Journal of Clinical Laboratory Analysis 21:382–386 (2007)

Flinders Technology Associates (FTA) Filter Paper–Based DNA Extraction With Polymerase Chain Reaction (PCR) for Detection of *Pneumocystis jirovecii* from Respiratory Specimens of Immunocompromised Patients

Surang Nuchprayoon,[1]* Wilai Saksirisampant,[1] Siraya Jaijakul,[1] and Issarang Nuchprayoon[2]

[1]*Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*
[2]*Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

We evaluated the diagnostic value of Flinders Technology Associates (FTA) filter paper together with polymerase chain reaction (PCR) for detection of *Pneumocystis jirovecii* (*carinii*) from induced sputum (IS) and bronchoalveolar lavage fluid (BALF) samples. The study involved 162 patients with clinical diagnosis of pneumocystis pneumonia (PcP) of human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) patients and other immunocompromised patients. *P. jirovecii* cysts or trophozoites were detected in IS and BALF by cytological method. The mitochondrial 5S ribosomal ribonucleic acid (rRNA) gene of *P. jirovecii* was amplified from these samples by using FTA filters together with a one-step PCR method (FTA-PCR). With the FTA-PCR method, the sensitivity and specificity of the test compared to microscopic examination were 67% and 90% for IS, while they were 67% and 91% for BALF, respectively. The sensitivity and specificity of the FTA-PCR test was also comparable to PCR with the conventional deoxyribonucleic acid (DNA) extraction method. We concluded that FTA-PCR is useful to detect *P. jirovecii* in noninvasive IS. J. Clin. Lab. Anal. 21: 382–386, 2007. © 2007 Wiley-Liss, Inc.

Key words: Pneumocystis pneumonia; polymerase chain reaction; FTA filter paper; sputum; bronchoalveolar lavage fluid

INTRODUCTION

Pneumocystis carinii, a fungus, has been recently renamed as *Pneumocystis jirovecii* (1,2). It was first identified as a human respiratory pathogen by Frenkel (3), and originally misclassified as a protozoa (4). The infection emerged as a significant cause of pneumonia in Europe after World War II (5). It is an opportunistic organism that causes high morbidity and mortality of pneumonia in neonates and in malnourished and immunocompromised individuals (e.g., human immunodeficiency virus/acquired immune deficiency syndrome [HIV/AIDS] patients). An airborne route is important for transmission (6). Prior to the use of chemoprophylaxis for pneumocystis pneumonia (PcP) in HIV-infected patients, 75% of these patients would develop PcP during their lifetime and this organism was responsible for two-thirds of AIDS-defining illnesses (7). The extensive use of chemoprophylaxis and the advent of highly active antiretroviral therapy (HAART) have made the incidence of PcP decline. However, PcP remains one of the most common opportunistic infections in HIV-infected patients (1,8,9). Studies from Thailand show a prevalence of 27–40% among HIV-infected patients (10–12).

*Correspondence to: Surang Nuchprayoon, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Rama IV Rd., Patumwan, Bangkok 10330, Thailand.
E-mail: fmedstt@md.chula.ac.th

Surang Nuchprayoon and Wilai Saksirisampant contributed equally to this work.

Grant sponsor: Rajadapiseak Sompoj Fund, Faculty of Medicine, Chulalongkorn University.

Received 14 May 2007; Accepted 12 July 2007
DOI 10.1002/jcla.20200
Published online in Wiley InterScience (www.interscience.wiley.com).

The diagnosis of PcP has undergone an evolution. At first, it was diagnosed basically on clinical presentations such as dyspnea, low grade fever, and cough, together with physical examination and chest radiographic demonstration of bilateral perihilar interstitial infiltrates. In addition, histological identification is made on the basis of frothy intraalveolar infiltrate in hematoxylin-eosin-stained section of open lung biopsy specimens or autopsy tissues (9). A variety of staining techniques, including Gomori's methenamine silver (GMS), Giemsa, Gram-Weigert, Papanicolaou, and O-toluidine blue stains, have been used to identify the cysts and (or) trophozoites. The biopsy specimens have recently been replaced by examination of induced sputum (IS) or bronchoalveolar lavage fluid (BALF) (13).

