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A highly sensitive novel PCR assay for detection of *Pneumocystis jirovecii* DNA in bronchoalveloar lavage specimens from immunocompromised patients

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Abstract

Pneumocystis jirovecii pneumonia (PCP) is a leading cause of morbidity and mortality in immunocompromised patients. Despite the sensitivity of the commonly used PCR for diagnosing *P. jirovecii* with primers pAZ102-H/pAZ102-E and pAZ102-X/pAZ102-Y derived from *mtLSU rRNA* (conventional PCR), some PCP patients who had demonstrable organisms by staining methods failed to give positive PCR results. Herein, we devised a more sensitive PCR assay derived from the same gene target to circumvent these false-negative tests. Single brochoalveolar lavage (BAL) samples were collected from human immunodeficiency virus (HIV)-infected (n = 66) and non-HIV (n = 36) immunocompromised patients presenting with fever, dyspnoea, cough and pulmonary infiltrates. *Pneumocystis jirovecii* was diagnosed with Giemsa-stained smear, immunofluorescence assay, conventional single-round and nested PCR, and new single-round and nested PCR in 46 (45.1%), 53 (52.0%), 69 (67.6%), 74 (72.6%), 87 (85.3%) and 91 (89.2%) patients, respectively. The new PCR could detect *P. jirovecii* DNA in BAL fluids two to three orders of magnitude more dilute than conventional PCR. Sequence analysis revealed one to three nucleotide substitutions within the primers for conventional PCR among clinical isolates. Although both conventional and new PCR assays were highly specific for diagnosing *P. jirovecii*, the new PCR yielded more positive results than conventional PCR among BAL samples that were negative by both Giemsa stain and immunofluorescence assay. Hence, the new PCR offered a more sensitive detection of *P. jirovecii* infection and colonization than conventional PCR.

Keywords: Diagnosis, human immunodeficiency virus, immunocompromised host, mitochondrial large subunit ribosomal RNA, *Pneumocystis jirovecii*, polymerase chain reaction

Original Submission: 15 February 2011; **Revised Submission:** 18 August 2011; **Accepted:** 19 August 2011

Editor: E. Roilides

Article published online: 31 August 2011

Clin Microbiol Infect 2012; **18:** 598–603

10.1111/j.1469-0691.2011.03656.x

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Introduction

One of the significant morbidity and mortality consequences in immunocompromised patients has been attributable to *Pneumocystis jirovecii* pneumonia (PCP) [1]. Despite its cosmopolitan distribution, the prevalence of PCP seems to exhibit geographic variation and depend on the population studied [2,3]. Although microscopy methods confirm both cystic and trophic stages of *P. jirovecii* and have the best correlation with diagnosis of PCP, PCR-based methods that only detect *P. jirovecii* DNA display greater sensitivity and correlate with PCP in patients with acquired immunodeficiency syndrome with an altered radiography [4]. Detection of *P. jirovecii* DNA is probably a good indicator of the presence of *P. jirovecii* cells in the lung. On the other hand, detection of *P. jirovecii* DNA does not necessarily imply the presence of viable and infectious *P. jirovecii* organisms. Meanwhile, the diagnostic performance of microscopy is variable and highly dependent on quality and type of sample, the number of organisms in the tested material and the experience of the microscopist [4].

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One of the attractive gene targets for diagnostic PCR is the mitochondrial large subunit ribosomal RNA locus (*mtLSU rRNA*) of *P. jirovecii* [4–7]. It is noteworthy that the outer pair of primers pAZ102-E and pAZ102-H, and the inner pair of primers pAZ102-X and pAZ102-Y by Wakefield and colleagues targeting *mtLSU rRNA* of *P. jirovecii* (henceforth conventional PCR) have been widely used as reference primers to evaluate the performance of other diagnostic methods [5–8]. Intriguingly, some isolates of *P. jirovecii* containing demonstrable organisms by staining methods failed to be amplified by conventional PCR [7,9]. Despite various possibilities generating false-negative results, sequence variation within the primers could hinder effective primer-template annealing in a PCR [10]. To address this issue, we determined the *mtLSU rRNA* sequences of *P. jirovecii* isolates spanning regions used for conventional PCR primers and assessed the diagnostic performance of our newly developed PCR assay using bronchoalveolar lavage (BAL) fluids from both human immunodeficiency virus (HIV) and non-HIV immunocompromised patients.

