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Detection of Opportunistic Fungus *Pneumocystis jirovecii* Major Surface Glycoprotein (MSG) gene in HIV-AIDS Patients with Pneumoniae in Jakarta

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Abstract

Pneumocystis jirovecii is known to cause opportunistic infections in the lower respiratory tract in individuals with low immune systems, especially patient with HIV infection. The prevalence of *P. jirovecii* pneumonia (PjP) in various countries show varying numbers. In Indonesia, HIV cases continue to rise. However, the data in Indonesia concerning the case of PjP is very limited. Until now the prevalence of PjP in Indonesia is only based on clinical symptoms of the patient. Currently, diagnosis of PjP relies on microscopic examination. The disadvantage of this examination is not easy to do and has a high negative predictive value. Thus, this study was conducted to develop a molecular test to diagnose PjP infection in HIV-AIDS suspected pneumonia. Molecular diagnostic test aimed for Major Surface Glycoprotein (MSG) gene of *P. jirovecii* detection was done through real-time PCR against 100 sputum samples. Demographic data show that the prevalence of PjP infection in HIV-AIDS suspected pneumonia patients in Jakarta is 20.0%, male 75% within 31-40 y.o (35%), dominant (80%) from patients with CD^{4+} T-lymphocytes of 200-349 cells/µL. Molecular real-time PCR methods were shown to give five times sensitivity higher than Giemsa stain.

Keywords: *P. jirovecii*, HIV, real-time PCR

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1. Introduction

Pneumocystis jirovecii is known to cause opportunistic infections in the lower respiratory tract in individuals with weakened immune systems, especially patients with infection HIV[1]. Individuals with an intact immune system will control the primary infection and the microorganisms will remain latent in the lung. Disease will occur when the immune system is disrupted or deficient. In this case, it is possible to find the cause of clinical pneumonia in respiratory tract[2,3].

International epidemiologic survey showed that pneumonia distributed worldwide with antibody prevalence varies among different geographical areas and can attack human at various ages[4,5]. The prevalence of PjP in various countries showed varying numbers. In Indonesia, HIV cases continue to rise. However, the data in Indonesia concerning the case of the PjP is very limited, until now the prevalence of PjP in Indonesia is only based on clinical symptoms of patients in the amount of 13.4% as reported by Lydia et al. (2009). In the same year at other hospitals in Indonesia (RS Persahabatan and the RSCM), 14.55% of the PjP was found in 55 patients studied. The figure is lower than the state other countries[6,7].

Inadequate diagnostic facilities cause PjP incidence data in Indonesia is not known with certainty. Currently in Indonesia, raw diagnostic standard for the diagnosis of PjP is not available, resulting in undetected cases. This can occur because, despite the many studies done, successful breeding of *Pneumocystis jirovecii* is very limited, so that its life cycle is not yet fully understood[8].

Until now, diagnosis of PjP relies on microscopic examination of respiratory specimens such as bronchial washings fluid (bronchoalveolar lavage/BAL), sputum induction or sputum[8]. The disadvantage of this examination is not easy to do and has a high negative predictive value.[9]

This study conducted real-time PCR detection for gene Major Surface Glycoprotein (MSG) as a rapid diagnosis of infection with *P. jirovecii*. Hopefully this research can be applied to diagnose infection with *P. jirovecii* thereby assisting in the management of HIV patients with pneumonia symptoms quickly and accurately.

2. Methods

Hundreds of sputum that have been obtained is mixed with ditiotreitol (Sputolysin; Behring Diagnostics), and vortexed for 30 seconds (Vortex Genie 2, Scientific Industries), followed by incubation in the incubator (Thermo Electron Corporation) with 35-37˚C temperature for 15 minutes. After incubation, the specimen is mixed with phosphate buffered saline (pH 7.4) until the turbidity is equivalent to 0.5 Mc. Farland. The mixture was then vortexed for another 15 seconds. Furthermore specimen was centrifuged at 3000 RPM (Universal 320 R, Hettich Zentrifugen) at a temperature of 25˚C for 5 minutes. The supernatant was discarded while the pellet (1 mL) was used for coloring Giemsa[10,11,12,13].

