ABSTRACT

HIV and opportunistic infections remain a big problem especially in developing country. Pneumocystis jiroveci pneumonia is a prevalent infection in HIV infected patient with high mortality rate. Diagnosis of Pneumocystis jiroveci pneumonia is mainly based on clinical evidence. Microbiological diagnosis is quite challenging since this microorganism cannot be cultured and is mainly based on microscopic examination. Microscopic examination with special staining is still a gold standard diagnosis for P. jiroveci infection. The objectives of this study was to describe CD4 lymphocyte profile and establish microbiological diagnosis with recent molecular method in PJP suspected HIV positive patients. Fiberoptic bronchoscopy of HIV infected patients with lower respiratory tract infection in Dr. Soetomo general hospital Surabaya were performed to collect bronchoalveolar lavage specimens from December 2016 to April 2017 for identification of Pneumocystis jiroveci using real time PCR assay. Positive samples were then evaluated for microscopic examination with Gomori Methenamine Silver staining for comparison. Patient’s CD4 lymphocyte count were gathered prior of admission. CD4 lymphocyte count from this study were very low with 61% of the patients were below 50 cells/µL. There were five of total thirteen patients (38,5%) with positive real time PCR assay (MSG gene) and one patient was also positive with GMS staining showing characteristic cysts shape with dark centered area of P. jiroveci. Patient with positive microscopic examination showed no history of prophylactic therapy. Low CD4 lymphocyte count remains a strong risk factor of P. jiroveci pneumonia in HIV/AIDS patients. Real time PCR assay shows high value in detection of P. jiroveci regarding patient’s prophylactic status.

Keywords: HIV/AIDS, Pneumocystis jiroveci, pneumonia, low CD4 count, Dr. Soetomo hospital Surabaya

ABSTRAK

INTRODUCTION

Human Immunodeficiency Virus (HIV) is a virus that attack immune cells and weaken the immune system. Cumulative data reported from Indonesia Ministry of Health showed that there were 191,073 persons with HIV infection and 77,940 persons with AIDS.\(^1\) By 2014, there were estimated 37,600 persons with new HIV infection in USA. Opportunistic infection rarely occur at early stage of HIV infection, the use of antiretroviral therapy can reduce the viral load in the patient and maintain immune system.\(^2\) In Indonesia, antiretroviral therapy coverage is quite low (11.67%) compared with high income countries where the coverage is expected to be more than 45%.\(^3\) Several studies shows that at least 33.3% HIV patients with antiretroviral therapy will experience at least one time opportunistic infection during the study period and the general prevalence of opportunistic infection in HIV persons is 42.8% including recurrent infection.\(^2,4\) Pneumonia is one of the most prevalent opportunistic infection in HIV patients, it covers around 22.1% of the total opportunistic infection.\(^4\) The frequency of opportunistic infection may vary on each country because differences in genetic factor, environmental factor, and the people social background such as discrimination and stigmata which remain a potential difficulty in diagnosis and treating infection.\(^5\)

_Pneumocystis jiroveci_ is an opportunistic pathogen that often occur in immunocompromised persons with high mortality rate and strongly related with HIV/AIDS condition.\(^6\) The chance of patient with HIV/AIDS will experience _Pneumocystis jiroveci_ Pneumonia (PJP) was 75% in their course of the disease.\(^7\) Multicenter study in Korea showed that prevalence of PJP in HIV/AIDS patient were 11.1%. PJP is the third most prevalent opportunistic infection in HIV patient after Candida infection and tuberculosis infection.\(^8\)

Chemoprophylaxis in PJP suspected HIV/AIDS patients is directed to prevent PJP infection but even with routine prophylaxis, the death of PJP related in HIV/AIDS patient is around 12% to 33% depending on resources and facility of the hospital where the patient admitted.\(^9\)

MATERIAL AND METHOD

Clinical Specimens

Fiberoptic bronchoscopy in order to collect bronchoalveolar lavage is an invasive and expensive procedure especially for patient with respiratory problem. They were performed by pulmonologist and to minimize the risk factor for the development of adverse effects, the patients should have had minimal prerequisite lung function status, arterial blood gas recent data, platelet count and prothrombin time. A total of 13 bronchoalveolar lavage specimens from HIV/AIDS patients with pneumonia were collected in 5 month period from December 2016 until April 2017. The patient’s blood was taken and sent to the Clinical Pathology laboratory for CD4 lymphocyte count using flowcytometry method. This research has been approved by the hospital ethic committee no. 401/Panke. KKE/VI/ 2017.