The parasitological diagnosis of PcP lacks sensitivity, especially for patients in whom the organism burden is low, or who have subclinical disease or extrapulmonary infections. In this circumstance, polymerase chain reaction (PCR) can enhance the detection of *P. jirovecii* in clinical specimens. This technique can be performed in large sample sizes and replaces the microscopic diagnostic method, which requires a highly trained specialist. Due to the limitation of currently available deoxyribonucleic acid (DNA) extraction methods, the simple filter-based technique (i.e., Flinders Technology Associates [FTA®] paper; Whatman Bioscience, Cambridge, UK) has been developed and demonstrated a high sensitivity for DNA detection by PCR (14,15).

We report here the use of FTA-PCR to detect the *P. jirovecii* mitochondrial 5S rRNA gene in IS and BALF specimens.

MATERIALS AND METHODS

Population and Clinical Specimens

Between January 2002 and June 2004, 162 samples were obtained from 162 patients aged 1 month to 81 years in King Chulalongkorn Memorial hospital. There were 74 HIV/AIDS patients and 88 patients with other immunosuppressed conditions. These specimens were 106 IS and 56 BALF. This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Each sputum specimen was diluted with distilled water (1:1) and mixed with dithiothreitol (DTT; Gibco BRL, Grand Island, NY) to a final concentration of 5 mM. After incubation at 37°C for 10 min, samples were centrifuged and pellets were washed with phosphate buffered saline (PBS). Then the pellets were processed for cytological method and DNA amplification. The BALF, which was normally clear, was first used for parasitological staining and the rest was kept at −20°C for further PCR assay.

Parasitological Staining

Giemsa staining was performed as previously described (11).

DNA Preparation on the FTA Filter Paper

The DNA was extracted with FTA filter paper as previously described (14,16). A 6-mm disk was punched out from FTA filter paper (Whatman Bioscience) by using a modified hole punch. A total of 15 μL of each pellet was applied directly onto the FTA disks, and air dried at room temperature. The FTA disk was washed twice with 200 μL of FTA purification buffer (Whatman Bioscience) for 15 min, and washed twice with 200 μL of TE-1 buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM ethylene diamine tetraacetic acid [EDTA], pH 8.0) for 5 min, and then again air dried as recommended by the manufacturer.

Amplification Primers

The primers were pAZ 102E-(5′GATGGCTGTTTCC AAGCCCA-3′) and pAZ 102H-(5′GTGTACGTTGC AAAGTACTC-3′). They were used to amplify a 346-bp segment from a specific region at the mitochondrial 5S ribosomal ribonucleic acid (rRNA) gene of *P. jirovecii* (17). Primers were synthesized by Invitrogen Life Technologies (Carlsbad, CA).

Amplification Protocol

The PCR reactions were performed in a DNA thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer, Norwalk, CT), as previously described (14). The PCR products were determined by submarine agarose gel electrophoresis, stained by ethidium bromide, and visualized under ultraviolet light. Normal human sputa were used as the negative control.

Analysis of PCR Data

All the PCR results were analyzed independently from the microscopic examination results. A positive result was accepted as agreement of two of the test determinations (cytological method and FTA-PCR method).

RESULTS

Detectability Rate of FTA-PCR and Cytological Method for the Detection of *P. jirovecii*

Of the 162 specimens, 74 were from HIV/AIDS patients and 88 were from other immunocompromised patients (Table 1). The typical cytological result of

TABLE 1. Microscopic and PCR results of *P. jirovecii* detection classified by disease

	Microscopic examination				
	Positive		Negative		
Associated disease	PCR+	PCR−	PCR+	PCR−	Total
HIV/AIDS	6	3	9	56	74
Other immunocompromised diseases	8	4	5	71	88
Total	14	7	14	127	162

*Values are the number of specimens in each category.