Materials and Methods

Clinical samples

From June 2006 to May 2009, single BAL samples were collected from 102 immunocompromised patients (46 men, 56 women; mean age 42.4 years) presenting with respiratory symptoms and pulmonary infiltrates at King Chulalongkorn Memorial Hospital in Bangkok. Of these, 66 patients had HIV infection with $CD4^+$ lymphocytes < 200/μL and 36 patients were non-HIV with underlying solid organ malignancies (n = 14), haematological malignancies (n = 11), systemic lupus erythematosus (n = 8) and organ transplantation (n = 3). Each BAL sample (~3–5 mL) was briefly vortex-mixed and divided into 1-mL aliquots. The ethical aspects of this study have been approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University (IRB No. 176/51).

Case definition

Because there is no reference standard for PCP diagnosis, we deployed both clinical and laboratory criteria for 'definite PCP' based on the following characteristics: (1) subtle onset of progressive dyspnoea, non-productive cough and pyrexia, (2) bilateral interstitial infiltration on chest roentgenogram, (3) clinical response to anti-*Pneumocystis* treatment, and (4) presence of *P. jirovecii* in respiratory samples by Giemsa stain or immunofluorescence assay (IFA). 'Probable PCP' included those who met all the criteria for 'definite PCP' except that both Giemsa stain and IFA gave negative results. Patients who (1) had respiratory symptoms not typical for PCP, (2) had variant lung infiltrations and (3) were responsive to treatment with other antimicrobial agents or had other definite diagnosis were defined as 'non-PCP'. Forty-seven HIV-positive patients and seven non-HIV patients met all the criteria for 'definite PCP', three HIV-positive patients and three non-HIV patients for 'probable PCP', and 16 HIV-positive patients and 26 non-HIV patients for 'non-PCP' (pulmonary tuberculosis, n = 16; bacterial pneumonia, n = 6; viral pneumonia, n = 4; lung cancer, n = 4; lymphoma, n = 4; aspergillosis, n = 2; fibrotic lung disease, n = 1; systemic sclerosis, n = 1; and unknown causes, n = 4).

Morphological diagnosis

Aliquots of BAL fluids from each patient were used for Giemsa stain and for IFA using the PneunoCell kit (Cellabs, Sydney, Australia) specific for trophic and cystic stages of *P. jirovecii*. Slides were examined by an experienced microscopist who was blinded from the results of other test methods.

DNA extraction

DNA was extracted from a pellet of each BAL sample using QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the instruction manual except for elution with 30 μL TE buffer.

PCR detection

Nested PCR targeting *mtLSU rRNA* of *P. jirovecii* was performed using primers pAZ102-H and pAZ102-E for primary PCR, and pAZ102-X and pAZ102-Y for nested PCR as described elsewhere [5,6] (Fig. 1) in a total volume of 20 μL containing template DNA, 2.5 mM each deoxynucleotide triphosphate, 1.5 μL 10× PCR buffer, 0.3 μM of each primer and 0.4 units Ex*Taq* DNA polymerase (Takara, Seta, Japan). Thermal cycling profile for primary PCR included a pre-amplification denaturation at 94°C, 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and final extension at 72°C for 5 min. Nested PCR was performed using the same thermal cycling profile except annealing at 50°C. All amplifications were performed in an Applied Biosystem GeneAmp® PCR System 9700 thermocycler (PE Biosystems, Foster City, CA) and were analysed by 2% agarose gel electrophoresis.

