

Genotypes of the DHPS resistance of *Pneumocystis jirovecii* and Variations of Nucleotides mtLSU-rRNA Genes Detected from HIV Positive Patients with Pneumonia in Jakarta, Indonesia

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Abstract

This study investigated the prevalence and genotype distribution of *Pneumocystis jirovecii* in HIV-positive patients with pneumonia in Jakarta, Indonesia. A total of 100 sputum samples were collected from two hospitals and analyzed using real-time PCR targeting the *P. jirovecii* MSG gene to detect the presence of the organism. Genotyping was performed through mtLSU ribosomal DNA sequencing and DHPS gene restriction fragment length polymorphism (RFLP) analysis. *P. jirovecii* DNA was detected in 20% of samples (20/100). DHPS analysis revealed that 58.3% (7/12) of patients with *P. jirovecii* pneumonia carried resistant genotypes, including 16.7% genotype 3 and 41.7% mixed genotypes. Sequencing of mtLSU identified seven variants, with variant 3 being the most common and showing a strong association with lower CD4+ T-lymphocyte counts. These results suggest that multiple resistant genotypes of *P. jirovecii* are circulating in Indonesia and that resistance is more closely linked to the patient's clinical condition and immune status than to mtLSU variant type. This study provides important molecular epidemiological data on *P. jirovecii* in HIV-positive patients in Indonesia and highlights the need for ongoing monitoring of genotypes and resistance markers to guide treatment strategies and improve patient outcomes.

Keywords: *Pneumocystis jirovecii*, DHPS, mtLSU, HIV

Резюме

Това проучване изследва разпространението и разпределението на генотипа на *Pneumocystis jirovecii* при HIV-позитивни пациенти с пневмония в Джакарта, Индонезия. Общо 100 проби от храчки са събрани от две болници и анализирани с помощта на PCR в реално време, насочени към гена MSG на *P. jirovecii*, за да се открие наличието на организма. Генотипизирането е извършено чрез секвениране на рибозомна ДНК на mtLSU и анализ на полиморфизъм на дължината на рестрикционните фрагменти (RFLP) с DHPS ген. ДНК на *P. jirovecii* е открита в 20% от пробите (20/100). DHPS анализът разкри, че 58.3% (7/12) от пациентите с пневмония, причинена от *P. jirovecii*, носят резистентни генотипове, включително 16.7% генотип 3 и 41.7% смесени генотипове. Секвенирането на mtLSU идентифицира седем варианта, като вариант 3 е най-често срещаният и показва силна връзка с по-нисък брой CD4+ Т-лимфоцити. Тези резултати показват, че в Индонезия циркулират множество резистентни генотипове на *P. jirovecii* и че резистентността е по-тясно свързана с клиничното състояние и имунния статус на пациента, отколкото с типа вариант на mtLSU. Това проучване предоставя важни молекулярно-епидемиологични данни за *P. jirovecii* при HIV-позитивни пациенти в Индонезия и подчертава необходимостта от непрекъснато наблюдение за разпространението на генотиповете и маркерите за резистентност, за да се насочат стратегиите за лечение и да се подобрят резултатите при пациентите.

Introduction

Pneumocystis jirovecii is a fungal organism responsible for pneumonia (*Pneumocystis pneumonia* [PCP]) in immunocompromised individuals, both with and without Human Immunodeficiency Virus (HIV) infection (Almaghrabi *et al.*, 2019; Bozzi *et al.*, 2022). This microorganism is ubiquitous, airborne, and primarily targets the respiratory system. It attaches to type I alveolar epithelial cells and triggers host inflammatory responses (Beard *et al.*, 2000). Under certain conditions, *P. jirovecii* can disseminate to extrapulmonary organs such as the liver, spleen, and kidneys (Witt *et al.*, 1991).

