morbidity and mortality in immunocompromised individuals, especially HIV-infected patients.[1] Pneumonia caused by *P. jirovecii* (PCP) can be diagnosed by demonstration of characteristic cystic and trophic stages in respiratory samples by staining methods such as Giemsa and methenamine silver stains. However, the diagnostic performance of these methods can be variable depending on quantity of organisms in the tested specimens and competency of microscopist.[2] For instance, the number of *P. jirovecii* in pneumonic non-HIV patients is usually fewer than that in HIV-infected patients, resulting in a lower detection rate in the former.[3] Furthermore, recent studies have revealed that *P. jirovecii* can colonize both upper and lower respiratory tracts of asymptomatic healthy individuals; thereby the paucity of organisms in these cases may not render effective detection by staining methods.[4 - 6] Meanwhile, application of immunofluorescence assay (IFA) has remarkably increased diagnostic yield. On the other hand, a number of samples containing submicroscopic level of *P. jirovecii* remained undiagnosed.[7]

PCR-based detection of *P. jirovecii* provides a higher diagnostic performance than conventional staining methods including IFA.[7 - 9] However, DNA extraction methods can be tedious, such as phenol/chloroform extraction, or costly, when commercial kits are deployed. Although a simple proteinase K treatment followed by boiling method has been applied in DNA preparation for PCR diagnosis of *P. jirovecii*, its efficiency seems to be variable comparing with other DNA purification procedures.[10] Our previous study has shown that direct boiling of sputum samples infected with *P. jirovecii* for 60 minutes was a suitable DNA template preparation for subsequent PCR-based diagnosis in which the method yielded 3.7 times more sensitive than conventional microscopy detection by Giemsa stain method.[11] Nevertheless, the sensitivity of PCR detection by use of DNA template prepared by simple boiling method was about 32% lower than that extracted by a commercial kit. One of the contributing factors that may affect to the success rate of PCR amplification is the duration of boiling. Therefore, an improved boiling procedure for a more efficient DNA preparation from sputum samples remained mandatory. Herein, we evaluated the effect of boiling time on *P. jirovecii* DNA extraction based on nested PCR amplification targeting the mitochondrial large subunit rRNA gene (mtLSU rRNA).[11]

Materials and Methods

Sputum samples

Paired induced sputum and bronchoalveolar lavage (BAL) were obtained from 51 HIV-infected patients (30 males, 21 females; mean age 44.2 years) who had clinical and chest roentgenogram compatible with PCP were recruited for analysis. Additionally, 20 paired sputum and BAL samples from patients (12 males, 8 females; mean age 39.5 years) who had final diagnosis of non-PCP were used for comparison.

Giemsa stain

About 20 μL of each sputum sample or BAL were smeared on a clean glass slide and allowed to dry at room temperature. After fixation with absolute methanol, the slide was stained with 20% Giemsa

(MERCK, Germany) in phosphate buffer saline solution (pH 7.2) for 30 minutes and washed with water until no excess dye remained on the slide. The stained slide was allowed to dry at ambient temperature. The slide was examined under light microscope (Olympus, BX50) with X100 objective lens. Diagnosis of *P. jirovecii* was based on characteristic size and structure of the organisms.

IFA for P. jirovecii

Sputum and BAL samples were centrifuged at 5,000 rpm for 10 minutes. Twenty five μL of the pellets were spread over the 12-well multi-test slide (ICN Biomedicals, Ohio, USA), allowed to dry at ambient temperature and fixed with acetone. The procedure for immunofluorescence detection of *P. jirovecii* was performed according to the manufacturer's protocol (Merifluor Pneumocystis, Meridian Bioscience, Ohio, USA). The detection reagent contains fluorescein isothiocyanate (FITC) labeled monoclonal antibodies directed against cell wall and matrix antigens of *P. jirovecii* cysts and trophic stages. Interpretation of results was done by examination under fluorescence microscope using wavelength 490 - 500 nm (Olympus BX50 fluorescence microscope).

