Evaluation of a Real-Time PCR Assay for the Diagnosis of *Pneumocystis* pneumonia

KOHJU ETOH

Department of Medicine, Kurume University School of Medicine, Kurume 830-0011, Japan

Received 3 March 2008, accepted 16 February 2009

Edited by KOICHI KUWANO

Summary: The aim of this study was to evaluate of the quantification of *Pneumocystis jiroveci* using a real-time PCR assay. We tried to verify whether quantification was really effective in differentiating between carriage and *Pneumocystis* pneumonia (PCP) using real-time PCR with or without sample species normalization for classifying each sample species (sputum, bronchoalveolar lavage (BAL), and total samples).

Twenty-two positive samples previously examined by conventional qualitative PCR were subjected to real-time PCR. Of these 22 lower respiratory tract specimens, 10 were BAL samples and 12 were (induced) sputum samples. According to our clinical diagnostic criteria, 17 were PCP and 5 were non-PCP. In the 12 sputum samples the concentrations of *Pneumocystis*-specific DNA detected in the non-PCP patients did not differ significantly from those in the PCP patients. The data were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene to exclude differences due to the number of human cells in collected samples. After normalization, the *Pneumocystis*-specific DNA/GAPDH-DNA ratio in the non-PCP patients was higher than that in the PCP patients. In the BAL samples (10 samples), the mean concentration of *Pneumocystis*-specific DNA detected in the PCP patients was 9.6 times higher than that in the non-PCP patients (P=0.058), and after normalization, the *Pneumocystis*-specific DNA/GAPDH-DNA ratio in the PCP patients did not differ significantly (P=0.19) from that in the non-PCP patients. Although the present study indicated that normalization using GAPDH might be not helpful but BAL specimens are recommended over sputum specimens for the diagnosis of *Pneumocystis* Pneumonia by quantification with real-time PCR.

Key words PCP, realtime PCR, normalization, GAPDH, BAL

INTRODUCTION

*Pneumocystis jiroveci*, formerly known as *Pneumocystis carinii* f. sp. *Hominis*, is an important cause of morbidity and mortality, causing *Pneumocystis* pneumonia (PCP) in patients whose immune systems have been compromised due to disease and/or immunosuppressive treatments. The standard method for the diagnosis of PCP is microscopic examination of stained (Giemsa and Grocott techniques) invasive lower respiratory tract specimens from bronchoalveolar lavage (BAL), lung biopsy, aspirates or induced sputum specimens. This method has relatively low sensitivity but high specificity [3,13,17,29,33]. Molecular detection systems such as PCR techniques have the potential to provide a higher degree of sensitivity than microscopic examinations [1-4,7,8,12,16,17,20-23,25,28,29-32]. Due to the high sensitivity of molecular methods, *Pneumocystis jiroveci* DNA has been detected in respiratory samples from patients without PCP, probably representing either colonization or subclinical infection, at a rate of 2.2-40% [7,9,15-22,24,25,27,28].

It is thought that real-time PCR, as recently described [5,14], might be useful in distinguishing between colonization and clinical disease. This sugges-
tion is based on the hypothesis that PCP patients should have a higher organism burden than colonized or subclinically infected patients, and that this would be reflected by a higher amount of *Pneumocystis jiroveci* DNA present in the extracted specimens from PCP patients. However, sample quality and quantity can vary significantly from person to person, and the quality of the sputum and the rate of retrieval of BAL fluid can differ each time a sample is collected.

Therefore, we postulated that normalization using GAPDH, a human house-keeping gene, might overcome these limitations, and evaluated the quantification of *Pneumocystis jiroveci* using a real-time PCR with sample species normalization.

**MATERIALS AND METHODS**

**Subjects**

Samples for this study came from all patients evaluated by the Department of Medicine, Division of Respiratory, Neurology, and Rheumatology, and other 2 divisions (Gastroenterology, Nephrology) and other 2 Departments (Pediatrics, Acute medicine), Kurume University School of Medicine and other hospital (Kurume University Medical Center) between May 2000 and August 2004 who presented with clinical symptoms of pulmonary infection associated with immunosuppression, thus justifying a search for *Pneumocystis* in BAL and induced sputum specimens.