Although the incidence of PCP among HIV-positive individuals has decreased due to the widespread use of antifungal prophylaxis and antiretroviral therapy, it remains one of the most common opportunistic infections with significant morbidity and mortality, especially in developing countries (Oladele *et al.*, 2018; Gingerich *et al.*, 2021). In Indonesia, HIV continues to represent an important public health problem, with an estimated 0.4% prevalence among people aged 15–49 years and approximately 510,000–570,000 people living with HIV (PLHIV) as of 2022 (HIV/AIDS Data Hub for the Asia Pacific, 2022). The epidemic is concentrated primarily among key populations such as men who have sex with men (MSM), sex workers, people who inject drugs, and transgender individuals (UNAIDS, 2024). Notably, more than 50% of new HIV infections occur in the 15–24-year-old age group, indicating a growing burden among younger populations.

Therapeutic failure and the high mortality rate of PCP have been associated with genetic polymorphisms in *P. jirovecii* strains (Singh *et al.*, 2019). One of the most studied polymorphisms involves mutations in the dihydropteroate synthase (DHPS) gene, resulting in amino acid substitutions at codons 55 and 57 (de la Horra *et al.*, 2021). These amino acid changes confer resistance to trimethoprim-sulfamethoxazole (TMP-SMX), the drug of choice for both prophylaxis and therapy (Salzer *et al.*, 2019).

DHPS is the enzymatic target of sulfonamides, the main class of drugs used for PCP prophylaxis and treatment. The combination of trimethoprim and sulfamethoxazole is widely employed as first-line therapy for pneumonia caused by *P. jirovecii* and other pathogenic microorganisms such as *Plasmodium falciparum*, *Toxoplasma gondii*, and *Streptococcus pyogenes* (de Armas *et al.*, 2012; Maitte *et al.*, 2013). Mutations in the DHPS gene can lead to resistance to TMP-SMX, and resistant strains have

been reported in several countries (Xue *et al.*, 2022).

DHPS gene resistance is commonly determined by restriction enzyme analysis, as described by Marijke *et al.* (2014), which classifies isolates into four genotypes based on *AccI* and *HaeIII* restriction patterns: wild-type (WT)-restricted by both enzymes; genotype 1-restricted by *HaeIII* but not *AccI*; genotype 2-restricted by *AccI* but not *HaeIII*; genotype 3- not restricted by either enzyme; and mixed genotypes (Szydlowicz *et al.*, 2019; Liu *et al.*, 2020).

In addition, *P. jirovecii* pathogenicity and disease severity are also associated with genetic diversity among strains. Several molecular markers have been used to identify genetic variations, including mitochondrial large subunit ribosomal RNA (mtLSU-rRNA), small subunit mitochondrial ribosomal RNA, internal transcribed spacer 1 (ITS1), superoxide dismutase (SOD), and cytochrome b (CYB) (Szydlowicz *et al.*, 2019; Liu *et al.*, 2020). Among these, mtLSU-rRNA is most frequently used (Gurbuz *et al.*, 2021; Rafat *et al.*, 2021; Xue *et al.*, 2022). Studies from various countries have reported different mtLSU-rRNA distribution patterns, indicating high genetic diversity among *P. jirovecii* strains (Dimonte *et al.*, 2013; Jarbouli *et al.*, 2013; Kim *et al.*, 2015).

To our knowledge, no study has yet reported the molecular characteristics of *P. jirovecii* strains in Indonesia based on DHPS and mtLSU-rRNA gene analysis. Therefore, this study aims to describe these genetic profiles for the first time. Such information is essential for improving PCP patient management, particularly among immunocompromised individuals with and without HIV infection.

P. jirovecii is a fungal organism responsible for pneumonia diseases (*Pneumocystis pneumonia* [PCP]) in immunocompromised people with Human Immunodeficiency Virus (HIV) and non-HIV (Almaghrabi *et al.*, 2019; Bozzi *et al.*, 2022). This microorganism is scattered everywhere, spreads through the air, attacks the respiratory system deposited in the alveoli, is attached to alveolar cell type 1, and triggers the host's inflammatory response (Beard *et al.*, 2000). Under certain conditions, *P. jirovecii* can spread to various locations in the extrapulmonary lung, such as the liver, spleen, and kidneys (Witt *et al.*, 1991).