Cloning and sequencing of partial mtLSU rRNA of *P. jirovecii*

Because the 5′ and 3′ sequences outside the outer PCR primers by Wakefield and colleagues remained unknown, we

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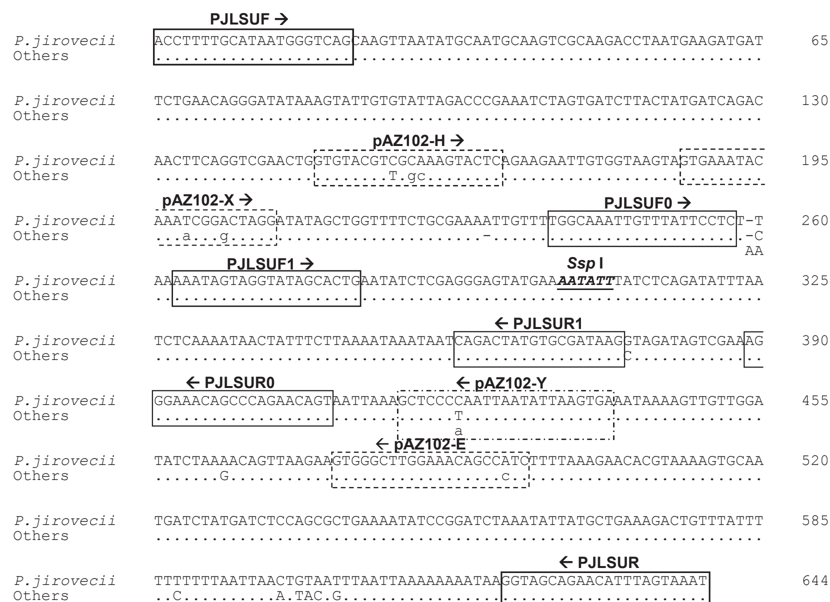

FIG. 1. Alignment of the mitochondrial large subunit ribosomal RNA locus (*mtLSU rRNA*) sequences of *Pneumocystis jirovecii* (PJ1 isolate) encompassing 644-base-pair fragment. 'Others' denotes sequences from 12 Thai isolates determined herein and 26 sequences available in the GenBank database (accession numbers AF337535, AJ608260, EF439813–EF439817, EU917445, EU979566, FJ357841-FJ357852, M58605, S42926, S77824, S77852 and S79766). Locations of primers for conventional PCR [5,6] and for new PCR are in broken lined and solid lined boxes, respectively. Dots represent identical nucleotides and dash es represent deletions. Upper case substituted nucleotides indicate occurrence in two or more sequences. Singleton substitutions are in lower case letters.

used PCR primers derived from *Pneumocystis carinii* (accession number GU133622) to obtain the orthologous sequence of *P. jirovecii*. The forward and reverse primers were PJLSUF: 5′-ACCTTTTGCATAATGGGTCAG-3′ (positions 21664–21684) and PJLSUR: 5′-ATTTACTAAATGTTCTGCTACC-3′ (positions 20986–21007), respectively. The DNA template was from a *P. jirovecii* isolate (PJ1 isolate) previously verified by the dihydropteroate synthase and dihydrofolate reductase sequences. Sequence was determined directly and bi-directionally from PCR-purified templates.

Development of a new PCR assay

A total of 26 partial *mtLSU rRNA* sequences of *P. jirovecii* retrieved from the GenBank database (Fig. 1) and 12 clinical isolates whose sequences were determined in this study were aligned using the Clustal X program [11]. The PCR primers were derived from conserved region as shown in Fig. 1. Primers for primary PCR (PJLSUF0: 5′-TGGCAAATTGTTTATTCCTC-3′ and PJLSUR0: 5′-ACTGTTCTGGGCTGTTTCCCT-3′) and nested PCR (PJLSUF1: 5′-AAATAGTAGGTATAGCACTG-3′ and PJLSUR1: 5′-CTTATCGCACATAGTCTG -3′) generated 171-base-pair (bp) and 113-bp products, respectively. Thirty-five cycles (94°C for 30 s, 55°C for 30 s and 72°C for 1 min) were performed for primary PCR and 25 cycles for nested PCR. The products were analysed by 2% agarose gel electrophoresis.