Specimens Processing

BAL specimens were centrifuged at 3000g for 15 minutes. Supernatant were removed. After the removal of supernatant, 1 ml sedimentation were resuspended with mixed pipetting, aliquote of the sediment were smear on an object glass for GMS staining and the rest of the sediment were transferred in a microcentrifuge tube and kept in -80°C freezer until DNA extraction were performed.

GMS Staining

The BAL smears were fixed in alcohol 95% overnight and then ready to be stained according to the staining procedure.

Real Time PCR Assay

Before the PCR assay, DNA extraction were done with QIAamp DNA mini kit (Qiagen) according to the manufacturer’s procedure. The real time PCR assay were performed by Roche molecular system using Roche light cycler 2.0. The primers used were specific for Major Surface Glycoprotein (MSG) gene. The sequence of the primers were as follows: forward primer 5’-CAAAATAACAYTSACATCAACRAGG-3’, reverse
primer 5'-AAATCATGAACGAAATAACCATTGC–3', and probe 5'–TGCACAACACAGTGACAGGACAGG–3'. Master mix was prepared, aliquot of the master mix was pipette 15 µl and added by 5 µl extracted sample. The reaction was consist of one cycle of denaturation in 95°C for 10 minutes, continue with 45 cycles of annealing at 95°C for 10 seconds and extention at 58°C for 1 minutes. All reactions were run simultaneously with positive and negative controls.

Prevention of Contamination

Prevention of contamination including the use of aerosol barrier pipette tips, the use of separate areas of the laboratory for master mix preparation and specimens DNA extraction.

RESULT AND DISCUSSION

Of the 13 specimens tested, 61% of the blood specimens showed very low CD4 lymphocyte count below 50 cells/µL (Figure 1). The mean CD4 lymphocyte value was 82.69 cells/µL (Table 1). This result is similar to a multicenter study in Korea which stated that 65% patient with PJP showed very low count below 50 cells/µL. Low CD4 lymphocyte count is a risk factor for PJP infection, other risk factor of PJP are P. jiroveci past infection, oral candidiasis, recurrent bacterial pneumonia, loss of body weight, high HIV viral load and genotypic relationship with mannose-binding lectin.

Real time PCR assay were performed on all BAL specimens and 5 (38.5%) were positives (Table 2). One specimen was positive with PCR assay and microscopy examination with GMS staining (Figure 2). BAL is the best specimen for detection of P. jiroveci cyst because lavage in each lung segment can overcome more than 1 million alveoli, it is estimated that up to 3% of the lung tissue can be sampled. Real time PCR assay has sensitivity up to 96% in detecting 70 cases of PJP with negative microscopy examination and 94% in detecting 71 cases of PJP with positive microscopy examination, this assay rarely resulting false positive. Positive results must be interpreted carefully since this assay has high sensitivity value, a positive result without evident clinical symptoms and negative microscopy examination might be colonization or asymptomatic carrier of this microorganism. Negative PCR assay can be concluded true negative. PJP-HIV/AIDS patients with negative microscopy examination often are colonized with P. jiroveci. This might be true in this research since among the positives PCR result, there is only 1 positive microscopy examination.

Real time PCR is a semi quantitative method. The result of this assay can be concluded negative when there are no DNA detected and no increase of cycle threshold (CT) value, on the other hand positive when the targeted DNA is detected and there is an increase of cycle threshold value below 45 cycles. The less cycle the increasing of CT indicated the more targeted DNA load in the sample.

Cycle threshold value positively correlated with the microorganism density in the sample. A cut off CT value of 32 cycle can be applied in differentiating colonization to P. jiroveci infection with sensitivity 72% and specificity 75%.

