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 non-specific 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 immunocompromised 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 1,000g 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´- GATGGCTGTTTCAAGCCCA- 3´) and pAZ102- L2 (5´- ATAAAGGTAGATAGTGGCAAGG- 3´) (Wakefield et al, 1990b). Cycling using Perkin Elmer GeneAmp 2400 Thermal Cycler parameters were as follows: 94°C for 5 minutes; 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds for 40 cycles; and 72°C 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 $\kappa$-value (Kappa statistics), an index that compares the agreement against that which might be expected by chance. $\kappa$-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.

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).

<table>
<thead>
<tr>
<th>IFA</th>
<th>Single-step PCR</th>
<th>Nested PCR</th>
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<tr>
<td>Positive</td>
<td>7 (7)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Negative</td>
<td>7 (7)</td>
<td>87 (83)</td>
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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.

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REFERENCES


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