Pneumocystis Jiroveci in HIV/AIDS Patients: Detection by FTA Filter Paper Together with PCR in Noninvasive Induced Sputum Specimens

Siraya Jaijakul MD*,
Wilai Saksirisampant MSc**, Juraratt Prownebon BSc* ,
Sutin Yenthakam BSc*, Mathirut Mungthin MD, PhD***,
Saovanee Leelayoova PhD***, Surang Nuchprayoon MD, MPH, PhD*,**

** Department of Parasitology, ** Lymphatic Filariasis Research Unit, Faculty of Medicine, Chulalongkorn University , *** Department of Parasitology, Phramongkutklao College of Medicine*

Objectives: *To detect P. jiroveci (previously named P. carinii) by PCR using FTA filter paper to extract the DNA, from noninvasive induced sputum samples of HIV/AIDS patients.*
Material and Method: *Fifty two HIV/AIDS patients suspected of Pneumocystis jiroveci pneumonia (PJP) in King Chulalongkorn Memorial Hospital were recruited. Both cytological method and PCR with FTA filter paper technique were performed to detect P. jiroveci from each specimen.*
Results: *The detectability rate of P. jiroveci infection was 21%. The PCR with FTA filter paper method was 4 folds much more sensitive than Giemsa staining technique. P. jiroveci was detected in 18% of the HIV/AIDS patients in spite of receiving standard PJP prophylaxis.*
Conclusion: *Detection of P. jiroveci by using FTA filter paper together with PCR in induced sputum samples could detect more cases of P. jiroveci infection than by using cytological method. DNA extraction using the FTA filter paper was more rapid and convenient than other extraction methods. The causes of failure of PJP prophylaxis should be evaluated.*

Keywords: *Pneumocystis jiroveci pneumonia, PJP, Polymerase chain reaction, FTA filter paper*

J Med Assoc Thai 2005; 88(Suppl 4): S294-9
Full text. e-Journal: *http://www.medassocthai.org/journal*

Pneumocystis pneumonia (PJP) is caused by *Pneumocystis jiroveci*, formerly named *Pneumocystis carinii*[1]. Although an incidence of this disease has declined as a result of highly active antiretroviral therapy (HAART)[2], PJP remains one of the most common opportunistic infection in patients infected with human immunodeficiency virus (HIV)[3]. The incidence of pneumocystis pneumonia in developing countries, including Thailand, has continuously increased[4].

Diagnosis of PJP is based on history, physical examination, and laboratory investigation. The risk of pneumocystis pneumonia among HIV/AIDS patients is greatly increased when the T-helper cell count (CD4+) is less than 200 cells per millimeter[5] because the host defence against *P. jiroveci* depends mainly on the cellular immunity. The dominant presentations of the disease are low-grade fever, progressive dyspnea, nonproductive cough, bilateral perihilar interstitial infiltrates, and hypoxemia[6]. Because these classic symptoms do not always appear[7], the diagnosis of *P. jiroveci* infection needs to be confirmed by laboratory investigation. Nevertheless, *Pneumocystis* spp. cannot be cultured; therefore, the current diagnostic method still requires direct microscopic examination after Giemsa, O-Toluidine, or Gomori methenamine silver (GMS) staining of respiratory specimens in order to identify the *Pneumocystis* organism[8,9].

Recently, the polymerase chain reaction (PCR) has been used to amplify *P. jiroveci*-specific DNA sequences from respiratory specimens, so as to increase the detection of the parasite. A single PCR has greater sensitivity and specificity than staining methods, when

Correspondence to : Nuchprayoon S, Lymphatic Filariasis Research Unit, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. Phone: 0-2256-4387, Fax: 0-2252-4963, E-mail: fmedstt@md2.md.chula.ac.th

the PCR primers for *Pneumocystis* mitochondrial large-subunit ribosomal RNA (rRNA) are used[10,11]. The induced sputum samples often contain many endogenous substances that will inhibit PCR, and yield false-negative results. In order to achieve appropriate DNA template preparations, the methods currently used for PCR to detect microorganisms often require multiple steps[12]. However, the simple filter-based technique (ie. Flinders Technology Associates; FTA® paper) has been developed to obtain DNA for PCR technique effectively[12,13].