Thirty-two positive samples, previously examined by conventional qualitative PCR, were utilized for real-time PCR. In 22 of these 32 samples, we were able to verify the data. Ten samples were excluded because the PCR would not be able to evaluate. Therefore 22 lower respiratory tract specimens were collected from 22 patients with immunosuppression due to various diseases and/or immunosuppressive treatments: 10 BAL samples and 12 induced sputum samples. The ten BAL samples comprised 5 specimens retrieved by washing with only 20 ml of 0.9% NaCl (BAL-20) due to the patient’s severe respiratory status. Four were retrieved by washing with 100 ml of 0.9% NaCl (BAL-100) and 1 was retrieved by washing with only 10ml of 0.9% NaCl (BAL-10) due to the patient’s age (only 2 months).

**Preparation of DNA specimens**

Patient sputum samples were incubated with an equal volume of Sputazyme (semialkaline proteinase, 200 mg/vial; Na$_2$HPO$_4$, 16.25 mg/ml; KH$_2$PO$_4$, 2.88 mg/ml [pH 7.2]; Kyokuto Pharmaceutical Co., Ltd. Tokyo, Japan) at 37°C for 10 min. The samples were centrifuged at 1,600 × g for 15 min. The pellets were resuspended in 0.5 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 7.5]) and then spun for 10 sec at 13,000 × g. This procedure was repeated twice, and the final pellet was resuspended in 200 µl of InstaGene DNA Purification Matrix (Bio-Rad Laboratories, Inc.). The mixture was incubated for 15 min at 56°C, vortexed for 10 sec, incubated for 8 min at 100°C, vortexed for 10 sec and then spun at 13,000 rpm for 2 min. The BAL fluid samples were centrifuged at 1,600 × g for 10 min. The pellets were resuspended in 1 ml of phosphate-buffered saline and then incubated with an equal volume of Sputazyme. These samples were treated in the same manner as the sputum samples. We used 2 µl of this supernatant as the DNA sample.

**Conventional PCR**

PCR was performed according to Honda et al. [11]. For the detection of Pneumocystis-specific DNA, the primers PAZ102-E (5’-GATGGCTGTTTCAAGCCCA-3’) and PAZ102-H (5’-GTGTACGGAAAGTACTC-3’), derived from the mitochondrial large subunit rRNA (mtLSUrRNA), and which amplified a 346 bp sequence, were used as reported previously [31].

**Real-time PCR with the LightCycler**

**PCR conditions:** The real time PCR assay involves LightCycler technology, which combines rapid thermocycling with on-line fluorescence detection of the PCR products. The reactions were performed in a volume of 20 µl of a mixture containing 0.01 mM of each primer and 2 µl of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH Roche Applied Science Nonnenwald 282372 Penzberg Germany) containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye, and 10 mM MgCl$_2$. The final concentration of MgCl$_2$ was adjusted to 3 mM. The samples were placed into LightCycler capillaries (20µl) (Roche Diagnostics GmbH Roche Applied Science Nonnenwald 282372 Penzberg Germany), capped, centrifuged for a few seconds in a micro-centrifuge using appropriate adapters, and placed into the LightCycler rotor. An initial preheating step of 10 min at 95°C was used to activate the DNA polymerase, deactivate UNG, and melt double-stranded DNA. Next, a touch-down procedure followed, consisting of 15 sec at 95°C, annealing for 10 sec at temperatures decreasing from 65 to 60°C during the first 11 cycles (with 0.5°C decremental steps in each cycle), and ex-
tension at 72°C for 15 sec, and acquisition of the fluorescent signal from the samples at 80°C for 2 sec to increase the specificity of PCP specific DNA. Acquisition of the fluorescent signal from the samples was performed at 82°C for 2 sec to increase the specificity of GAPDH DNA. The transition rate of temperature was set at 20°C/sec for denaturation to annealing and from annealing to extension and extension to denaturation. A total of 45 cycles were performed. The PCR products were subjected to analysis by electrophoresis on a 2% agarose gel to confirm the efficiency of the melting curve analysis.

**Standards and external controls:** To obtain standards for real-time PCR, *P. jiroveci*-positive BAL samples (by Grocott smear) were used. The extracted DNA (described above in Preparation of DNA specimens) was used for amplification of the major surface glycoprotein (MSG) gene of *Pneumocystis* [14]. A PCR method previously described [11] was modified for the real-time assay. The commercially synthesized primers (Operon Biotechnologies, Inc. Tokyo, Japan) JKK14/15 (5'-GAA TGC AAA TCY TTA CAG ACA ACA G-3') and JKK17 (5'-AAA TCA TGA ACG AAA TAA CCA TTG C-3') amplified a 250-bp segment of the multicopy MSG gene family [14]. The amplified products were subjected to electrophoresis in agarose gels, and bands were visualized with UV light following ethidium bromide staining.