DNA preparation

One hundred ตL of each induced sputum sample or BAL fluid were used for DNA extraction by each method. The final volume of ~50 - 70 μL of DNA solution was obtained from each procedure. To determine the appropriate boiling time for DNA preparation, we recruited 2 induced sputum samples and 4 BAL fluids from PCP patients who met all the following criteria: (i) clinical symptoms of atypical pneumonia characterized by fever, non-productive cough and dyspnea; (ii) diffuse interstitial pulmonary infiltration on chest roentgenogram; (iii) demonstration of *P. jirovecii* by Giemsa stain or IFA; and, (iv) clinical responses to co-trimoxazole treatment. Because accurate enumeration of *P. jirovecii* in clinical samples could not be reliably determined, we sampled 200, 100, 20 and 4 μL from each specimen and the final volume was adjusted to 200 μL with TE (10 mM Tris.HCl pH 8.0, 1 mM EDTA) buffer before boiling. The duration of boiling for each dilution of sample was 10, 20, 30, 60 and 90 min. After boiling at 100 ^{O}C in a water-bath, samples were centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to the new tubes and stored at -20 ^{O}C until use. To further evaluate the performance of DNA extraction using optimum boiling period for 20 min comparing with that using a commercial DNA extraction kit (QIAamp DNA mini kit, Hilden, Germany).

PCR detection of P. jirovecii

Primers PJLSUF0 and PJLSUR0 were used in a primary PCR, and PJLSUF1 and PJLSUR1 in a nested PCR as described, generating 171 and 113 bp products, respectively.[7] PCR was performed in a total volume of 20 μL containing 3 μL of template DNA, 2 μL of 10x PCR buffer, 2.5 mM each deoxynucleotide triphosphate, 0.3 μM of each primer and 0.4 units Dream *Taq* DNA polymerase (ThermoFischer Scientific, USA). PCR was performed in an Applied Biosystem GeneAmp® PCR system 9700 thermocycler (PE Biosystems, Foster City, USA) using a preamplification denaturation at 94 ^{O}C, 1 min; 35 cycles of 94 ^{O}C for 30 s, 55 ^{O}C for 30s and 72 ^{O}C

for 1 min, and a final extension at 72 ^{O}C for 5 min for primary PCR. As for nested PCR, one μL of primary PCR product was used as template. Nested PCR essentially followed the same conditions except that the PCR was done for 30 cycles.[7] The PCR products were analyzed by 2% agarose gel electrophoresis. All procedures including preparation of each sample dilution, boiling at varying period and PCR assays were done twice. Therefore, a total of 350 PCR assays were performed. Evaluation of positive control *P. jirovecii* DNA samples for PCR assay was performed by dilution of samples with sterile water at 1:1, 1:10 and 1:50. Genomic DNA samples of *Plasmodium vivax*, *Plasmodium falciparum*, *Toxoplasma gondii*, *Cystoisospora belli*, *Cyclospora cayetanensis*, *Cryptosporidium hominis*, *Leishmania donovani*, *Trypanosoma brucei*, *Candida albicans*, *Histoplasma capsulatum*, *Aspergillus fumigatus* and *Vittaforma corneae* were used as templates to assess specificity of the test methods. Water was used as negative control in the PCR assay.

Ethical aspects

This study has been reviewed and approved by the Institutional Review Board of Faculty Medicine, Chulalongkorn University (IRB No. 176/51 and IRB No. 142/54).

Statistical analysis

Comparison of diagnostic performance of each assay was done in terms of sensitivity, specificity and accuracy. PCR results from DNA templates containing *P. jirovecii* extracted by use of the QIAamp DNA minit kit were set as gold standard. Performance indices were indicated by the number of true positive (TP), number of true negative (TN), number of false positive (FP) and number of false negative (FN). Sensitivity was determined from TP/(TP+FN) and specificity from TN/(TP+FP). Accuracy was as calculated by (TP+TN)/(TP+TN+FP+FN).

Results

Microscopy and IFA detection of P. jirovecii

Of 51 sputum samples, *P. jirovecii* was demonstrated by Giemsa stain method in 18 (35.29%) samples. Giemsa stained slides from BAL samples yielded positive results in 33 (64.71%) of 51 tested samples. Meanwhile, 33 (64.71%) sputum samples and 39 (76.47%) BAL samples gave positive tests for *P. jirovecii* by IFA method. Therefore, IFA offered 4.13 fold higher positive rate than Giemsa stain in sputum samples. However, IFA was only slightly superior to Giemsa stain when applied on BAL samples in which 1.18 fold differences was observed (Table 1).

PCR from standard genomic DNA extraction

Genomic DNA extracted from sputum samples using the QIAgen DNA mini kit has yielded 48 (94.12%) and 51 (100%) positive tests during primary and nested PCR assays for *P. jirovecii*, respectively. Likewise, genomic DNA from BAL samples extracted by the same method has shown positive results in 49 (96.08%) and 51 (100%) samples for primary and nested PCR, respectively (Table 1). No cross-reactivity of the nested PCR method was detected when non-*P. jirovecii* DNA samples were deployed as DNA templates. No potential cross-contamination was observed because none of the negative control tests showed positive results.

