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Research Report

LOW CD4 LYMPHOCYTE COUNT RELATED RISK TO *Pneumocystis jiroveci* PNEUMONIA IN HIV/AIDS PATIENTS FROM BRONCHOALVEOLAR LAVAGE SPECIMENS USING REAL TIME PCR DETECTION

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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 Gommori 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

HIV dan infeksi oportunistik masih merupakan masalah yang banyak ditemui terutama pada negara berkembang. Pneumocystis jiroveci pneumonia merupakan infeksi yang banyak ditemui pada pasien HIV dan memiliki angka kematian yang tinggi. Diagnosis Pneumocystis jiroveci pneumonia terutama berdasarkan gejala klinis. Diagnosis mikrobiologi merupakan suatu tantangan tersendiri karena mikroorganisme ini tidak dapat dikultur dan diagnosis utamanya bergantung dari pemeriksaan mikroskopis. Pemeriksaan baku emas untuk diagnosis infeksi P. jiroveci adalah dengan pemeriksaan mikroskopis dengan pewarnaan khusus. Tujuan dari studi ini adalah untuk menggambarkan profil jumlah hitung limfosit CD4 pada pasien HIV dan menegakkan diagnosis mikrobiologis PJP dengan metode molekular terbaru pada pasien yang dicurigai infeksi PJP. Pemeriksaan bronkoskopi fiber optik dilakukan pada pasien HIV dengan infeksi saluran nafas bawah di RSUD Dr. Soetomo untuk mengumpulkan spesimen broncoalveolar lavage, spesimen klinis dikumpulkan dalam rentang waktu Desember 2016 hingga April 2017 untuk dilakukan identifikasi P. jiroveci dengan pemeriksaan real time PCR. Sampel dengan hasil positif kemudian dilakukan pemeriksaan mikroskopis dengan pewarnaan Gommori Methenamine Silver sebagai perbandingan. Pemeriksaan hitung jumlah limfosit CD4 dilakukan pada awal pasien masuk rumah sakit. Hitung jumlah yang didapatkan pada studi ini didapatkan hasil sangat rendah, yaitu 61% didapatkan hitung jumlah limfosit CD4 di bawah 50 sel/ µL. Lima

dari total 13 pasien didapatkan hasil positif pemeriksaan real time PCR (gen MSG) dan salah seorang diantaranya didapatkan hasil positif dari pemeriksaan mikroskopis yaitu ditemukannya bentukan karakteristik kista dengan area kehitaman pada bagian tengah dengan pewarnaan GMS. Pasien dengan hasil positif pemeriksaan mikroskopis tidak memiliki riwayat terapi profilaksis sebelumnya. Hitung jumlah limfosit CD4 merupakan faktor risiko yang kuat terhadap terjadinya P. jiroveci pneumonia pada pasien HIV/AIDS. Pemeriksaan real time PCR menunjukkan nilai yang tinggi dalam mendeteksi P. jiroveci tanpa memandang status profilaksis pasien.

Kata kunci: *HIV/AIDS, Pneumocystis jiroveci, pneumonia, hitung jumlah limfosit CD4 rendah, RSUD Dr Soetomo Surabaya*

INTRODUCTION

Human Immunodeficiency Virus (HIV) is a virus that attack immune cells and weaken the immune system. Cummulative 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]

Pneumocystic 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'-CAAAAATAACAYTSACATCAACRAGG-3', reverse

primer 5'-AAATCATGAACGAAATAACCATTGC–3', and probe 5'–TGCAAACCAACCAGTGCACGACAGG – 3'. Master mix was prepared, aliquote 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.[8] 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[10] and genotipic relationship with mannose-binding lectin.[11]

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.[12] 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.[13] 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.[11] Negative PCR assay can be concluded true negative.[14] PJP-HIV/AIDS patients with negative microscopy examination often are colonized with P. jiroveci.[15] 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.[16] Cycle threshold value positively correlated with the microorganism density in the sample.[17] A cut off CT value of 32 cycle can be applied in differentiating colonization to P. jiroveci infection with sensitivity 72% and specificity 75%.[17]

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

	n	Mean	Median
CD4 lymphocyte count	13	82.69 cells/ µL	15 cells/µL

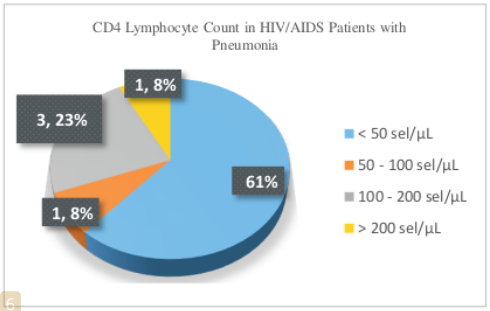

Figure 1. Distribution of CD4 lymphocyte count in HIV/AIDS patients with pneumonia from December 2016 to April 2017

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

Real Time PCR PJP	n	%
Positive	5	38.5
Negative	8	61.5
Total	**13**	**100**

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

	Real Time PCR *P. jiroveci*		Total
	Positive	Negative	
CD4 < 200	5	0	5
CD4 > 200	0	8	8
Total	5	8	13

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.[18,19] It is clinically evident that CD4 lymphocyte count can be use

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

	n	Nilai rt PCR Positif	Nilai rt PCR Negatif
Terapi Cotrimoxazole profilaksis	4	3 / 75%	1 / 25%
Terapi Cotrimoxazole definitif	3	1 / 33.3%	2 / 66.7%
Tidak mendapatkan terapi	6	1 / 16.7%	4/ 83.3%

as a biomarker for immunodeficient condition and related to opportunistic infection.[19] Yanagisawa and Nojima also stated that PJP prevalency in HIV/AIDS patient is 50% higher in CD4 lymphocyte count below 200 cells/ µL.[11]

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.

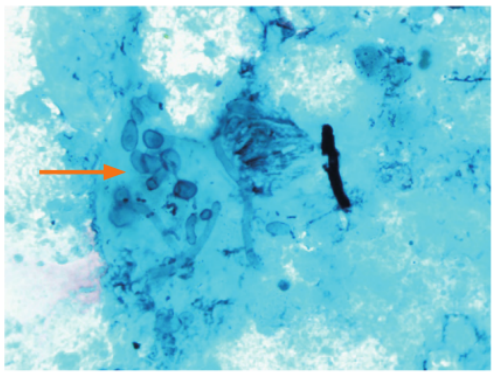

Figure 2. GMS staining of BAL smear from positive real time PCR assay showing characteristic cyst shape with dark centered area

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.[20] Microscopy examination with GMS staining has the same sensitivity on BAL or induction sputa, this specimens are specimen of choice in establishing microbiological diagnosis of PJP.[21,22] *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.[23] Trimethoprim-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.[16]

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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