The appropriate specimens for detection of *P. jeroveci* are induced sputum, bronchoalveolar fluid (BALF), or lung tissue. BALF has traditionally been the definitive clinical sample. However, the procedure to obtain the specimen is invasive while the induced sputum sample is not sensitive compared to BALF and lung tissue for detection of *P. jiroveci* [14,15]. The collection methods of sputum specimens are less invasive, and more convenient than those of BALF or lung tissue. Therefore, increasing sensitivity and specificity for PJP detection assay using sputum samples should be collected in the laboratory.

This study is the first report to evaluate the usefulness of FTA filter paper together with PCR to detect *P. jiroveci* in HIV/AIDS patients.

Material and Method

Study population and clinical specimens

Respiratory specimens were induced sputum samples from 52 patients with HIV/AIDS and suspected of *Pneumocystis jiroveci* pneumonia in King Chulalongkorn Memorial hospital, between January 2002 and June 2004. The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Each sputum specimen was treated with mucolytic agent (6.5 mM dithiothreitol, Gibco, Grand Island, USA) and followed by centrifugation. The pellet was processed for cytological method and DNA extraction.

Cytology

The pellet was directly smeared onto a slide pellet, dried and fixed with methanol. Giemsa staining was performed using methodology as described previously[16]. The slides were examined by two individuals independently.

DNA extraction and detection of P. jirovecii

The DNA was extracted from the sample by using FTA filter paper (FTA, Whatman Inc, Kent, UK) as previously described[12,13]. Each specimen was spotted onto the FTA paper, air dried at room temperature, and washed twice with 200 ml of FTA purification buffer for 15 min. The repeated washing was performed twice with 200 ml of TE-1 buffer (10mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0) for 5 min. The product was used as a DNA template for the PCR amplification.

The primers used were pAZ 102E-(5'GATGGCTGTTTCCAAGCCCA-3') and pAZ 102H-(5'GTGTACGTTGCAAAGTACTC-3') (Invitrogen, Gibco,Tokyo, Japan) in order to amplify a 346-bp segment from a specific region of a gene coding for a large subunit of mitochondrial rRNA of human *P. jiroveci*[10].

The DNA was amplified in PCR buffer (10mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$) containing 200 µM dNTPs, 1 pmol of each primer, and 0.5 units of Taq DNA polymerase. The PCR was performed by predenaturation at 94°C for 3 min and followed by denaturation at 94 °C for 1 min, annealing at 52 °C for 30 sec, and extension at 72 °C for 1 min, for 35 cycles, by using a DNA Thermocylcer (GeneAmp® PCR system 2400, Perkin Elmer, USA). The PCR products were determined by 0.8% agarose gel electrophoresis (Gibco Grand Island, USA) with Tris-acetate EDTA buffer (Tris 0.04M, Acetic acid 0.02M, EDTA, disodium 0.001M). The gel was stained with ethidium bromide and visualized by using ultraviolet light. Negative control (normal human sputum samples) and positive control (sputum of HIV/AIDS patient with PJP) were included in each experiment.

Results

Of all 52 HIV/AIDS patients, 29 were female and 23 were male patients. Cytological staining and PCR were performed for detection of *P. jiroveci* in induced sputum samples. The 346-bp band of *P. jiroveci* DNA from some patients was showed as a single band (Fig. 1). Eleven (21%) cases were positive for *P. jeroveci* infection by PCR, 8 were female and the rest (3) were male (data not showed). Three HIV/AIDS patients were under age of 15, and 1 among them was positive for PJP detection. The rest (49) were more than 15 years old, while 10 of them were positive for *P. jeroveci* (data not showed)

The result of *P. jiroveci* detection is summarized in Fig. 2 and Table 1. Of the total 52 specimens tested, 11 were positive by PCR, giving the detectability rate of *P. jiroveci* infection to 21%. Only 3 (6%) samples were positive by cytology. Eight samples negative by cytology were positive by PCR, while none of the cytology-positive specimens were negative by

PCR. Therefore, the use of FTA filter paper together with PCR could detect more cases of *P. jiroveci* 4 folds as much compared to staining method.