Table 1. Characteristic of CD4 lymphocyte count in HIV/AIDS patients with pneumonia in Dr. Soetomo Hospital Surabaya from December 2016 to April 2017

<table>
<thead>
<tr>
<th>CD4 Lymphocyte Count</th>
<th>n</th>
<th>Mean</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 &gt; 200</td>
<td>13</td>
<td>82.69</td>
<td>15 cells/µL</td>
</tr>
<tr>
<td>&lt; 50 sel/µL</td>
<td>3</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td>50 - 100 sel/µL</td>
<td>1</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>100 - 200 sel/µL</td>
<td>6</td>
<td>61%</td>
<td></td>
</tr>
<tr>
<td>&gt; 200 sel/µL</td>
<td>1</td>
<td>8%</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Distribution of CD4 lymphocyte count in HIV/AIDS patients with pneumonia from December 2016 to April 2017

Table 2. Real time PCR assay of BAL specimens from HIV/AIDS patients with pneumonia in Dr. Soetomo Hospital, period December 2016 – April 2017

<table>
<thead>
<tr>
<th>Real Time PCR PJP</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>5</td>
<td>38.5</td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>61.5</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3. Association of CD4 lymphocyte count with real time PCR P. jiroveci from HIV/AIDS patients with pneumonia in Dr. Soetomo Hospital, period December 2016 – April 2017

<table>
<thead>
<tr>
<th>Real Time PCR P. jiroveci</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 &lt; 200</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>CD4 &gt; 200</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>8</td>
<td>13</td>
</tr>
</tbody>
</table>

CD4 lymphocyte count and P. jiroveci as an agent of pneumonia show significant relationship with p value 0.002. Opportunistic infection with low CD4 count below 200 cells/µL was dominated with P. jiroveci infection compare to other causes such as Cryptococcus and toxoplasmosis. It is clinically evident that CD4 lymphocyte count can be use
as a biomarker for immunodeficient condition and related to opportunistic infection. Yanagisawa and Nojima also stated that PJP prevalence in HIV/AIDS patient is 50% higher in CD4 lymphocyte count below 200 cells/µL.

Trimethoprim-sulfamethoxazole is one of few prophylactic therapy used for PJP, prophylactic is usually started when HIV/AIDS patient with low CD4 lymphocyte count is admitted in the hospital, especially when this patient come with clinical symptom and radiologic supporting pneumonia. Of all 13 patients, more than 50% already administered trimethoprim-sulfamethoxazole for prophylactic therapy, the fiber optic bronchoscopy procedure was performed after more than 2 days of prophylactic therapy. Among the 7 patients with therapy, positive PCR result was found in 4 patients (Table 4). One positive PCR result was found in non prophylactic patient.

Table 4. Real time PCR positivity value against prophylactic therapy in HIV/AIDS patients with pneumonia in Dr. Soetomo Hospital Surabaya

<table>
<thead>
<tr>
<th></th>
<th>Nilai rt PCR Positif</th>
<th>Nilai rt PCR Negatif</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terapi Cotrimoxazole profilaksis</td>
<td>4 / 75%</td>
<td>1 / 25%</td>
</tr>
<tr>
<td>Terapi Cotrimoxazole definitif</td>
<td>3 / 33.3%</td>
<td>2 / 66.7%</td>
</tr>
<tr>
<td>Tidak mendapatkan terapi</td>
<td>6 / 16.7%</td>
<td>4 / 83.3%</td>
</tr>
</tbody>
</table>

The diagnosis of PJP relies on microscopy detection of characteristic shape of *P. jiroveci* with special staining such as GMS, giemsa or immunofluorescence, this microscopy examination is difficult and quite challenging especially when the fungal load is low, false negative result is often detected. Microscopy examination with GMS staining has the same sensitivity on BAL or induction spu,

this specimens are specimen of choice in establishing microbiological diagnosis of PJP. *P. jiroveci* can colonize patient with high risk condition such as chronic obstructive pulmonary disease, it is important to differentiate *P. jiroveci* as a infective agent or colonizer. Trimevoprim-sulfamethoxazole therapy might interfere with microscopy examination result because non viable microorganism might be not detected since the characteristic cyst shape is destroyed during therapy. The *P. jiroveci* DNA can be detected even after prophylactic therapy but the microscopy examination can be difficult to achieve.

CONCLUSION

The majority CD4 lymphocyte count in HIV/AIDS patient in Dr. Soetomo Hospital is below 200 cells/µL. Lower CD4 lymphocyte count is a strong risk factor of *P. jiroveci* pneumonia in HIV/AIDS patients. Real time PCR *P. jiroveci* is a valuable diagnostic method with 57% positivity detection for *P. jiroveci* on patients receiving prophylactic and definitive therapy.

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REFERENCES