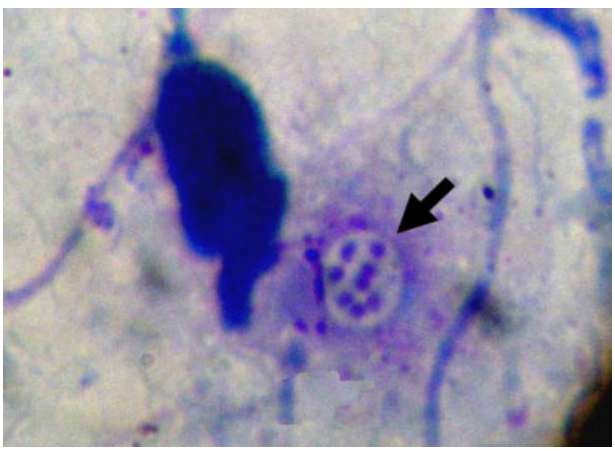

Fig. 1. *P. jirovecii* cyst by Giemsa staining. The cysts appeared as clear areas containing eight dots of nuclei of the trophozoites within the cyst wall (arrows).

P. jirovecii by Giemsa staining is shown in Fig. 1. The rounded cysts were 4–7 μm in diameter and contained eight intracystic bodies, with nuclei stained by the dye. The well-defined band of *P. jirovecii*-specific mitochondrial 5S DNA migrated at the anticipated size of 346 bp (Fig. 2). An example of *P. jirovecii*-specific mitochondrial 5S DNA fragment in HIV/AIDS and other immunocompromised patients is shown. In our study, if both tests were positive, the result was defined as true-positive. Therefore, six (8%) and eight (9%) of the HIV/AIDS and other immunocompromised patients, respectively, were positive for *P. jirovecii* (Table 1). Among all the specimens, 28 (17%) were positive by PCR (Table 1), compared to 21 (12%) positive by cytology (CYT). A total of 35 (21%) samples were positive by either PCR or CYT.

Sensitivities and Specificities of FTA-PCR

Sputum

Of the 106 IS specimens (Table 2), nine were positive for *P. jirovecii* by CYT and 16 were positive by PCR. Of these nine CYT-positive specimens, six were positive by PCR. The three PCR-negative specimens that yielded a CYT-positive were from two HIV/AIDS patients and one non-HIV-infected patient.

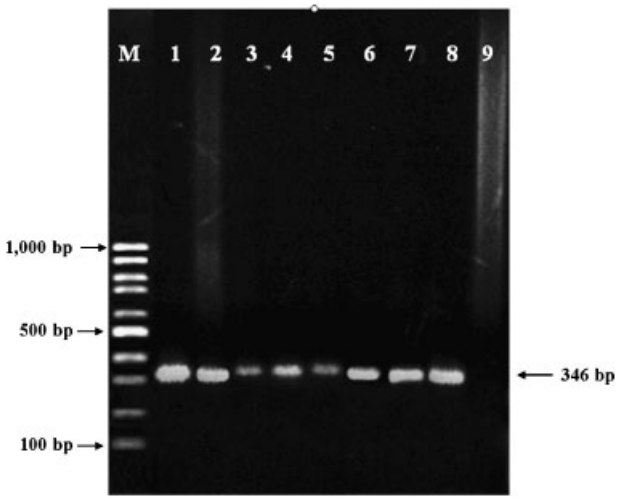

Fig. 2. PCR detection for the *P. jirovecii* mitochondrial 5S DNA from specimens. The well-defined band of *P. jirovecii*-specific DNA migrated at the anticipated size of 346 bp (arrow). Lane M: 100-bp DNA marker; Lane 1: positive control; Lanes 2–5: *P. jirovecii* positive in HIV/AIDS patients; Lanes 6–8: *P. jirovecii* positive in other immunocompromised patients; Lane 9: negative control.

TABLE 2. FTA-PCR and microscopic examination for *P. jirovecii* detection in induced sputum (IS) and bronchoalveolar lavage fluid (BALF) specimens

	Microscopic examination			
	IS		BALF	
PCR result	Positive	Negative	Positive	Negative
Positive	6	10	8	4
Negative	3	87	4	40

*Values are the number of specimens in each category.

BALF

Of the 56 BALF specimens, 12 were positive for *P. jirovecii* by CYT. There were four BALF specimens that were negative by CYT but positive by PCR. An additional four specimens that were positive by CYT, but negative by PCR, were from patients who were under treatment prior to the specimen collection.