To detect amplicons (*Pneumocystis* DNA) in agarose gels (TBE buffer), trimmed gel slices and the chopped pieces were used for DNA extraction with Quantum Prep Freeze’N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad Laboratories, Inc.) according to the manufacturer’s recommendations. The extracted DNA was amplified using JKK14/15 and JKK17 primers, and the amplicon was confirmed as a 250 bp sequence by electrophoresis in agarose gels (described above). The GAPDH samples were confirmed in the same manner as the respective *Pneumocystis* samples. The commercially synthesized primers (Operon Biotechnologies, Inc. Tokyo, Japan) GAPDH-sense (5'-CTT CAC CAC CAT GGA GAA GGC-3') and JKK17 (5'-GTC ATG GAC TGT GGT CAT GAG-3') amplified a 260-bp segment. The concentration was determined using a ND-1000 Spectrophotometer (NanoDrop Technologies) and the A260 and A280 optical density measurements. Sample species normalization was calculated as the ratio of *Pneumocystis*-specific DNA/GAPDH-DNA. GAPDH was used as a housekeeping gene.

**Data analysis**

Diagnosis of PCP based on microscopic examination of stained samples is difficult because of the low sensitivity for detection of *Pneumocystis* cells. The criteria that we used for confirming ongoing pneumocystosis were clinical findings of PCP with characteristic X-ray findings (new bilateral diffuse interstitial infiltrates), dyspnoea with/without arterial hypoxaemia (partial pressure of arterial oxygen <70 mmHg) as well as response to anti-*Pneumocystis* treatment but resistance to other antibiotics or response to both anti-*Pneumocystis* treatment and other antibiotics. Based on these diagnostic criteria, the quantities and normalized quantities were evaluated. Statistical assessment of these characteristics was performed using the Fisher’s test and G test (P<0.05 was considered significant). Statistical assessment of differences of the mean of *Pneumocystis* DNA concentration [×1/10fg/μl] from PCP or non-PCP cases was performed by the unpaired Student’s t-test (P<0.05 was considered significant).

**RESULTS AND DISCUSSION**

According to our diagnostic criteria, of the 22 patients profiled, 17 had developed PCP. In the remaining 5, the diagnosis of PCP was not confirmed.

**Quantitative PCR**

**Total samples (sputum and BAL)**

In the 22 samples, the mean concentrations (×1/10fg/μl) of *Pneumocystis*-specific DNA detected in the non-PCP patients and PCP patients were 3.85×10⁶ and 3.51×10⁷, respectively (Fig. 1-a). The mean concentration of the non-PCP patients was significantly higher than that of the PCP patients (p<0.05). The mean concentrations (×1/10fg/μl) of GAPDH-DNA detected in the non-PCP patients was significantly higher than that in the PCP patients (P<0.05). The mean concentrations of GAPDH-DNA in the non-PCP patients and PCP patients were 8.64×10⁷ and 1.23×10⁸, respectively (Fig. 1-b). After normalization, the ratio of Pneumocystis-specific DNA/GAPDH-DNA in the non-PCP patients did not differ significantly (p=0.41) from that of the PCP patients (Fig. 1-c; the mean ratio of the non-PCP patients was 0.26 while the mean ratio of the PCP patients was 0.23). Normalization was considered necessary because the qualities and quantities of the various samples were variable, and the quality of the sputum and the rate of retrieval of BAL differed from...
Normalization was performed using the ratio of *Pneumocystis*-specific DNA/GAPDH-DNA. GAPDH is a housekeeping gene that is stably expressed with little variation in human cells. Therefore, we used GAPDH for normalization in order to calculate the amount of PCP DNA relative to the number of the cells calculated. However, our results showed no significant difference between PCP and non-PCP patients after normalization. In microscopic examinations of stained sputum, the smear positive rate with instruction was higher than that without instruction (according to the classification of Miller and Jones)\[10\], and according to the classification of Geckler et al. \[6\], microscopic screening along with the rejection of unsatisfactory specimens can minimize the unreliability of sputum culture. We therefore considered that the evaluation of sputum quality and preparation might be required for the quantification of PCR studies. In the BAL samples, the amount that was retrieved by washing with 100 ml of 0.9% NaCl (BAL-100) was variable in each sample.