The PCP cases in people with HIV are decreasing because of the prophylactic fungal medications and antiretroviral therapy; however, it continues to be the most common infection with significant morbidity and mortality, particularly in

developing countries (Oladele *et al.*, 2018; Gingerich *et al.*, 2021). The therapeutic failure and high mortality could be correlated with genetic polymorphisms of *P. jirovecii* strains (Singh *et al.*, 2019). One of the polymorphisms was a mutation of the DHPS (dihydropteroic synthase) gene leading to the amino acid change of the dihydropteroic synthase enzyme at codons 55 and 57 (de la Horra *et al.*, 2021). The amino acid changes caused *P. jirovecii* to be resistant to trimethoprim-sulfamethoxazole [TMP-SMX], as the drug of choice for prophylaxis and therapy (Salzer *et al.*, 2019).

Dihydropteroate synthase (DHPS) is an enzymatic target or gene target for sulfonamides, the main drug for prophylaxis or the treatment of Pneumocystis pneumonia. The trimethoprim-sulfamethoxazole combination is widely used for the first line of prophylaxis and treatment of pneumonia caused by *P. jirovecii* and other pathogenic microbes such as *P. falciparum*, *T. gondii*, and *S. pyogenes* (de Armas *et al.*, 2012; Maitte *et al.*, 2013). The DHPS gene is a gene that has the potential to undergo mutations that result in resistance to trimethoprim-sulfamethoxazole. Several researchers have reported the presence of a resistant DHPS gene in some countries (Xue *et al.*, 2022).

Resistance to the DHPS gene is determined based on the results of enzyme cleavage, classified by Marijke *et al.* (2014). There are 4 genotypes based on differences in the cutting of these enzymes, namely: WT (wild type) is restricted by both *AccI* and *HaeIII* enzymes; genotype 1 is restricted by *HaeIII* but not restricted by *AccI*; genotype 2 is restricted by *AccI* but not restricted by *HaeIII*; genotype 3 is not restricted by *HaeIII* or *AccI*, and mixed genotypes (Szydlowicz *et al.*, 2019; Liu *et al.*, 2020).

On the other hand, the *P. jirovecii* pathogenicity and the disease severity were also associated with genetically different strains. To identify the *P. jirovecii* genetic variations, mitochondrial large subunit ribosomal RNA (mtLSU-rRNA), small subunit mitochondrial ribosomal RNA, internal transcribed spacer 1 (ITS1), superoxide dismutase (SOD), and cytochrome b (CYB) have been used (Szydlowicz *et al.*, 2019; Liu *et al.*, 2020), and among those, the mtLSU-rRNA was frequently used by many researchers (Gurbuz *et al.*, 2021; Rafat *et al.*, 2021; Xue *et al.*, 2022). Studies from many countries reported different patterns of mtLSU-rRNA distributions, indicating high genetic diversity of *P. jirovecii* strains (Dimonte *et al.*, 2013; Jarboui *et al.*, 2013; Kim *et al.*, 2015).

To our knowledge, there is no report regarding the DHPS and mtLSU-rRNA-based molecular characteristics of *P. jirovecii* strains detected in Indonesia; therefore, here, we report it as the first study. This information is very useful to evaluate PCP patient management, particularly immunocompromised people with HIV and non-HIV.

Materials and Methods

Characteristics of patients

A total of one hundred patients who met the inclusion criteria were tested using a real-time RT-PCR assay targeting the mSG gene of *P. jirovecii*. Among them, twenty hospitalized HIV-positive patients with acute pneumonia from RSCM and RSAL Hospitals, Jakarta, collected between November 2015 and June 2016, were confirmed positive for *P. jirovecii*, as previously reported (Tjampakasari *et al.*, 2019). All patients provided written informed consent and fulfilled the study's inclusion criteria. Demographic data, including age, sex, and ethnicity, were recorded.