Positive and negative controls

DNA of the PJ1 isolate was used as positive control and sterile water as negative control. Both these controls were incorporated in separate reaction tubes for every ten tested samples. All PCR-negative samples were re-amplified by adding positive control to exclude the presence of PCR inhibitor.

SspI restriction analysis

To reaffirm that all PCR products were authentic *mtLSU rRNA* of *P. jirovecii*, the PCR amplified products were digested with *Ssp*I under the recommended optimal condition (Takara, Shiga, Japan) and analysed by 3% agarose gel electrophoresis.

Specificity

Specificities of conventional and new PCR assays were verified for the lack of cross-amplification when DNA of *Pneumocystis carinii*, *Absidia corymbifera*, *Candida albicans*, *Histoplasma capsulatum* and *Rhizopus oryzae* was used as template DNA.

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Comparative sensitivities

Ten-fold serial dilutions of *P. jirovecii* genomic DNA (PJ1 isolate) were used as templates to assess the efficiency of amplifications of both conventional and new PCR assays. The PCR were performed in quadruplicate for each dilution of template. Results were determined by analysis of the amplified products on 2% agarose gel electrophoresis.

Results

Partial mtLSU rRNA sequence

Primers derived from *mtLSU rRNA* of *P. carinii* could successfully amplify the orthologous gene fragment of *P. jirovecii* generating a 643-bp PCR product that spanned 147 bp upstream to the primer pAZ102-H and 126 bp downstream to the primer pAZ102-E (Fig. 1). Sequence analysis of 12 clinical isolates in this study and 26 sequences available in the GenBank database has revealed 16 single nucleotide polymorphisms and two insertions/deletions (Fig. 1). Intriguingly, all four primers conventionally used for PCR assay were located in regions containing single nucleotide polymorphisms, i.e. three, two, one and one single nucleotide polymorphism in primers pAZ102-H, pAZ102-X, pAZ102-Y and pAZ102-E, respectively (Fig. 1). By contrast, no single nucleotide polymorphism was observed among Thai isolates and those in the GenBank database for primers PJLSUF0, PJLSUF1, PJLSUR1 and PJLSUR0 developed in this study.

Comparative sensitivity

Under the optimal condition for each PCR assay, the minimum dilutions of genomic DNA of the PJ1 isolate that gave reproducible positive results for conventional single-round and new single-round PCR assays were 10^{-6} and 10^{-8}, respectively. The minimum dilutions of the same DNA template that conferred consistent positive results for conventional nested and new nested PCR were 10^{-8} and 10^{-11}, respectively. These data were essentially the same for all four repeated assays.

Specificity of the PCR assays

No cross-amplification was obtained from both conventional and new PCR when genomic DNA of *P. carinii* and some representative fungi were used as templates. The *mtLSU rRNA* sequence of *P. jirovecii* determined in this study was distinct from that of *P. carinii* with 22% nucleotide differences. Therefore, substantial nucleotide mismatches between these diagnostic primers and *mtLSU rRNA* of *P. carinii* plausibly precluded cross-hybridization in the PCR assay. The amplified products from all positive samples exhibited specific restriction fragments upon *Ssp*I digestion (132-bp and 120-bp fragments for conventional nested PCR and 69-bp and 44-bp fragments for new nested PCR), indicating no false amplification.