For real-time PCR test, the pellets were then centrifuged with a speed of 12000 RPM (Sorvall Biofuge Primo) at a temperature of 25˚C for 5 minutes. Furthermore, the

supernatant was discarded while the pellet as genomic DNA stored in a refrigerator at a temperature of -80˚C to be used for the analisys[14,15,16].

2.1. Test microscopy with Giemsa Staining

Staining was done by using Giemsa staining Modification[17]. Microscopic tests were done by spreading the suspension of pellets above the glass pedestal to form a thin layer. This was then dried through aeration in open air. Following this, 95% ethyl alcohol was poured until it covered the entire surface of the preparation, and this was then left to dry. Furthermore Sulphation reagent is dripped onto the preparation and allowed to stand for 10 minutes, then washed with running water for 5 minutes. After this, the preparations were stained with Giemsa 10% for 30 minutes, before being washed with water and allowed to dry in the air. After drying, a drop of emersion oil was dripped on the preparation and then viewed under microscope with magnification of 10 x 100[18].

2.2. DNA extraction *Pneumocystis jirovecii*

Extraction of specimens was performed in accordance with the procedures on extraction kit QIAmp DNA Mini Kit (Qiagen) as follows: Pellet from the processing of specimens was removed from the refrigerator to a temperature corresponding to room temperature. Furthermore 20 mL of Qiagen protease (proteinase K) and 180 mL of aqueous Tissue Lysis buffer (ATL) was added into pellets, homogenized using a vortex (Maxi Mix II) for 15 seconds and incubated at 56˚C overnight for the lysis of cells. After 24 hours, the sample was centrifuged at a speed of 12000 RPM for 15 seconds to remove fluid around the tube eppendorf. Then added to 200 mL of Aqueous Lysis buffer (AL), hereinafter homogenize using a vortex for 15 seconds, and incubation at temperature of 70˚C for 10 minutes. The next step centrifugation at a speed of 12000 RPM for 15 seconds to remove fluid tube wall, followed by addition of 200 mL of ethanol (96-100%). The specimen was then homogenized using a vortex for 15 seconds and re-centrifuged with the speed of 12000 RPM for 15 seconds. The next step was to move 600 mL of solution to QIAmp DNA mini spin column without wetting the rim, and centrifuged at 12000 RPM for 1 minute. The QIAmp DNA mini spin column was then transferred to a clean collection tube and added 500 mL of Aqueous Washing buffer 1 (AW1), followed by centrifugation at 12000 RPM for 1 minute. Furthermore, DNA QIAmp mini spin column was transferred back to the new receiver tube and have the filtered receiver tube removed. After this, a 500 mL of Aqueous Washing buffer 2 (AW 2) was added and then re-centrifuged

(12000 RPM) for 3 minutes. Next, the collected filtrate was discarded, and DNA QIAmp mini spin tube was put back in the same tube. After this, centrifugation was re-performed at 12000 RPM for 1 minute. After this, the QIAmp DNA mini tube was placed on clean microsentrifuge 1.5 ml tube, followed by adding 70 mL of aqueous elution buffer (AE) and incubated at room temperature for 5 minutes, then re-centrifuged (12,000 RPM) for 2 minutes. In the final step, the filtrate was collected in microcentrifugated tubes and stored at -35˚C until used for the reaction real-time PCR[19,20,21].

2.3. Real-time PCR

Pneumocystis jirovecii in samples was detected by using kappa probe Fast qPCR Master Mix kit (Bio-Rad iCyclerTM). Primers and probes were used in this study as reported by Gupta *et al* (2008) [22] (Table 1). The composition of the real-time PCR reaction (20 µL) is a follows: 1x Kapa Fast Probe Enzyme, 0.5 µM Primary (MSG forward), 0.5 µM Primary (reserve MSG), 0.5 µM Probe and 4 µL DNA. The real-time PCR reaction was carried out using Bio-Rad iCyclerTM (Bio-Rad) with the following conditions: 95˚C for 3 minutes (enzyme activation) and 45 cycles at 95˚C for 15 seconds and 58 ˚C for 1 minute.