Clinical samples

Standard or induced sputum samples were collected from patients by specialist doctors. The samples were transported to the laboratory using a cool box with a 2-8°C condition, and the samples were immediately processed by adding 1 ml of 1 M dithiothreitol and incubated at 37°C for 15 min. The mixture was added by 1 ml of 1x phosphate buffer saline (PBS), pH 7.4. One ml of the mixture was centrifuged at 14000g for 5 min. The supernatant was discarded, and the pellet was stored at -80°C until used.

DHPS-based genotype

Nested PCR generated 370 bp product using primers from Monroy-Vaca *et al.* (2014) and Lane *et al.* (1997). PCR I (20 µl) and PCR II (30 µl) contained 1× PCR Buffer, 1× Q Solution, 0.2 mM dNTP, 2.5 mM MgCl₂, primers (0.4 µM for PCR I, 0.3 µM for PCR II), 0.5 U HotStar Taq, and DNA template (PCR I: 5 µl, PCR II: 4 µl of PCR I product). Thermal cycling was 95°C for 15 min; 35–42 cycles of 94°C for 30 s, 52–55°C for 1 min, 72°C for 1 min; and 72°C for 10 min. PCR II products were gel-purified using QIAEX II and analyzed by RFLP with *AccI* and *HaeIII* to determine DHPS polymorphisms at codons 55 and 57 (Helweg-Larsen *et al.*, 2000).

mtLSU-rRNA characterization

Nested PCR produced a 251 bp product using primers from Helweg-Larsen *et al.* (2000) and

Wakefield (1996). PCR I (40 μ l) and PCR II (50 μ l) contained 1 $\times$ PCR Buffer, 1 $\times$ Q Solution, 0.2 mM dNTP, 2.5 mM MgCl₂, primers (0.4 μ M), and HotStar Taq (1–1.25 U). DNA template: PCR I 10 μ l, PCR II 5 μ l of PCR I product. Thermal cycling: 95°C for 15 min; 40–42 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min; and 72°C for 10 min. PCR II products were gel-purified with QIAEX II and sequenced using the same primers. Sequences were analyzed with SeqScape v2.7 and deposited in GenBank (Accession No: PQ892037–PQ892056).

Statistical test

The Kruskal-Wallis statistical test was performed to examine the relationship between DHPS and mtLSU genes with genotype, age, and CD4+ count, respectively.

Results

Patient characteristics

Of 20 patients, the ages ranged from 20 to 66 years (mean $\pm$ 37.76) with three ethnicities (9 Javanese [45%], 9 Sumatrans [45%], and 2 Sulawesi [2%]). Most of the patients were male (15 [75%]), while the rest were female (5 [25%]). The patient CD4+ counts were 200–349 (16 [80%]), $\geq$ 350 (2 [10%]), 50–199 (1 [5%]), and $<$ 50 (1 [5%]) cells/mm³.

DHPS-based genotype

Of the 20 samples that were positive for *P. jirovecii*, only 12 were positive for the DHPS gene. The genotypes were conducted by DNA restriction using *HaeIII* and *AccI* enzymes (Fig. 1 and 2). Of the 12 samples, 5 (41.67%) were wild type (WT), 2 (16.67%) were 3, and 5 (41.67%) were mixed (WT and 1) genotypes (Table 1). The patients infected by a mixture of genotype WT and 1 had 150–350 cells/mm³ at the ages of 34–49; those infected by genotype WT had 280–381 cells/mm³ at the ages of 24–48, and those infected by genotype 3 had 267–300 cells/mm³ with ages of 31 and 66.