The specimens of patients who were subsequently positive for *P. jirovecii* by both CYT and PCR were considered true-positive specimens. Compared to CYT, the sensitivity and specificity of the PCR assay for the

detection of *P. jirovecii* from IS were 67% and 90% and from BALF specimens were 67% and 91%, respectively.

DISCUSSION

PcP remains one of the most common opportunistic infections in HIV/AIDS patients (18). The detectability rate of *P. jirovecii* in this study was 8% for HIV/AIDS patients and 9% for other immunocompromised patients. Therefore, apart from HIV/AIDS patients, PcP should also be concern of in other immunocompromised patients.

To determine the etiology of interstitial pneumonia is a very important issue in the treatment of immunocompromised patients. We used FTA-PCR of *P. jirovecii* mitochondrial 5S rRNA gene to detect *P. jirovecii* in samples from invasive (BALF) and noninvasive (IS) procedures. Detection of *P. jirovecii* in lung tissue, IS, and/or BALF specimens has classically required direct visualization of cysts or trophozoites by special staining methods. The efficiency of this technique is limited not only by the skill of the observer, but also by the organism burden in the specimens. Compared with PCR, diagnosis by direct examination is much more subjective; for example, this organism has approximately the same size and pattern as the yeast form of *Candida albicans*, which can result in its misdiagnosis (19).

PCR has been widely used to increase the detection of parasites including *P. jirovecii* (20–23). The technique has greater sensitivity and specificity than cytological methods for detection of *P. jirovecii* (14,17,24). Our results also showed that PCR for detection of the *P. jirovecii* mitochondrial large-subunit rRNA gene in clinical respiratory samples has a higher detectability rate than CYT. However, the conventional DNA extraction technique, the phenol-chloroform method, takes several steps and all manipulations should be carried out in a chemical hood because phenol is highly corrosive (25). Furthermore, other commercially available DNA extraction kits (i.e., QIAamp) are also time-consuming (16). Therefore, the more convenient and safer DNA extraction method of FTA filter paper has been developed.

Currently, the PCR-band detection of *P. jirovecii* from sputum samples is not as sensitive as BALF samples (26,27). We showed that the sensitivity and specificity of the FTA-PCR assay for *P. jirovecii* detection from IS (67% and 90%, respectively) were comparable to that of BALF specimens (67% and 91%, respectively). Moreover, using FTA filter paper, the performance of the assay in our study from IS specimens was comparable with other published studies that showed the sensitivity of PCR for IS (71–100%) and BALF (85–100%), as well as the specificity for IS (94–100%) and BALF (77–100%) (27–29). The use of extraction-free FTA is very simple and it is easy to transport because the FTA filter paper can be stored at room temperature for over 14 years. FTA purification buffer is a nontoxic and nonorganic reagent, thus the FTA technique is safe to perform. Furthermore, this method can be used with a large number of specimens, and does not require experienced personnel to handle it (16).

Moreover, because the negative predictive value of the test in IS sample is high (97%), a negative PCR test could rule out PcP. This is a key advantage because it could decrease the need for bronchoscopy. Obtaining an IS is less costly, less invasive, and has lower risk of complication than bronchoscopy.

In conclusion, we emphasize the usefulness of FTA filter paper with PCR-based detection of *P. jirovecii*, using IS specimens, because of its high sensitivity and specificity, and its ease of handling, compared to BALF specimens. This technique could improve the diagnosis of PcP from respiratory material obtained noninvasively from immunocompromised patients. However, unfortunately, we could not obtain both IS and BALF specimens from each patient because we could not perform bronchoscopy, which is a practical invasive procedure, in every suspected patient. Therefore, further study to improve diagnostic yield is still needed.

ACKNOWLEDGMENTS

We thank Dr. Saovanee Leelayoova, Dr. Mathirut Mungthin, Mr. Suradej Siripattanapipong, Ms. Alisa Junpee, Ms. Sutin Yenthakam, and Ms. Jarurat Paonibol for their technical advice and assistance.

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