Table 1. Detection of *P. jirovecii* in induced sputum and bronchoalveloar lavage by Giemsa stain, immunofluorescence test (IFA) and nested PCR.

Procedure	Sputum		BAL	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Giemsa stain	18 (35.29)	33 (64.71)	33 (64.71)	18 (35.29)
IFA	33 (64.71)	18 (35.29)	39 (76.47)	12 (23.53)
Primary PCR*	48 (94.12)	3 (5.88)	51 (100)	0 (0)
Nested PCR*	49 (96.08)	2 (3.92)	51 (100)	0 (0)

*Genomic DNA extracted by use of QIAamp DNA mini kit.

Effect of boiling time on DNA templates

Both sputum samples gave positive tests by nested PCR for *P. jirovecii* when genomic DNA was prepared by boiling for 20, 30 and 60 minutes. Negative results were observed for all dilutions of templates when sputum #1 was boiled for 10 and 90 minutes. On the other hand, sputum #2 gave positive tests when DNA samples were prepared by boiling for 10, 20, 30, 60 and 90 minutes while samples boiling for 20 and 30 minutes gave positive tests at 1:50 dilution of the templates. Therefore, it is likely that sputum #2 contained more organisms than those in sputum #1. Likewise, BAL #1 and BAL #4 seem to contain more *P. jirovecii* DNA than BAL #2 and BAL #3. A consistent superiority in positive rates of PCR detection was found when DNA from either sputum samples or BAL specimens was prepared by boiling for 20 minutes that outperformed DNA preparation by boiling for 30 minutes (Table 2).

Table 2. PCR detection of *P. jirovecii* LSU rRNA gene fragment using DNA extracted from sputum and BAL fluids by boiling method.

Samples/Dilution*	Boiling period (minutes)*				
	10	20	30	60	90
Sputum #1					
Undiluted	-,-	+,+	+,+	+,+	-,-
1:1	-,-	+,+	+,+	+,+	-,-
1:10	-,-	+,+	+,+	-,-	-,-
1:50	-,-	-,-	-,-	-,-	-,-
Sputum #2					
Undiluted	+,+	+,+	+,+	+,+	+,+
1:1	+,+	+,+	+,+	+,+	+,+
1:10	+,+	+,+	+,+	+,+	+,+
1:50	-,-	+,+	+,+	-,-	-,-

Table 2. (Con) PCR detection of *P. jirovecii* LSU rRNA gene fragment using DNA extracted from sputum and BAL fluids by boiling method.

Samples/Dilution*	Boiling period (minutes)*				
	10	20	30	60	90
BAL #1					
Undiluted	+,+	+,+	+,+	+,+	+,+
1:1	+,+	+,+	+,+	+,+	+,+
1:10	+,+	+,+	+,+	+,+	-,-
1:50	-,-	+,+	+,-	-,-	-,-
BAL #2					
Undiluted	+,+	+,+	+,+	+,+	+,+
1:1	+,+	+,+	+,+	+,+	+,+
1:10	-,-	+,+	+,+	-,-	-,-
1:50	-,-	+,+	-,-	-,-	-,-
BAL #3					
Undiluted	+,-	+,+	+,+	+,+	-,-
1:1	-,-	+,+	-,-	-,-	-,-
1:10	-,-	-,-	-,-	-,-	-,-
1:50	-,-	-,-	-,-	-,-	-,-
BAL #4					
Undiluted	+,+	+,+	+,+	+,+	+,+
1:1	+,+	+,+	+,+	+,+	+,+
1:10	+,+	+,+	+,+	+,+	-,-
1:50	-,-	+,+	-,+	-,-	-,-
Total positives	23	42	36	28	18

- = negative PCR; + = positive PCR * All tests were done twice.

Application of 20-minute boiling method to clinical samples

Diagnostic performance of nested PCR using DNA templates prepared by 20-minute boiling was assessed by comparison with the same PCR method using DNA templates extracted by QIAamp DNA mini kit. Of 51 sputum samples; DNA prepared by 20-minute boiling gave 31 and 47 positive tests from primary and nested PCR, respectively. Meanwhile, 46 and 49 DNA samples from BAL prepared by 20-minute boiling yielded positive results from primary and nested PCR, respectively. When results from nested PCR were considered, sputum and BAL samples prepared by 20-minute boiling offered sensitivity of 92.16% and 96.08%, respectively; whereas both samples gave 100% specificity.

Table 3. Diagnostic performance of detection methods for diagnosing *Pneumocystis jirovecii* in clinical samples.