Figure 1 shows the visualization of the restriction (enzyme cleavage) results of the *DHPS* gene fragments using the *AccI* enzyme, while Figure 2 shows the results using the *HaeIII* enzyme. For patient samples numbered 41, 49, 9, 24, and 26, two bands were observed, indicating that the fragments were successfully cut (restricted) by both *AccI* and *HaeIII*. In contrast, samples from patients 31 and 37 were not cleaved by either enzyme. Meanwhile, samples from patients 6, 7, 29, 43, and 44 appeared to be partially digested by the enzyme, producing

three bands, suggesting a mixture +/- with the *AccI* enzyme.

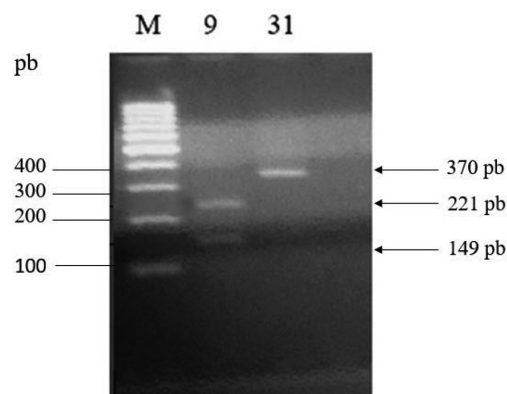


Fig. 1. Example of the visualization results of DHPS gene fragment restriction using the *AccI* enzyme. Column M: DNA marker 100 pb. Columns 9, 31: patient identification number. No. 9 is restricted by the *AccI* enzyme. No. 31 is not restricted by *AccI* enzymes.

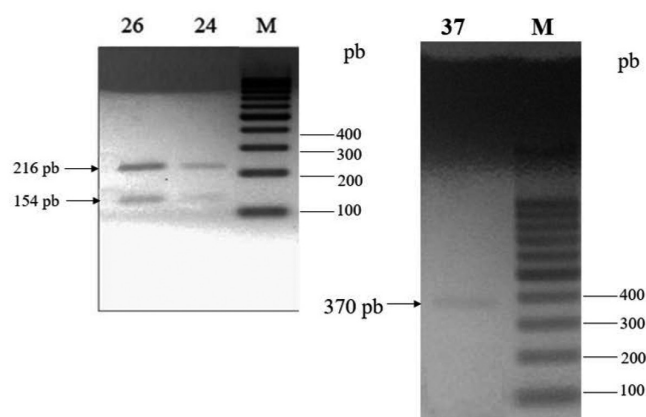


Fig. 2. Example of the visualization results of DHPS gene fragment restriction using the *HaeIII* enzyme. Column M: DNA marker 100 pb. Columns 24, 26, and 37: patient identification number. No. 24 and 26 are restricted by the *HaeIII* enzyme. No. 37 is not restricted by *HaeIII*.

The percentage of genotypes with patient demographics can be seen in Table 1. *P. jirovecii* WT genotype was detected in patients with an age range of 31–50 years by 80%, while the age range of 20–30 years was 20%. Genotype 3 was detected in just as many (50%) patients with an age range of 41–50 years and over 60 years. For the mixed genotype, dominance (60%) was detected in the age range of 31–40 years. The table also shows the relationship between genotype and clinical patients obtained based on CD4+ T lymphocyte cell values. Based on these data, the value range of CD4+ T lymphocyte cells in patients with detected DHPS genes ranges from 150 to 381 cells/mm³.

The association between *P. jirovecii* genotypes (WT, Genotype 3, and Mixed [1 + WT]) and patients' CD4+ counts and agewas evaluated using the Kruskal-Wallis test. No significant differences were observed in CD4+ counts ($H=4.12$, $p=0.127$)

or age ($H=2.34$, $p=0.31$) among the genotypes, indicating that genotype variation was not associated with immunology status or age in this cohort.