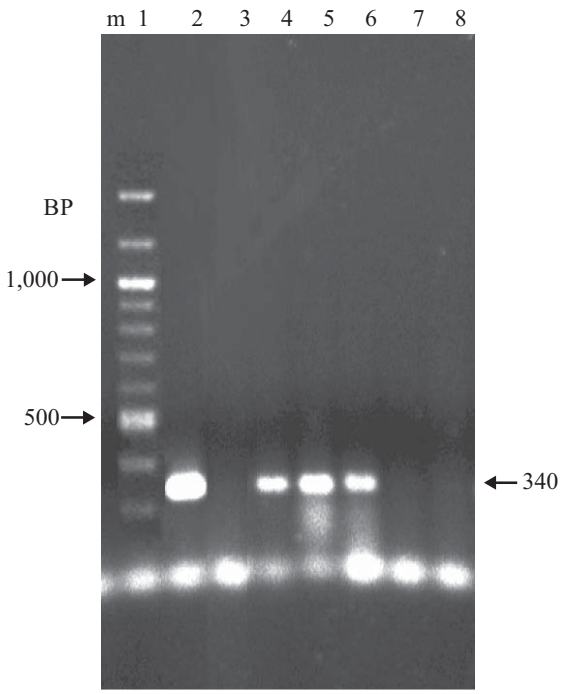
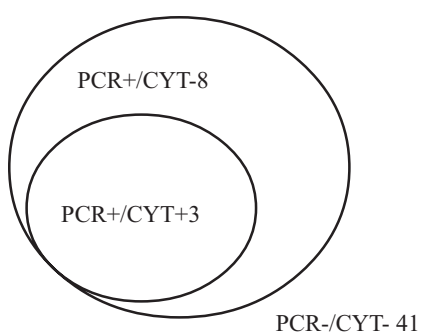


Fig. 1 PCR detection for *P. jiroveci* DNA from induced sputum specimens. Ten microliters of PCR products were subjected to electrophoresis in 0.8% agarose gel. The 346-bp band was confirmed a positive result in both positive control and patients' specimens containing *P. jiroveci*. LaneM: 100 bp DNA marker; Lane 1: positive control; Lane 2: negative control; Lane 3-5: HIV +ve /*P. jiroveci* +ve; Lane 6-7: HIV +ve/ *P. jiroveci* -ve



+ = positive result - = negative result

Fig. 2 Venn diagram of Cytology (CYT) and PCR to detect *P. jiroveci* in 52-induced sputum samples obtained from HIV/AIDS-infected patients

Table 1. Comparison of PCR with cytological staining method for detection *P. jiroveci*

A.

Sputum	No. of samples		Detectability (%)
	Positive	Negative	
CYT	3	49	6
PCR	11	41	21

B.

		PCR detction	
		Positive	Negative
Microscopic examination	Positive	3	0
	Negative	8	41

Table 2. Correlation of pneumocystis pneumonia prophylaxis and the *P. jiroveci* detection

		PJP prophylaxis		Total
		+	-	
P. jiroveci	+	9	2	11
Detection	-	40	1	41
Total		49	3	52

Among the total 52 HIV/AIDS patients, 94% (49/52) received prophylactic drug against the *P. jiroveci* infection (Table 2). We found that 9 (18%) patients given prophylaxis were still positive for detection of *Pneumocystis*. However, one of three HIV/AIDS patients who did not receive the prophylaxis remained negative for the organism, while the other two became positive.

Discussion

Pneumocystis jiroveci is an important opportunistic fungus that causes potentially fatal pneumonia in HIV/AIDS patients. This pathogen cannot be cultured. Therefore, the diagnosis of PJP is based on direct microscopic examination to visualize cysts or trophozoites by special stains of respiratory specimens (ie. induced sputum, BALF and lung tissue). Identification of the organism is still considered to bethe gold standard for dianosis of *P. jiroveci*. Giemsa stain is routinely used to detect trophozoites, while Gomori methinamine silver (GMS) and O-Toluidine is used to detect cysts. As Giemsa stain is simple, inexpensive and familiar to most microbiological laboratories, this staining method is widely used for screening samples suspected of PJP[17]. However, Giemsa cytological detection still presents some disadvantages. Direct microscopic examination requires enough burden of the organisms in picked-up specimens to visualize.