From previous reports on qualitative PCR, the sensitivity of BAL samples was higher than that of sputum samples [4,23,29], or was nearly equal [26,30]. In a qualitative PCR study Larsen et al. \[14\] reported that sputum samples were PCR positive but the differences between the PCP and non-PCP patients in the quantitative PCR results from the induced sputum did not reach the level of significance. Therefore we analyzed our data after classifying the sample specimens (sputum and BAL).

**Sputum samples (Fig. 2)**

In the sputum samples (12 samples), the concentration of *Pneumocystis*-specific DNA detected in the non-PCP patients (n=3) did not significantly differ from that of the PCP patients (n=9). The mean concentrations (×1/10fg/μl) were 6.41×10⁷ and 6.24×10⁶ in the non-PCP and PCP patients, respectively (Fig. 2-a). The concentrations of GAPDH-DNA detected in the non-PCP patients were higher than those in the PCP patients (P<0.05). The mean concentrations (×1/10fg/μl) were 1.42×10⁸ and 1.35×10⁷ in the non-PCP and PCP patients, respectively (Fig. 2-b). After normalization, the ratio of *Pneumocystis*-specific DNA/GAPDH-DNA in the non-PCP patients was significantly higher (P<0.05) than that in the PCP patients. The mean ratios were 0.41 and 0.19 in the non-PCP and PCP patients, respectively (Fig. 2-c).
Fig. 2. Quantity of DNA in the sputum samples by clinical category.

**Fig. 2-a:** PCP-DNA concentrations in the PCP cases [n=9] and the non-PCP cases [n=3]. The mean concentrations (×1/10^6/μl): The mean PCP-DNA concentration in the PCP positive patients was 6.24×10^6; the mean PCP-DNA concentration in the non-PCP patients was 6.41×10^7; P=0.13.

**Fig. 2-b:** GAPDH-DNA concentrations in the PCP cases [n=9] and the non-PCP cases [n=3]. The mean concentrations (×1/10^6/μl): The mean GAPDH-DNA concentration in the PCP positive patients was 1.35×10^7; the mean GAPDH-DNA concentration in the non-PCP patients was 1.42×10^8; P<0.01.

**Fig. 2-c:** PCP/GAPDH ratios in the PCP cases [n=9] and the non-PCP cases [n=3]. The mean ratios: The mean PCP/GAPDH ratio in the PCP positive patients was 0.19; the mean PCP/GAPDH ratio in the non-PCP patients was 0.41; P<0.05. Symbols: =, mean; *, P<0.05.

Fig. 3. Quantity of DNA in the BAL samples by clinical category.

**Fig. 3-a:** PCP-DNA concentrations in the PCP cases [n=8] and non-PCP cases [n=2]. The mean concentrations (×1/10^6/μl): The mean PCP-DNA concentration in the PCP positive patients was 4.40×10^4; the mean PCP-DNA concentration in the non-PCP patients was 4.59×10^5; P=0.058.

**Fig. 3-b:** GAPDH-DNA concentrations in the PCP cases [n=8] and the non-PCP cases [n=2]. The mean concentrations (×1/10^6/μl): The mean GAPDH-DNA concentration in the PCP positive patients was 1.08×10^7; the mean GAPDH-DNA concentration in the non-PCP patients was 3.14×10^6; P=0.099.

**Fig. 3-c:** PCP/GAPDH ratios in the PCP cases [n=8] and the non-PCP cases [n=2]. The mean ratios: The mean PCP/GAPDH ratio in the PCP positive patients was 0.27; the mean PCP/GAPDH ratio in the non-PCP patients was 0.039; P=0.19. Symbols: =, mean; *, P<0.05. 
**BAL samples (Fig. 3)**

In the BAL samples (10 samples), the mean concentration of *Pneumocystis*-specific DNA detected in the PCP patients was 9.6 times higher than that in the non-PCP patients (P=0.058). The mean concentrations (×10⁵ fg/μL) were 4.40×10⁵ and 4.59×10⁴ in the PCP and non-PCP patients, respectively (Fig. 3-a). After normalization, the ratio of *Pneumocystis*-specific DNA/GAPDH-DNA in the PCP patients did not differ significantly from that in the non-PCP patients (Fig. 3-c). In the PCP patients, lung tissue is often injured and a large number of human cells are obtained from the BAL samples. GAPDH quantity might reflect the amounts of human cells in samples. The samples from inflammatory lesion might have more inflammatory cells than those from non-inflammatory did. Therefore, the ratios of *Pneumocystis*-specific DNA/GAPDH-DNA may not reflect the concentration of PCP.