Comparative detection of P. jirovecii in BAL samples

Of 102 BAL fluids, Giemsa stain demonstrated characteristic trophic forms and octonucleate cysts of *P. jirovecii* in 40 (60.6%) HIV-infected patients and six (16.7%) non-HIV-infected individuals. The IFA did not increase diagnostic yield from BAL samples from non-HIV patients whereas seven additional positive cases were found among HIV-infected patients (Table 1). Conventional nested PCR gave positive results 1.40 times more than IFA (1.23 and 2.67 times more

TABLE 1. Comparative detection of *Pneumocystis jirovecii* by Giemsa stain, immunofluorescence assay (IFA), conventional PCR and new PCR in bronchoalveolar assay fluids from 102 patients

		Positive results (%)					
				Conventional PCR		New PCR	
Result	*n*	Giemsa stain	IFA	Single-round	Nested	Single-round	Nested
HIV-positive patients	66	40 (60.6)	47 (71.2)	56 (84.9)	58 (87.9)	64 (97.0)	65 (98.5)
Definite PCP	47	40 (85.1)	47 (100)	46 (97.9)	47 (100)	47 (100)	47 (100)
Probable PCP	3	0	0	2 (66.7)	2 (66.7)	3 (100)	3 (100)
Non-PCP	16	0	0	8 (50.0)	9 (56.2)	14 (87.5)	15 (93.7)
Non-HIV patients	36	6 (16.7)	6 (16.7)	13 (36.1)	16 (44.4)	23 (63.9)	26 (72.2)
Definite PCP	7	6 (85.7)	6 (85.7)	7 (100)	7 (100)	7 (100)	7 (100)
Probable PCP	3	0	0	3 (100)	3 (100)	3 (100)	3 (100)
Non-PCP	26	0	0	3 (11.5)	6 (23.1)	13 (50.0)	16 (61.5)
Total cases	102	46 (45.1)	53 (52.0)	69 (67.6)	74 (72.6)	87 (85.3)	91 (89.2)
Efficacy ratio							
Positives by a given test/Giemsa positives		1	1.15	1.50	1.61	1.89	1.98
Positives by a given test/IFA positives		0.87	1	1.30	1.40	1.64	1.72

HIV, human immunodeficiency virus; PCP, *Pneumocystis jirovecii* pneumonia.

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than IFA for HIV-infected and non-HIV patients, respectively) whereas new nested PCR offered positive tests 1.72 times more than IFA (1.36 and 4.33 times more than IFA in HIV-infected and non-HIV immunocompromised patients, respectively). The diagnostic efficacies of conventional nested PCR and new nested PCR assay conferred 61% and 98% more positive results than Giemsa stain, respectively (Table 1). All tests used in this study gave concordant results in 55 BAL samples (53.9%). New nested PCR yielded the highest positive results compared with other tests whereas single-round new PCR offered 13 more positive tests than conventional nested PCR. No negative PCR samples contained PCR inhibitors because positive results upon re-amplifications were obtained when positive control DNA was added.

Clinical relevance of diagnostic methods

Conventional nested PCR and new single-round and nested PCR assays could detect all samples that were positive by Giemsa stain or IFA, which could be because of the presence of *P. jirovecii* DNA above the PCR detection threshold. However, conventional single-round PCR failed to detect one isolate that was positive by both staining methods. Sequence analysis of this sample has shown a C/T substitution in the annealing region for primer pAZ102-H as shown in Figure 1.

Discussion

Confirmation of *P. jirovecii* by microscopy has been hampered by the low sensitivity of conventional staining methods such as Giemsa and methenamine silver stains. Although subsequent application of immunofluorescence stain has remarkably increased diagnostic yield, a number of samples containing a submicroscopic level of *P. jirovecii* remained undiagnosed [9,12,13]. Background fluorescence of some samples under IFA could lead to false identification of the trophic stage of *P. jirovecii* [7]. In this study, IFA seems to be more sensitive than Giemsa stain in HIV-infected patients with pulmonary symptoms than in non-HIV immunocompromised hosts. Despite a limited number of samples in this analysis, a higher proportion of false-negative results from detection methods based on visualization of organisms were more pronounced in non-HIV patients than in HIV-infected patients with definite PCP (Table 1). These findings were in good agreement with previous reports that PCP in non-HIV patients usually had fewer organisms than in HIV-infected patients [14,15].

