RESEARCH NOTE

SENSITIVE DETECTION OF *PNEUMOCYSTIS JIROVECII* DNA IN GASTRIC WASH USING NESTED POLYMERASE CHAIN REACTION

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Abstract. One hundred and five samples of gastric washes were obtained from 52 pediatric patients. Eleven of the 105 samples (10%) gave positive results using immunofluorescence antibody test (IFA) for *Pneumocystis jirovecii*. Single-step polymerase chain reaction (PCR) produced 13 % (14 samples), whereas detection by nested PCR was increased to 65 samples (62%). Moderate agreement (kappa = 0.5) was found between test results of IFA and single-step PCR, but no agreement was found between the results of IFA and nested PCR (kappa = 0.1).

INTRODUCTION

Pneumocystis jirovecii, formerly known as *Pneumocystis carinii* f. sp. *hominis*, is a fungal pathogen that causes *Pneumocystis* pneumonia (PCP). The dramatic rise in the incidence of PCP associated with human immunodeficiency virus (HIV) infection in the last decade has led to a recognition of PCP as a major medical and public health problem, especially in immunocompromized patients (Morris *et al,* 2004). In Thailand, epidemiological studies revealed that PCP is one of the leading causes of admission of HIV/AIDS patients (Suwanagool *et al,* 1997; Chokephaibulkit *et al,* 1999; Anekthananon *et al,* 2004). Studies of hospitalized adult HIV-infected patients admitted to Siriraj Hospital, Mahidol University, Thailand, from January 2003 to December 2003 showed that PCP (14.7%) is one of the three most common opportunistic infections, second to tuberculosis (42.6%) and cryptococcosis (13.1%) (Ratanasuwan *et al,* 2005).

Diagnosis of PCP is difficult due to nonspecific symptoms and signs, use of prophylactic drugs in the treatment of HIV-infected patients and simultaneous infection with multiple organisms (such as cytomegalovirus) in an immunocompromized host. Diagnosis of PCP, therefore, requires microscopical examination in order to identify *Pneumocystis* in clinically relevant sources such as sputum, bronchoalveolar fluid, or lung tissue (Cushion and Beck, 2001). A variety of stains have been used to identify *P. jirovecii* in respiratory tract secretions, including Giemsa or Giemsa-like rapid stains (*eg*, Diff-Quik®), Gomori methenamine silver stain, toluidine blue O stain, and fluorescein-conjugated monoclonal antibody (Kroe *et al,* 1997).

Immunofluorescence antibody assay (IFA) has been the most widely used immunologic technique for *P. jirovecii* diagnosis (Cregan *et al,* 1990); it is more sensitive than histologic

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stains, which is considered the "gold standard" for *P. jirovecii* detection (Elvin *et al,* 1988; Radio *et al,* 1990).

Polymerase chain reaction (PCR) has introduced a new level of sensitivity in *P. jirovecii* detection (Olsson *et al,* 1996; Agostini *et al,* 1997; Hanano *et al,* 1998). PCR technique has been compared with Wright-Giemsa (WG) and Gomori methenamine silver (GMS) stains and with IFA for detection of *P. jirovecii* in immunocompromized patients and shown to be an important tool in the epidemiological study of *P. jirovecii* of high-risk individuals (Pinlaor *et al,* 2004).

To date, attempts have failed to culture *P. jirovecii,* thus, the detection of this organism should be as accurate as possible. Collection of specimens that accurately reflect the disease process in the lungs is an essential component of the diagnostic evaluation of patients with suspected PCP. Bronchoalveolar lavage (BAL) collection procedures are usually used in adult patients. However, in pediatric patients, BAL may be harmful, compared with the technique of collection of gastric washes which is more safe and less invasive. In the present study, PCR technique was compared with IFA for detecting *P. jirovecii* in gastric washes.

MATERIALS AND METHODS

Clinical specimens

This study was approved by the Ethics Committee for Human Research, Faculty of Medicine Siriraj Hospital, Mahidol University. One hundred and seventy-four samples of gastric washes were obtained from 52 patients. The specimens were centrifuged at 1,000*g* for 5 minutes to pellet cells and organisms.

Immunofluorescence assay

IFA was performed using mouse anti-*P. carinii* monoclonal antibodies (clone 3F6; DAKO AS, Glostrup, Denmark) that reacts with an antigenic epitope highly specific for *P. jirovecii* (Elvin *et al,* 1988; Radio *et al,* 1990). In brief, gastric wash samples were mixed with 0.25% trypsin in phosphate buffered saline (PBS), incubated at room temperature for 5 minutes, and then sedimented. Indirect immunofluorescence on acetone-fixed smears was performed using mouse anti-*P. carinii* monoclonal antibodies, followed by rabbit anti-mouse FITC labeled IgM according to instructions provided by the manufacturer.

PCR

In order to extract *Pneumocystis* DNA, 200 µl of resuspended sediment were used with QIAamp DNA Mini kit (Qiagen) according to the manufacturer's recommendation.