Although we thought that sample normalization with a housekeeping gene might be effective in helping to determine a cut-off value, it turned out to yield a meaningless result. This might have been due to the underlying primary disease. In particular, results might differ between HIV-positive and -negative cases. Such a difference has been found in previous studies, which have indicated the difficulty of confirming a PCP diagnosis in non-HIV-infected cases due to the lack of sensitivity of PCR methods (20-25%), even in BAL specimens [5,23,25,32]. The PCR positive rate for PCP in the HIV positive patients was higher than that in the HIV negative patients (6/8:75% > 2/8:25%) [7].

In our cases, no HIV positive patient was PCP

<table>
<thead>
<tr>
<th>Case</th>
<th>Sample</th>
<th>Primary disease</th>
<th>Age (year)</th>
<th>Gender</th>
<th>WBC (/μL)</th>
<th>Lymphocyte (/μL)</th>
<th>CRP (mg/dL)</th>
<th>Beta-D-glucan (pg/mL)</th>
<th>Quanti-tave PCR (PCP)</th>
<th>Quanti-tave PCR (GAPDH)</th>
<th>PCP/GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BAL-20</td>
<td>SLE</td>
<td>37</td>
<td>F</td>
<td>3800(6)</td>
<td>228</td>
<td>6.3</td>
<td>86.9</td>
<td>1.78×10⁶</td>
<td>8.92×10⁴</td>
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</tr>
<tr>
<td>2</td>
<td>BAL-10</td>
<td>SCID</td>
<td>2 months</td>
<td>F</td>
<td>1900(36)</td>
<td>684</td>
<td>0.3</td>
<td>3.5</td>
<td>1.15×10⁵</td>
<td>6.70×10⁶</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>3</td>
<td>BAL-100</td>
<td>RPGN due to MPA</td>
<td>71</td>
<td>F</td>
<td>10500(15.5)</td>
<td>1627.5</td>
<td>5.9</td>
<td>181.1</td>
<td>3.03×10⁴</td>
<td>8.52×10⁴</td>
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<tr>
<td>4</td>
<td>Sputum</td>
<td>MG/Thymoma (invasive type)</td>
<td>71</td>
<td>F</td>
<td>6700(6.0)</td>
<td>402</td>
<td>9.1</td>
<td>563.1</td>
<td>8.79×10⁵</td>
<td>2.67×10⁵</td>
<td>0.3</td>
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<tr>
<td>5</td>
<td>Sputum</td>
<td>Lung cancer</td>
<td>74</td>
<td>M</td>
<td>6200(2.0)</td>
<td>124</td>
<td>6.6</td>
<td>204.4</td>
<td>4.43×10⁴</td>
<td>1.77×10⁴</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>6</td>
<td>Sputum</td>
<td>Lung cancer</td>
<td>69</td>
<td>F</td>
<td>6900(2.0)</td>
<td>138</td>
<td>28.1</td>
<td>395.7</td>
<td>2.79×10⁵</td>
<td>6.85×10⁴</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>7</td>
<td>Sputum</td>
<td>Lung cancer</td>
<td>71</td>
<td>M</td>
<td>5300(16.1)</td>
<td>853.3</td>
<td>13.9</td>
<td>3.5</td>
<td>1.15×10⁵</td>
<td>5.22×10⁴</td>
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<tr>
<td>8</td>
<td>Sputum</td>
<td>Multiple Myeloma</td>
<td>62</td>
<td>F</td>
<td>700(34)</td>
<td>238</td>
<td>24.0</td>
<td>2.9</td>
<td>1.71×10⁵</td>
<td>3.89×10⁵</td>
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<td>BAL-100</td>
<td>MPA</td>
<td>67</td>
<td>F</td>
<td>10400(2.5)</td>
<td>260</td>
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<td>214.5</td>
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<td>4.64×10⁴</td>
<td>&lt;0.1</td>
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<td>10</td>
<td>BAL-20</td>
<td>RPGN due to mPA/Psoriasis</td>
<td>60</td>
<td>M</td>
<td>6800(15)</td>
<td>1020</td>
<td>6.1</td>
<td>104.2</td>
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<td>4.58×10⁵</td>
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<tr>
<td>11</td>
<td>BAL-20</td>
<td>CRF/cryoglobulinemia/LC-C</td>
<td>72</td>
<td>F</td>
<td>8200(2)</td>
<td>/</td>
<td>2.9</td>
<td>117.7</td>
<td>2.31×10⁵</td>
<td>8.64×10⁴</td>
<td>&lt;0.1</td>
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<tr>
<td>12</td>
<td>Sputum</td>
<td>Lung cancer,SVC syndrome</td>
<td>74</td>
<td>M</td>
<td>8600(3)</td>
<td>258</td>
<td>1.6</td>
<td>11.5</td>
<td>4.52×10⁵</td>
<td>4.55×10⁵</td>
<td>1.0</td>
</tr>
<tr>
<td>13</td>
<td>BAL-20</td>
<td>Lung cancer</td>
<td>69</td>
<td>M</td>
<td>8500(1)</td>
<td>85</td>
<td>30.6</td>
<td>13.1</td>
<td>1.46×10⁵</td>
<td>9.47×10⁴</td>
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<tr>
<td>14</td>
<td>Sputum</td>
<td>PN/pulmonary tuberculosis/Post MVR</td>
<td>59</td>
<td>M</td>
<td>5900(2)</td>
<td>118</td>
<td>6.4</td>
<td>100.8</td>
<td>3.77×10⁵</td>
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<tr>
<td>15</td>
<td>Sputum</td>
<td>Systemic Sclerosis</td>
<td>64</td>
<td>F</td>
<td>6400(2)</td>
<td>/</td>
<td>3.9</td>
<td>165.2</td>
<td>1.66×10⁵</td>
<td>2.34×10⁴</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>16</td>
<td>Sputum</td>
<td>Malignant Lymphoma relapse</td>
<td>82</td>
<td>M</td>
<td>1900(29.5)</td>
<td>560.5</td>
<td>5.8</td>
<td>36</td>
<td>5.68×10⁶</td>
<td>6.13×10⁶</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>17</td>
<td>BAL-20</td>
<td>SLE + chronic thyroiditis</td>
<td>48</td>
<td>M</td>
<td>10000(17)</td>
<td>1700</td>
<td>6.7</td>
<td>134.7</td>
<td>6.46×10⁵</td>
<td>2.03×10⁶</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* BAL-100, BAL fluid specimen after washing with 100 ml 0.9% NaCl; BAL-20, BAL fluid specimen after washing with 20 ml 0.9% NaCl; BAL-10, BAL fluid specimen after washing with 10 ml 0.9% NaCl.