Detection method	Specimen Result	Nested PCR (kit)* Positive	Nested PCR (kit)* Negative	Sensitivity (%)	Specificity (%)	Accuracy (%)
Giemsa	Sputum					
	Positive	8	0	15.69	100	39.44
	Negative	43	20			
BAL						
	Positive	33	0	64.71	100	74.65
	Negative	18	20			
IFA	Sputum					
	Positive	33	1	64.71	95	73.24
	Negative	18	19			
BAL						
	Positive	39	1	76.47	95	81.69
	Negative	12	19			
Nested PCR (Boiling)**	Sputum					
	Positive	47	0	92.16	100	94.37
	Negative	4	20			
BAL						
	Positive	49	0	96.08	100	97.18
	Negative	2	20			

* DNA template extracted using the QIAamp DNA mini kit is considered reference.

**DNA template prepared by boiling sample for 20 min.

Discussion

It has been widely perceived that molecular diagnostics of pathogens in clinical specimens including *P. jirovecii* remarkably outperformed conventional microscopy and immunofluorescence methods.[7] However, in low- or middle-income countries molecular diagnostics of pathogens were relatively costly, impeding general laboratory application of the methods in most hospitals. Furthermore, the procedure of DNA extraction by using commercial kits could be complicated processes or time-consuming. An alternative to the use of commercial DNA extraction kits, DNA could be prepared directly from unprocessed or with little manipulation of clinical samples such as boiling methods. Simple boiling procedure or modification

of DNA extraction prior to boiling method has been reportedly an efficient strategy to lower the cost and potentially shorten the time required for DNA extraction. Successful DNA extraction by inclusion of boiling step during DNA extraction has been reported for various pathogens ranging from bacteria, e.g. *Staphylococcus aureus*,[12] *Campylobacter jejuni* [13] and *Mycobacterium tuberculosis*,[14] protozoa, e.g. *Babesia bigemina*[15] and *Plasmodium falciparum*,[16] helminths' larvae and eggs, e.g. *Ostertagia ostertagi*,[17] *Wuchereria bancrofti* [18] and *Loa loa*,[19] and yeasts, e.g. *Candida* species[20]

Our previous study has shown that boiling of sputum samples for 60 minutes was an alternative *P. jirovecii* DNA extraction for subsequent PCR detection. However, the sensitivity of the method was only 67.87% in comparison with DNA extraction by using a commercial kit. Furthermore, increase in boiling time seems to deteriorate the quality of DNA samples because lower PCR positive rates were found when sputum samples were boiled for 90 minutes.[11] Herein, we have determined the appropriate boiling period that could offer higher positive rates of PCR detection of *P. jirovecii* in sputum and BAL samples. Importantly, boiling of unprocessed sputum or BAL specimens for 20 minutes gave the highest positive rates for PCR assay. Meanwhile, boiling for a shorter or a longer duration resulted in inferior results that could be from inadequate release of DNA from the cells or degradation of released DNA in solution, respectively (Table 2). Although IFA provided better diagnostic yields than Giemsa stain, ~35% of sputum samples and ~24% of BAL fluids gave negative results. DNA extracted from BAL fluids and sputum samples by boiling for 20 minutes gave 96.08% and 92.16% sensitivity, respectively while both yielded 100% specificity in nested PCR assays.

Although boiling method for DNA preparation of other organisms such as parasitic larvae or eggs, bacteria and fungi may require addition of cell lysis buffer or vigorous mechanical manipulation. Requirements for pre-boiling preparation of samples of these organisms could stem from differential biological characteristics of each pathogen. For instance, the cuticle of heminthic larvae could be resistance to boiling *per se*; thereby, pre-treatment with zinc BB disruption was mandatory to obtain better diagnostic PCR test.[19] Meanwhile, pre-treatment of bacterial cells with lysis buffer or proteinase K was required before boiling step on the one hand.[12] On the other hand, *P. jirovecii* exists as tropic and cystic stages, both are extracellular stages whose cellular structure could be easily disrupted during the heating process. Therefore, release of *P. jirovecii* DNA from sputum and BAL specimens by simple boiling seems to be an efficient method without addition of lysis or pre-treatment with proteolytic enzymes. Furthermore, simple boiling method with immediate use of DNA samples seems to offer the best diagnostic performance by using *P. jirovecii*-specific PCR detection. The method described herein should be applicable to molecular diagnosis of pneumocystosis in clinical practice.

Conclusion

P. jirovecii DNA could be feasibly and economically prepared from BAL fluids and sputum samples by boiling for 20 minutes that offered efficient nested PCR detection, comparable to DNA extraction by using a commercial kit.

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