Moreover, technical expertise is also needed to determine and differentiate *P. jiroveci* from artifacts and other organisms, such as yeast form of *Candida albican* which has approximately the same size as *P. jiroveci* cyst. Therefore, the microscopic examination is rather uncertain of both sensitivity and specificity.

The polymerase chain reaction (PCR) technique has been widely used as a tool for detection of *Pneumocystis* organisms. PCR has been found more sensitive than cytological staining with senstitivity of 84-100 % and specificity of 95-100%[15,18,19] . Our study also suggested that PCR was superior to cytological method in the detection of *P. jiroveci* in induced sputum samples. By using PCR, the detectability rate was about 4 folds as much as using cytological method.

BALF has been considered to be the definitive sample for detection of *P. jiroveci*. The *P. jiroveci* detection in BALF is more sensitive than that in induced sputum samples, with sensitivity of 100% and 84.62% respectively[15]. However, the procedure obtaining the BALF specimens is invasive. The more sensitive method to detect *Pneumocystis* organisms from noninvasive induced sputum should be useful and more practical for the physicians and patients. The FTA filter paper is a filter-based template. It has been used to extract DNA with more DNA recovery compared to phenol-chloroform method which is widely used as a standard method[13] . When organisms contact with this paper, they will be lysed after solubilized in FTA purification buffer, and their DNA will be trapped on the matrix. Other debris then will be removed by washing. Using FTA filter paper, our PCR result showed comparable detectability rate (4 folds as much as the rate of Giemsa staining) to previous PCR studies (2-6 times as much)[15,20]. Moreover, using the FTA filter paper does not involve several steps as the phenol-chloroform method. Furthermore, the FTA technique is a simple and rapid test that can be used with a large number of samples, as well as does not require experienced personnel to handle[12].

The US Public Health Service Task Force has recommended cotrimoxazole, the combination of trimetroprim (TMP) and sulfamethoxazole (SMZ), to be used as the first-line drug for the prophylaxis against *P. jiroveci* infection. Dose of cotrimoxazole recommended are 1 double-strength tablet daily or 1 single-strength tablet daily[21]. HIV/AIDS patients should receive primary prophylaxis against PJP when the CD4+ count is less than 200 cells per millimeter, or if there is a history of oropharyngeal candidiasis. Secondary prophylaxis should begin in patients who have ever had been infected with *Pneumocystis* organisms. SMZ is an inhibitor of dihydropterate synthase (DHPS), while TMP inhibits dihydrofolate reductase (DHFR). Our data showed 18% (9/49) failure rate in the HIV/AIDS patients receiving PJP prophylaxis. Although the patients provided the information of good drug compliance, the possibility of drug allergy and lacking adherence could not be ruled out. Another explanation for failure of PJP prophylaxis is that the drug resistance might occur in Thai-HIV/AIDS patients. Both DHPS and DHFR are enzymes involved in the biosynthesis of folic acid of *P. jiroveci*. The emergence of *P. jiroveci* drug resistance has been suggested[22-24]. The mechanism of drug resistance may result from the alteration of DHPS or DHFR genes. Further study of drug resistance will answer the question. There were two of three patients, who did not receive PJP prophylaxis, infected with *P. jeroveci*. This result suggests that criteria for PJP prophylaxis in HIV/AIDS patients may be needed to be reevaluated.

Acknowledgements

This study was supported by Rajadapisek Sompoj Fund, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. The authors wish to thank Mr. Suradej Siripattanapipong (Department of Microbiology, Faculty of Science, Mahidol University), Miss Alisa Junpee, Miss Piyachat Surattanasing, Miss Jatuporn Sutjitkul, and the staffs at Department of Parasitology, Faculty of Medicine, Chulalongkorn University, for their technical assistance.