* SLE: systemic lupus erythematosus; SCID: severe combined immunodeficiency; RPGN: rapidly progressive glomerulonephritis; MPA: microscopic polyangiitis; MG: myasthenia gravis; CRF: chronic renal failure; SVC syndrome: superior vena cava syndrome; PN: polyarteritis nodosa; MVR: mitral valve replacement

**TABLE 1. Comparison of each parameters in Pneumocystis pneumonia (PCP) patients**
positive (Table 1 and Table 2). This may have influenced our results. We believe that, in BAL samples, normalization using GAPDH is not useful for the diagnosis of Pneumocystis pneumonia. On the other hand, for HIV negative patients, BAL specimens are recommended rather than sputum specimens for the diagnosis of Pneumocystis pneumonia by quantitation with real-time PCR.

**Patient profiles (Table 1 and Table 2)**

The 22 specimens examined in this study were divided into two groups, PCP and non-PCP patients. No difference between the two groups regarding sample species (sputum and BAL) was demonstrated, and they were therefore analyzed together. There were no significant differences in any parameter (sample specimen/primary diseases without HIV/WBC/lymphocyte/CRP/beta-D-glucan) between the PCP and non-PCP patients. When separated according to sample specimens (sputum and BAL), none of the parameters (WBC/lymphocyte/CRP/beta-D-glucan) were significantly different between the PCP and non-PCP patients. In the case of Pneumocystis pneumonia, it will be difficult to reach a definitive diagnosis based solely on the patient’s clinical condition and laboratory data.

The present study indicated that normalization using GAPDH might be not helpful. However, more studies will be needed to distinguish between PCP, subclinical infection or colonization, and to determine cut-off values for each.

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Kurume Medical Journal Vol. 55, No. 3, 4, 2008