TABLE 1: Primer and probe sequences *Pneumocystis jirovecii* [23,24].

Primer	Sequences (5'-3')	Position
PjP Forward	CAAAAATAACAYTSACATCAACRAGG	223-248
PjP Reverse	AAATCATGAACGAAATAACCATTGC	378-354
Probe	Sequences	
PjP probe	FAM-TGCAAACCAACCAAGTGTACGACAGG-TAMRA	252 -277

3. Results

3.1. Subjects demographics

A total of 100 HIV-infected patients with pneumoniae symptoms were observed in this study. Symptoms and clinical signs found in patiens are low-grade fever, shortness of breath and non-productive cough.

Characteristics of subjects by sex, age and number of CD^{4+} T (cell/µL) can be seen in Table 1. Data obtained through anamnesa and confirmatiom of the patient's medical record status. The patients consisted of 68 male and 32 female. The age range from 20-66 years-olds. Data on the number of CD^{4+} T lymphocytes shows that there are 25

Table 2: Subjects demographics (n=100).

Variable		Total
Sex	Male	68
	Female	32
	Total	100
Age	20-30	12
	31-40	57
	41-50	19
	51-60	9
	>60	3
	Total	100
Number of CD^{4+} T lymphocytes (cell/µL)	<50	2
	50-199	11
	200-349	62
	≥350	25
	Total	100

patients with CD^{4+} ≥350, 62 patients with CD^{4+} 200-349, 11 patients with CD^{4+} 50-199 and CD^{4+} 50-199 as many as 2 patients.

3.2. Detection of *P. jirovecii* using the real-time PCR method

From 100 samples examined, 20 samples gave positive results of *P. jirovecii*. DNA amplification curves using real-time PCR can be seen in Figure 1. The figure shows that positive controls form a sigmois curve while negative controls do not form a sigmoid curve. One sample shows positive, and negative for the other.

The relationship between sex, age and CD^{4+} T-lymphocyte cell with a positive real-time PCR of *P. jirovecii* can be seen in Table 2. The table shows that 20 positive patients was consisted of 15 (75%) men and 5 (25%) women. The predominant range of age is 31-40 year old (30%). Analysis of number of CD^{4+} T-lymphocyte cells showed predominantly in CD^{4+} T-lymphocyte cells 200-349 with 16 patients (80%).

3.3. Detection of P. jirovecii using Giemsa Staining

Microscopic examination was performed with Giemsa staining modified with sulphation. We obtained 3 positive samples of *P. jirovecii* (Table 3). One of the samples found a

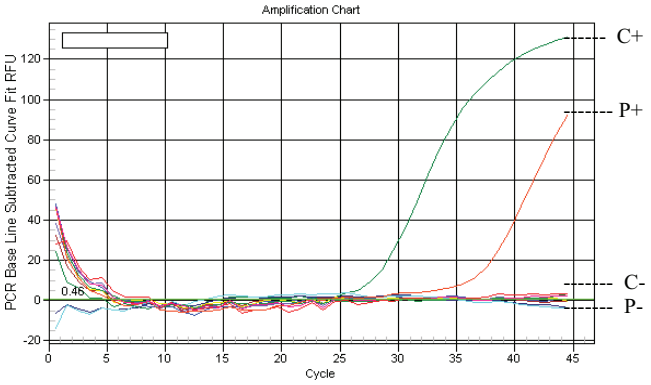

Figure 1: Example of *P. jirovecii* DNA detection results using real-time PCR. Horizontal line are real-time cycles. The vertical line is a fluorescent signal (*cycle threshold/*Ct). C+: positive control, C-: negative control, P+: positive patient, P-: negative patient.

Table 3: Demographics and characteristics of real-time PCR result.