References

1. James RS, Charles BB, Robert FM, Ann EW. A New Name (Pneumocystis jiroveci) for Pneumocystis from Humans. Emerg Infect Dis 2002; 8: 891-6.
2. Kaplan JE, Hanson D, Dworkin MS, Frederick T, Bertolli J, Lindegren ML, Holmberg S, Jones JL. Epidemiology of human immunodeficiency virus-associated opportunistic infections in the United States in the era of highly active antiretroviral therapy. Clin Infect Dis 2000; 30 (Suppl 1): S5-14.
3. Morris A, Lundgren JD, Masur H, Walzer PD, Hanson DL, Frederick T, Huang L, Beard CB, Kaplan JE. Current epidemiology of Pneumocystis pneumonia. Emerg Infect Dis 2004; 10: 1713-20.
4. Ruxrungtham K, Phanuphak P. Update on HIV/AIDS in Thailand. J Med Assoc Thai 2001; 84 (Suppl 1): S1-17.
5. Phair J, Munoz A, Detels R, Kaslow R, Rinaldo C,

Saah A. The risk of Pneumocystis carinii pneumonia among men infected with human immunodeficiency virus type 1. Multicenter AIDS Cohort Study Group. N Engl J Med 1990; 322: 161-5.
6. Thomas CF Jr, Limper AH. Pneumocystis pneumonia. N Engl J Med 2004; 350: 2487-98.
7. Wilkin A, Feinberg J. Pneumocystis carinii Pneumonia: A Clinical Review. Am Fam Physician 1999; 60: 1699-1708, 1713-4.
8. el-Nassery SM, Rahmy AE, el Gebaly WM, Sadaka HA. Pneumocystis carinii: recognition of the infection in albino rats using different stains. J Egypt Soc Parasitol 1994; 24: 285-94.
9. Savoia D, Caramello P. The microscopic identification of Pneumocystis carinii. J Protozool 1989; 36: 72S-4S
10. Wakefield AE, Guiver L, Miller RF, Hopkin JM. DNA amplification on induced sputum samples for diagnosis of Pneumocystis carinii pneumonia. Lancet 1991; 337: 1378-79
11. Ribes JA, Limper AH, Espy MJ, Smith TF. PCR detection of Pneumocystis carinii in bronchoalveolar lavage specimens: analysis of sensitivity and specificity. J Clin Microbiol 1997; 35: 830-5
12. Orlandi PA, Lampel KA. Extraction-free, filter-based template preparation for rapid and sensitive PCR detection of pathogenic parasitic protozoa. J Clin Microbiol 2000; 38: 2271-7.
13. Subrungruang I, Mungthin M, Chavalitshewinkoon-Petmitr P, Rangsin R, Naaglor T, Leelayoova S. Evaluation of DNA extraction and PCR methods for detection of Enterocytozoon bienuesi in stool specimens. J Clin Microbiol 2004; 42: 3490-4.
14. Mirdha BR, Guleria R. Comparative yield of different respiratory samples for diagnosis of Pneumocystis carinii infections in HIV-seropositive and seronegative individuals in India. Southeast Asian J Trop Med Public Health 2000; 31: 473-7.
15. Pinlaor S, Mootsikapun P, Pinlaor P, Phunmanee A, Pipitgool V, Sithithaworn P, Chumpia W, Sithithaworn J. PCR diagnosis of Pneumocystis carinii on sputum and bronchoalveolar lavage samples in immuno-compromised patients. Parasitol Res 2004; 94: 213-8.
16. Saksirisampant W, Eampokalap B, Chantharodevong R, Changthong R. Comparison of methods for identification of Pneumocystis carinii in bronchoalveolar lavage fluid. J Med Assoc Thai 2002; 85 (Suppl 1): S407-14.
17. Holten-Andersen W, Kolmos HJ. Comparison of methenamine silver nitrate and Giemsa stain for detection of Pneumocystis carinii in bronchoalveolar lavage specimens from HIV infected patients. APMIS 1989; 97: 745-7.
18. Ishimine T, Nakamoto A, Higa F, Koide M, Inadome J, Kawakami K, Fukuhara H, Kusano N, Kitsukawa K, Saito A. Usefulness of polymerase chain reaction method in the early diagnosis of Pneumocystis carinii pneumonia. Kansenshogaku Zasshi 1994; 68: 751-8.
19. Hua L, Qin S, Wang A, Sheng R, Zhang K. The diagnostic value of polymerase chain reaction for the detection of Pneumocystis carinii DNA from induced sputum samples. Zhonghua Nei Ke Za Zhi 2002; 41: 610-2.
20. Leibovitz E, Pollack H, Moore T, Papellas J, Gallo L, Krasinski K, Borkowsky W. Comparison of PCR and standard cytological staining for detection of Pneumocystis carinii from respiratory specimens from patients with or at high risk for infection by human immunodeficiency virus. J Clin Microbiol 1995; 33: 3004-7.
21. Centers for Disease Control and Prevention (CDC). Recommendations for Prophylaxis Against Pneumocystis carinii Pneumonia for Adults and Adolescents Infected with Human Immunodeficiency Virus. MMWR 1992; 4; 41(RR-4); 001.
22. Sritangratanakul S, Nuchprayoon S, Nuchprayoon I. Pneumocystis pneumonia: An update. J Med Assoc Thai 2004; 87 (Suppl 2): S309-17.
23. Nahimana A, Rabodonirina M, Bille J, Francioli P, Hauser PM. Mutations of Pneumocystis jirovecii dihydrofolate reductase associated with failure of prophylaxis. Antimicrob Agents Chemother 2004; 48: 4301-5.
24. Huang L, Crothers K, Atzori C, Benfield T, Miller R, Rabodonirina M, Helweg-Larsen J. Dihydropteroate synthase gene mutations in Pneumocystis and sulfa resistance. Emerg Infect Dis 2004; 10: 1721-8.