Fifty µl of the DNA sample were added to 50 µl of PCR master mix containing Taq enzyme (Gibco BRL) and dNTP. Amplicons were generated by single-step and nested PCR. In single-step PCR primer pair was pAZ102- E (5′- GATGGCTGTTTCCAAGCCCA- 3′) and pAZ102- H (5′- GTGTACGTTGCAAAGTACTC- 3′) (Wakefield *et al,* 1990b). For nested PCR, the product of the first round PCR was amplified with pAZ102- E (5′- GATGGCTGTTTCC AAGCCCA- 3′) and pAZ102- L2 (5′- ATAAG GTAGATAGTCGAAAG- 3′) (Wakefield *et al,* 1990b). Cycling using Perkin Elmer GeneAmp 2400 Thermal Cycler parameters were as follows: 94ºC for 5 minutes; 94ºC for 30 seconds, 56ºC for 30 seconds, and 72ºC for 30 seconds for 40 cycles; and 72ºC for 7 minutes. To avoid contamination, each step (reagent preparation, extraction and amplification) was performed in different rooms with different sets of micropipettes and using filter tips. PCR mixtures and the extraction step were prepared in a laminar-flow cabinet. To monitor for possible contamination, negative control (ultra-pure distilled water) was included in each PCR experiment.

The PCR products were separated by

electrophoresis in 2% agarose gel, and stained with ethidium bromide and visualized under UV-light. The expected size of the amplicon was 346 bp for single-step PCR and 120 bp for nested PCR.

Data analysis

Agreement between the IFA, single-step PCR and nested PCR tests was expressed as κ-value (Kappa statistics), an index that compares the agreement against that which might be expected by chance. κ-values between 0.4 and 0.6 indicate a moderate agreement, values between 0.6 and 0.8 good agreement, and values between 0.8 and 1.0 very good or high agreement.

RESULTS

Because of the varying volumes of the specimens, not all samples were included in the IFA and PCR assays. Only 105 samples were subjected to both IFA and PCR assays. Single-step and nested PCR results are shown in Fig 1. The IFA and PCR results of the 105 specimens tested are given in Table 1. Eleven of the 105 samples gave positive results by IFA (10%). Single-step PCR produced 13 % (14 samples) positive results and detection of *P. jirovecii* was increased by nested PCR to 62% (65 samples). Moderate agreement (kappa = 0.5) was found between test results of IFA and single-step PCR. As 54 samples (51%) were negative by IFA but positive by nested PCR, no agreement (kappa = 0.1) was found between the results of IFA and nested PCR.

Table 1
Results of IFA, single-step PCR and nested PCR for gastric washes.

IFA	No. (%) of gastric washes			
	Single-step PCR		Nested PCR	
	Positive	Negative	Positive	Negative
Positive	7 (7)	4 (4)	11 (10)	0 (0)
Negative	7 (7)	87 (83)	54 (51)	40 (38)

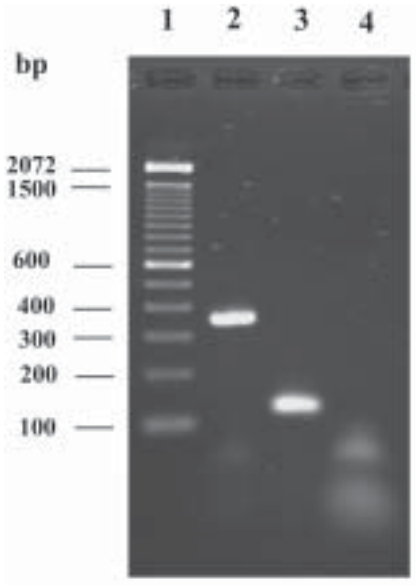

Fig 1–*P. jiroveccii* amplicons produced by single-step and nested PCR. PCR products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Lane 1: molecular weight markers; lanes 2 and 3, positive specimen from single step and nested PCR, respectively; lane 4, negative control (water).

DISCUSSION

The primers described by Wakefield *et al* (1990a,b) were chosen because investigators have shown that mitochondrial rRNA gene is the most specific and sensitive target for single-step PCR detection of *P. jirovecii* (De Luca *et al,* 1995; Lu *et al,* 1995).

P. jirovecii DNA was detected by nested PCR in 54 specimens (51%) that were negative by IFA staining. In the absence of clinical data and a sensitive gold standard, such results should not be considered as being false-positive, as high degree of sensitivity of PCR has permitted detection of *P. jirovecii* several weeks before or after an episode of micro-

scopically proven PCP (Wakefield *et al,* 1990a; Olsson *et al,* 1993; Cartwright *et al,* 1994). In experimental pneumocystosis, PCR becomes positive before the results of cytologic and histologic studies are positive (Sepkowitz *et al,* 1993) and remains positive much longer (Vargas *et al,* 1995).

The presence of a positive PCR result in a specimen that cannot be confirmed by other methods of detection presents a diagnostic dilemma. Such a situation might have resulted from the recent administration of anti-*P. jirovecii* drugs or may represent subclinical infection. In the latter case, some patients have gone on to acquire *P. jirovecii* pneumonia (Olsson *et al,* 1996; Agostini *et al,* 1997). Another consideration is that positive results may arise from nonviable microorganisms. *P. jirovecii* often persists from three to six weeks after successful therapy (Colangelo *et al,* 1991). However, the level of detectable *P. jirovecii*-associated DNA appears to drop rapidly following therapy (Leigh *et al,* 1993). Due to these considerations, the role of PCR in the diagnosis of *P. jirovecii* needs more clinical data for confirmation of diagnosis.

In summary, PCR assays for the detection of *P. jirovecii* has a higher sensitivity than IFA. Nested PCR has a higher sensitivity than single-step PCR. Single-step PCR shows agreement with IFA but nested PCR shows no agreement with IFA.

ACKNOWLEDGEMENTS

This study was part of "Development of techniques for routine diagnostic test of *Pneumocystis carinii*" project made possible by a Siriraj Grant for Research Development and Medical Education, Faculty of Medicine Siriraj Hospital, Mahidol University.

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