Variable		Real-time PCR result			
		Negative (n=80)		Positive (n=20)	
		Total	(%)	Total	(%)
Sex	Male	53	(66.2)	15	(75.0)
	Woman	27	(33.8)	5	(25.0)
	Total	**80**	**(100.0)**	**20**	**(100.0)**
Age Number of CD^{4+} T lymphocytes	20-30	10	(12.5)	2	(10.0)
	31-40	50	(62.5)	7	(35.0)
	41-50	13	(16.2)	6	(30.0)
	51-60	5	(6.2)	4	(20.0)
	>60	2	(2.5)	1	(5.0)
	Total	**80**	**(100.0)**	**20**	**(100.0)**
	<50	1	(1.2)	1	(5.0)
	50-199	10	(12.5)	1	(5.0)
	200-349	46	(57.5)	16	(80.0)
(cell/µL)	≥350	23	(28.8)	2	(10.0)
	Total	**80**	**(100.0)**	**20**	**(100.0)**

cyst form of this microorganism (Figure 2). Table 3 shows 2 (66,66%) male patients was positive Giemsa staining. The age range 31-40, 41-50 and 51-60 years-old occupy proportionately, with each age group at 33,33%. Analysis of number of CD^{4+} T-lymphocyte cells showed predominantly in CD^{4+} T-lymphocyte cells 200-349 (100%).

Table 4: Demographics and characteristics of Giemsa staining.

Variable		Giemsa staining			
		Negative (n=97)		Positive (n=3)	
		Jumlah	(%)	Jumlah	(%)
Sex	Male	66	(68.0)	2	(66,66)
	Female	31	(32.0)	1	(33,33)
	Total	**97**	**(100.0)**	**3**	**(100.0)**
Age	20-30	12	(12.4)	0	(0.00)
	31-40	56	(57.7)	1	(33.3)
	41-50	18	(18.6)	1	(33.3)
	51-60	8	(8.20)	1	(33.3)
	>60	3	(3.10)	0	(0.00)
	Total	**97**	**(100.0)**	**3**	**(100.0)**
Number of CD^{4+} T lymphocytes (cell/µL)	<50	2	(2.10)	0	(0.00)
	50-199	11	(11.3)	0	(0.00)
	200-349	59	(60.80	3	(100.0)
	≥350	25	(25.8)	0	(0.00)
	Total	**7**	**(100.0)**		**(100.0)**

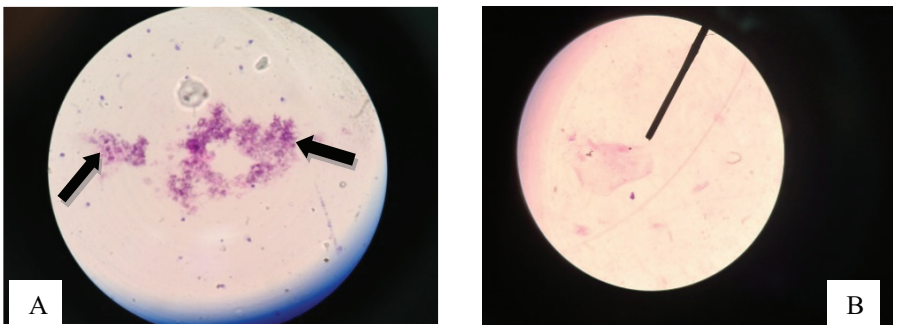

Figure 2: Giemsa staining results were modified with sulphation. A. Microscopic results of positive Giemsa. The arrow on the left shows the cyst stage, while the arrow on the right shows the trophozoite stage with a magnification of 1000x. B. Microscopic results of negative Giemsa.

3.4. Comparison of real-time PCR and Giemsa microscopic

Table 4 shows 3 out of 100 samples gave positive results both real-time PCR and Giemsa staining (15%). Seventeen samples that were detected as negative by microscopic gave positive result on real-time PCR (17%). This result shows that real-time PCR increases the positive value more than 5 times compared to microscopic test. Another 80 samples gave the same (negative) results both with real-time PCR and Giemsa staining.

Table 5: Comparison of real-time PCR and Giemsa microscopic.