Table 1. Genotype of *P. jirovecii* based on the DHPS gene

ID Patients	Restriction enzymes		Genotype	CD4+ (cell/mm ³)	Age
	AccI	HaeIII			
41	+	+	WT	350	24
49	+	+	WT	280	33
09	+	+	WT	320	33
24	+	+	WT	300	48
26	+	+	WT	381	31
37	-	-	3	267	66
31	-	-	3	300	42
06	+/-	+	Mix (1+WT)	200	45
07	+/-	+	Mix (1+WT)	200	48
29	+/-	+	Mix (1+WT)	320	34
43	+/-	+	Mix (1+WT)	150	49
44	+/-	+	Mix (1+WT)	300	35

+, -, and +/-: restricted, not restricted, and partly restricted by the indicated enzymes, respectively. WT: wild type.

mtLSU-rRNA characteristic

For mtLSU-rRNA, all 20 *P. jirovecii* positive samples were PCR (251 bp) positive. The two positions (85 and/or 248) are highly varied and can be used to differentiate the fungal strains (18, 23). After deducting the primer sequences, the mtLSU-rRNA sequences that can be analyzed are 207 bp from nucleic acids at positions 35 to 241. Therefore, a polymorphism at position 85 could be analyzed, while another polymorphism at position 248 could not be analyzed because it was not spanned by the primers used in this study. Based on variation at position 85, 11 (55%) of 20 strains had a nucleotide adenine (A), 6 (30%) had a nucleotide mixture of cytosine (C/A), 1 (5%) had a nucleotide mixture of C/thymine (T), 1 (5%) had nucleotide T, and 1 (5%) had nucleotide C (Table 2). Nucleotide A at position 85 was obtained from 11 patients with 150–492 CD4+ counts and 20–56-year-olds. Mixtures of C/A were obtained from 6 patients with 21–320 CD4+ counts and 14–58-year-olds. Nucleotide C, T, and a mixture of C/T were obtained from patients with CD4+ counts of 200 (39 years old), 200 (47 years old), and 320 (34 years old), respectively. Kruskal-Wallis analysis shows a significant difference in CD4+ counts among nucleotide genotypes at position 85 ($p < 0.05$), with patients carrying genotype A exhibiting higher CD4+ counts than those with C, T, or C/A genotypes. No significant differences were observed in patient age across genotypes ($p > 0.05$),

indicating that genotype at position 85 is not associated with age.

Table 2. Variations of nucleotides at position 85 of *Pneumocystis jirovecii* mtLSU-rRNA

ID Patient	Nucleotide at position 85	CD4+ count (cell/mm ³)	Age
01	C	200	39
02	T	200	47
06	A	280	45
08	A	381	31
09	A	492	33
07	A	300	48
13	A	350	56
15	A	350	24
16	A	150	49
17	A	300	35
20	A	300	58
21	A	275	20
22	A	280	33
03	C/A	200	58
04	C/A	320	41
05	C/A	280	43
12	C/A	300	42
14	C/A	267	66
18	C/A	21	21
10	C/T	320	34

C: cytosine. T: thymine. A: adenine.

Discussion

In contrast to most bacteria, the fungus *P. jirovecii* cannot be cultured. Therefore, genomic typing must be conducted directly from clinical samples. DNA amplification from clinical samples has certain obstacles because of the small amount of DNA fragments. In this study, amplification of DHPS DNA fragments showed less sensitive results. Of 20 *P. jirovecii*-positive samples, only 12 were DHPS-positive. For the mtLSU-rRNA DNA fragment, all 20 *P. jirovecii* positive samples were positive. The different sensitivity between DHPS and mtLSU-rRNA might be caused by different amounts of DNA molecules in the genome. Of the two targets, the mtLSU-rRNA is preferred because it exists in multiple copies of mitochondrial organelles; therefore, the mtLSU-rRNA can be detected more easily than a single copy of the DHPS gene (Wakefield *et al.*, 1990; Montes-Cano *et al.*, 2004).