The PCR-based method has remarkably facilitated diagnostic efficiency, resulting in an increase in the number of documented PCP cases worldwide [4]. It seems that the sensitivity of PCR detection depends on copy number of the gene target; thereby PCR assays that amplify single copy genes offered similarly concordant results and were less sensitive than the *mtLSU rRNA*-based PCR assay [7]. However, the sensitivity of single-round PCR targeting *mtLSU rRNA* using the same primer pairs was reportedly lower than nested PCR targeting ITS and 18S rRNA that are single copy genes [16]. The extended sequences of *mtLSU rRNA* of *P. jirovecii* obtained in this study have led to identification of nucleotide substitutions in primers pAZ102-H, pAZ102-E, pAZ102-X and pAZ102-Y among Thai isolates and those reported in the GenBank database. Importantly, new single-round and nested PCR were capable of amplifying DNA template that were two and three orders of magnitude more diluted than conventional single-round and nested PCR, respectively. Although nucleotide substitutions were not observed at the 3′ residue of the primers that could be crucial for primer–template annealing before DNA polymerization, the occurrence of two or more primer–template mismatches reportedly reduced the PCR product yield varying from two-fold to ten-fold compared with the PCR product generated from perfectly matched sequences between primer and template [10,17]. In this study, the sensitivity of conventional nested PCR containing at least one primer–template mismatch was 18.7% (17/91) less than that of new nested PCR whose primers were derived from conserved sequences (Table 1). Furthermore, a shorter span of target sequence of new PCR could permit more efficient amplification than conventional PCR. Meanwhile, failure to detect *P. jirovecii* DNA by PCR in samples that are positive by staining methods does not necessarily reflect sequence variation in the template DNA because the amount of organisms could vary among different sources of samples and the methods for sample processing in the laboratory in addition to the status of the patients. Furthermore, stochastic distribution of *P. jirovecii* in each aliquot of the same sample could cause such discrepant results.

Multiple lines of evidence indicate that *P. jirovecii* can colonize the mucosal epithelium of both healthy individuals and those with compromised immunity [18–20]. Our study has shown that new nested PCR could detect *P. jirovecii* DNA in BAL fluids from non-PCP patients with higher positive rates among HIV-infected patients than non-HIV patients, suggesting colonization or subclinical infection among these patients. However, it remains unknown whether *P. jirovecii* colonizes HIV-infected patients more often than non-HIV-infected individuals in a larger sample size and in a different population. Our PCR assays *per se* could not differentiate true PCP from *P. jirovecii* colonization whereas quantitative real-time PCR assay has been proven to be useful in discriminating these

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conditions in immunocompromised patients with or without HIV infection [21,22]. Alternatively, when quantitative real-time PCR is not available, it would be practical to make a diagnosis of PCP based on both laboratory results along with compatible clinical evidence. Taken together, the highly sensitive new PCR assay developed herein could be useful for reaffirming a clinical diagnosis of PCP in immunocompromised patients with consistent respiratory symptoms and radiographic changes when microscopy fails to detect the organisms. Furthermore, the method could also be useful for detecting a very low level of colonization in patients or otherwise healthy subjects. Deployment of a highly sensitive diagnostic tool is crucial for fine epidemiological surveillance and understanding the dynamic of spread of this medically important ubiquitous microfungus.

Acknowledgements

The authors thank Urassaya Pattanawong for assisting in the compilation of data and Ariya Chindamporn for providing some representative fungal samples.

Transparency Declaration

This study was supported by The National Research University Project of CHE and the Ratchadapiseksompot Endowment Fund (grant number HR1161A). All authors declare no dual or conflicting interests.

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