Total (%) Specimen						
P. jirovecii	rPCR+ M+	rPCR+ M-	rPCR- M-	rPCR- M+	Total M+	Total rPCR+ PCR+
	3 (15%)	17 (17%)	80 (50%)	0 (0%)	3 (15%)	20 (20%)

rPCR: real-time PCR, -: negative

M: microscopic test, +: positive

4. Discussion

4.1. Subject Characteristics

The real-time PCR test results show that the infection rate of *P. jirovecii* is 20%. Our study provides the most recent data for the prevalence of PjP infections in Indonesia. Until now the data reported only based on patient clinical symptoms which is 14,45% as reported in several hospitals in Jakarta[10]. Compared to other countries in Southeast Asia, infection of *P. jirovecii* obtained are lower but close to neighboring Malaysia which is 22.7%[5,8,9]. The differences in infection rates can be caused by host immunity infection, various diagnostic facilities and different geographical factors.

Male occupies a dominant position compared to women with a percentage of 75% and 25%, respectively. The age range of patients is between 20 -- 66 years. These results indicate differences compared with The Republic of Indonesia Ministry of Health 2013, that the highest percentage of cases was found in the range age of 25-49 years (70.4%) which is the most productive age group[25,26]. This age differences needs to be further examined as this study provide evidence of detection in both early and old age.

Based on the number of CD^{4+} T- lymphocytes, the range of 200-349 occupies the highest percentage (80%). This is an interesting finding in this study beacuse many other studies mentioned that Pneumocystis pneumonia generally occurs in HIV patients with CD^{4+} T-lymphocytes <200 cells/ µL[27,28,29,30,31], whereas in this study, the number CD^{4+} T-lymphocytes did not affect the presence of *P jirovecii* in patients. *P jirovecii* can be obtained from birth and occur through reactivation of altent infections when the immune system decrease. Several researchers have revealed that *P. jirovecii* transmission can be obtained from the enviroment, from infected people, and can also be transmitted from someone who has *P. jirovecii* colonization[32,33,34].

This study revealed thet even though anti-retroviral (ARV) treatment caused an increase in CD^{4+} T-lymphocytes levels, *P. jirovecii* remained an important opportunistic

infection in HIV-AIDS patients such as stated by Moris and Norris (2012) and Khalife *et al.* (2015)[6,7].

Giemsa staining that was modified using sulphation was found to improve the visualization of cyst shapes that was not detected in conventional Giemsa. This research has succeeded in getting a cyst shape in 1 of the 3 positive samples. Addition of sulphation which is addition of a mixture of sulfuric acid and glacial acetic acid before staining helped the Giemsa polychrome dye to react to opening the fungal cell wall and absorbsing dye so that the cyst can be clearly seen.

In terms of patients' gender distribution, male was found to be higher than female, with 66.66% and 33.33%, respectively. Distribution of patient's age was found to be proportionate, with 33.33% for each age range of 31-40, 41-50 and 51-60 years old. Analysis of the CD^{4+} T-lymphocytes showed that all patients were in the range of 200-349 cells/ µL.

4.2. Comparison of real-time PCR and Giemsa staining

Three samples gave positive results both on microscopic and real-time PCR, whereas 17 samples that were stated negative by microscope, gave positive results on real-time PCR. A total of 80 samples gave the same negative results, both with microscopic and real-time PCR.

Analysis of the results shows that real-time PCR increases of positive by more than 5 times compared to microscopic tests. This results are in accordance with research conducted by Pierre *et al* (2004), his research shows real-time PCR increases sensitivity by up to 60% compared to Giemsa staining. Tia *et al* (2011) and Florence *et al* (2014) showed real-time PCR increased positive results compared to microscopy, respectively at 41% and 19%[35,36,37]. Major surface glycoproteins were chosen as targets because they have the most varied sequences found on the surface of fungal cells and are sustainable. Additionally, the MSG gene can increase sensitivity so that it is used by many researchers for detection[38,39,40,41].

5. Conclusion

The developed real-time PCR detection was able to provide better diagnostic value than Giemsa staining. Demographically, the prevalence of *P. jirovecii* in HIV-AIDS with pneumonia in Jakarta reached 20%, dominant in the range of CD^{4+} T-lymphocytes 200-349 cells/ µL.

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