The characteristic of the DHPS gene, polymorphisms at codons 55 and 57, can affect the patient's response to antifungal drug therapy (Salzer *et al.*, 2018; de la Horra *et al.*, 2021). To know the

codon polymorphisms, the RFLP method can be used by using *AccI* (codon 55) and *HaeIII* (codon 57) enzymes. The method is simple, rapid, and cost-effective for the detection of point mutations at codons 55 and 57 (Ma *et al.*, 2018). In this study, of 12 samples, 41.67% (5/12) were WT, 16.67% (2/12) were 3, and 41.67% (5/12) were mixed (WT and 1) genotypes. This result showed a higher prevalence than in some countries in Asia. In Hangzhou, China, Zhu *et al.* (2020) found only one case (1.67% [1/163]) of mutation at codon 55, and the majority of cases were the WT genotype (Siripattanapipong *et al.*, 2008; Singh *et al.*, 2019). In Guangzhou, China, Deng *et al.* (2014) reported that of 25 patients, 22 (88%) showed the WT genotype, 2 (8%) showed a mixture of WT and mutant in codons 55 and 57, and the remaining 1 (4%) showed a mixture of WT and mutant in codon 55 (30). The mutation prevalence (codon 55 and 57) was also low in Thailand (11.7% [2/17]) and Iran (6.25% [1/16]), and there were no cases of the mutations in India (0% [0/14]), Turkey (0% [0/28]), and Korea (0% [0/95]) (Tyagi *et al.*, 2008; Deng *et al.*, 2014; Mane *et al.*, 2015; Zhu *et al.*, 2020).

Be aware of the data obtained; the results of the *P. jirovecii* study in Indonesia showed a high rate of gene mutations, that is, 7 of 12 patients (58.34%). This should be cautioned because gene mutations are associated with resistance to trimethoprim-sulfamethoxazole (TMP-SMX) and result in treatment failure. Genotypes do not change over time. The occurrence of the resistance genotype is due to increased exposure to TMP-SMX (Siripattanapipong *et al.*, 2008; Ozkoc *et al.*, 2018). Statistical analysis showed no significant differences in CD4+ counts among *P. jirovecii* genotypes (WT, Genotype 3, Mixed [1 + WT]). These results suggest that genotype variation is not associated with immune status or patient age in this cohort.

Analysis of polymorphism in the hot spot region of the mtLSU gene nucleotide positions 35 to 241 (205 PB) was obtained in 20 patients with 30 strains of *P. jirovecii*, where some patients were infected by 2 different strains (mixed infections). Seven variants were found in this study, and the 3rd variation type is the type of nucleotide variation that is widely found, which is 16 strains; the 2nd variation type is 2 strains, and 1 strain for each type of 4th, 5th, 6th, and 7th nucleotide variation. Of the 30 strains that were detected, mutations at nucleotide position 49 (C→T) were found in 1 strain. Mutations in nucleotide position 85 (C→T) were found in 2 strains, while mutations from C to

A were found in 20 strains. Mutations at nucleotide positions 100 (T→C), 119 (G→A), and 203 (A→G) were also found in 1 strain, respectively. So far, the 7 variants we found have not been reported by other researchers.

The results of our study are different from the results of Ernesto *et al.* (2014) study in Cuba; they found 10 variants with polymorphism from nucleotide positions 25 to 258 with changes from (A→T) at positions 25, 252, and 258. The 1st type of variation is the type of nucleotide variation that is widely found, which is as many as 31 strains. The differences in nucleotide variation between our study and Ernesto *et al.* (2014) were influenced by differences in disease, specimens, and subjects studied; transmission; and circulation of different *P. jirovecii* genotypes and geographical locations. Ernesto *et al.* (2014) examined infants and toddlers with cough symptoms as research subjects with nasopharyngeal swabs as the specimens studied (Sheikholeslami *et al.*, 2015).

The mtLSU gene is a gene that resides in the fungal ribosome, located on ribosomal RNA that is not directly involved in the formation of proteins (Sheikholeslami *et al.*, 2015). Therefore, the nucleotide variation in the mtLSU gene does not indicate any change in the amino acid arrangement that can be transformed into a new protein. Changes in ribosome structure due to nucleotide variations tend to change the structure of the ribosomal RNA itself. The existence of 7 variants of *P. jirovecii* found in this study shows the high genetic variation of the mtLSU gene in Jakarta.