การตรวจหาเชื้อ Pneumocystis jiroveci จากเสมหะของผู้ป่วยติดเชื้อเอชไอวี/เอดส์โดยการใช้ปฏิกิริยาลูกโซ่โพลีเมอเรสร่วมกับการสกัดสารพันธุกรรมโดยกระดาษกรองเอฟทีเอ

ศิรญา ไชยะกุล, วิไล ศักดิ์ศิริสัมพันธ์, จารุรัตน์ เภานิบล, สุทิน เย็นท่าข้าม, มฑิรุทธ มุ่งถิ่น, เสาวนีย์ ลีละยูวะ, สุรางค์ นุชประยูร

วัตถุประสงค์: *เพื่อประเมินผลการตรวจหาเชื้อ Pneumocystis jiroveci จากเสมหะของผู้ป่วยติดเชื้อโรคเอดส์โดยการใช้ปฏิกิริยาลูกโซ่โพลีเมอเรสร่วมกับการสกัดสารพันธุกรรมโดยกระดาษกรอง FTA*

วัสดุและวิธีการ: *ทำการศึกษาผู้ป่วยติดเชื้อเอดส์จำนวน 52 คนที่สงสัยว่าติดเชื้อปอดอักเสบ P. jiroveci และเข้ารับการรักษาที่โรงพยาบาลจุฬาลงกรณ์ โดยนำเสมหะของผู้ป่วยแต่ละรายมาตรวจโดยการย้อมสี และทำปฏิกิริยาลูกโซ่โพลีเมอเรสร่วมกับการสกัดสารพันธุกรรมโดยกระดาษกรอง FTA*

ผลการศึกษา: *อัตราการตรวจพบเชื้อ P. jiroveci ในการศึกษานี้ คือ 21% การตรวจโดยการใช้ปฏิกิริยาลูกโซ่โพลีเมอเรสร่วมกับการสกัดสารพันธุกรรมโดยกระดาษกรอง FTA สามารถตรวจพบเชื้อได้มากกว่าการย้อมสี Giemsa ถึง 4 เท่า พบว่า 18% ของผู้ป่วยติดเชื้อเอดส์ที่ได้รับยาป้องกันโรคยังมีการติดเชื้อโรคปอดอักเสบ P. jiroveci*

สรุป: *การใช้ปฏิกิริยาลูกโซ่โพลีเมอเรสร่วมกับการสกัดสารพันธุกรรมโดยกระดาษกรอง FTA สามารถตรวจพบเชื้อ P. jiroveci ในเสมหะของผู้ป่วยติดเชื้อโรคเอดส์ได้ดีกว่าการย้อมสี Giemsa และการสกัดสารพันธุกรรมด้วยกระดาษกรอง FTA มีความสะดวกและรวดเร็วกว่าวิธีอื่น นอกจากนี้ยังคงจำเป็นต้องศึกษาถึงสาเหตุของความล้มเหลวของวิธีการป้องกันโรคต่อไปในอนาคต*