This study analyzed the mtLSU gene hotspot area. Some researchers use the mtLSU gene as a genotyping study for their country, and some relate it to the patient's clinical condition to determine the severity of the disease (CDC, 2020). Therefore, it is necessary to characterize the genotype of the mtLSU gene to determine the severity of the disease. Genotype at nucleotide position 85 was significantly associated with CD4+ count, with A carriers showing higher levels than C, T, or C/A genotypes (Kruskal-Wallis, $p < 0.05$). No differences in age were observed ($p > 0.05$), suggesting that this variant affects immune status independently of age.

Regarding PCP prophylaxis in HIV-positive patients based on CD4 count, fungal drugs are recommended when CD4 counts are less than 200 cells/mm³ and discontinued when CD4 counts are sustained at > 200 cells/mm³ for more than 3 months (Witt *et al.*, 1991). If ART initiation must be delayed and frequent monitoring of CD4 counts

(e.g., every 3 months) is impossible, some experts recommend starting *P. jirovecii* Pneumoniae prophylaxis at CD4 counts ≥ 200 cells/mm³ to ≤ 250 cells/mm³. In one study, the *P. jirovecii* pneumonia case occurred in an HIV-infected patient with an undetectable viral count and a significant CD4 count (>487 cells/mm³) (Sullivan *et al.*, 2020).

Several intrinsic factors affect the characteristics of resistance and virulence of *P. jirovecii* with CD4⁺ T lymphocytes, including gender, age, and clinical condition of the patient. In addition to intrinsic factors, other causative factors that play a role are the risk of therapy and the presence of other diseases.

Based on gender, men differ significantly from women. Although age does not show significance, in this study, the productive age range (31-40 years) dominates. The patient's condition was traced based on the CD4⁺ T lymphocyte cell value and treatment status. Based on the data search, it was known that inpatients were indicated with more severe infections than outpatients.

Therapy plays an important role in the management of infection. When used correctly, antibiotics provide undoubted benefits. However, when used or prescribed inappropriately, it can cause widespread harm in terms of health, economy, and even for future generations. The use of antibiotics has increased tremendously over the past 5 decades; this is not only the case in Indonesia but also a problem in developed countries such as the United States (Ernesto *et al.*, 2014).

Another thing that needs to be followed up on is regular CD4⁺ T lymphocyte examinations to monitor the patient's clinical condition, accompanied by viral load examinations to monitor virus particles in the patient's blood. The higher the number of virus particles in the blood, the higher the risk of transmitting them and developing an opportunistic infection. It is crucial to link the data to the DHPS resistance gene to determine whether the genotype directly affects the patient's clinical condition. The information must also be linked to the different forms of the mtLSU gene so that we can figure out which form is most contagious based on the patient's health.

This study has several strengths. It employed sensitive molecular techniques, including real-time PCR, mtLSU sequencing, and DHPS RFLP analysis, allowing accurate detection and genotyping of *P. jirovecii*. The focus on HIV-positive patients provides clinically relevant insights into prevalence, genotype distribution, and potential antibi-

otic resistance, while linking genotype data with CD4⁺ counts adds an immunological perspective. Additionally, the study represents one of the first molecular investigations of *P. jirovecii* in Indonesia. However, the study also has limitations, including a relatively small sample size from only two hospitals and a cross-sectional design, which may limit generalizability and prevent causal inferences. Genotyping was restricted to mtLSU and DHPS RFLP analysis, and the observed association between genotype and CD4⁺ count is correlative rather than causal.

Conclusion

The study identified three DHPS genotypes (wild type, genotype 1, and genotype 3), with resistant genotypes being predominant. Seven mtLSU variants were detected, dominated by variant 3. Overall, DHPS resistance appears to be more closely associated with the patient's clinical condition than with mtLSU variant type.

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