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ORIGINAL ARTICLE

Detection of Epstein-Barr Virus in Saliva and Gen LMP1 among HIV-Infected Patients

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ABSTRACT

Epstein-Barr virus (EBV) is also called human herpes virus 4 (HHV-4), has detected 95% of the population and shows an asymptomatic state. EBV is etiological agent of oral hairy leukoplakia (OHL) in HIV patients. Latent membrane protein 1 (*LMP1*), an integral EBV protein can modulate growth, differentiation, induce the expression of several cells, activation of antigens, and adhesion molecules. The *LMP1* gene has been associated with OHL. **Objectives:** to determine the prevalence of EBV in saliva and the *LMP1* gene in HIV/AIDS patients with EBV positive. **Methods:** A cross-sectional was conducted on HIV/AIDS patients. The presence of EBV in saliva was done by microarray PCR. *LMP1* is examined by using nested PCR. **Results:** The research subjects involved 30 HIV/AIDS patients consisting 70% men and 30% women, with 50 % age group of 31-40 years old and 40% had CD4 counts <200 cells/mm³ (40%). EBV in saliva was found in 26 out of 30 (87%) HIV patients and *LMP1* was detected in 17 patients (65.38%). **Conclusion:** The high prevalence of EBV in saliva and the *LMP1* gene may increase the risk of OHL. Early screening for EBV infection in patients with HIV/AIDS is important to reduce the risk of EBV-associated diseases.

Key words: EBV, HIV/AIDS, *LMP1*, OHL

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INTRODUCTION

Human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) are challenges in health care today. Data from the World Health Organization (WHO) show that around 36 million people have been infected with HIV.^{1,2} HIV/AIDS has developed into one of the major and important health, and social problems in Indonesia.³ In Indonesia, the first HIV/AIDS case was found in the province of Bali in 1987. Until now, HIV/AIDS has spread in 386 districts/cities in all provinces in Indonesia. Every year, the population of Indonesia infected with HIV/AIDS is increasing.^{4,5}

HIV-infected patients are at high risk of developing opportunistic diseases related to Epstein-Barr virus (EBV).⁶ EBV, also called human herpes virus 4 (HHV-4), is the most common gamma-herpesvirus virus in

humans. About 95% of the world's population shows asymptomatic conditions for life. The virus infects through saliva or oral cavity contact, but transfusion transmission has also been reported. EBV can infect B cells and epithelial cells, and it is generally believed that epithelial cells in the oropharynx are the primary location and the location of EBV.⁷ EBV infection causes mononucleosis infection, associated with malignancy, including nasopharyngeal carcinoma (NPC), Burkitt lymphoma, and Hodgkin's lymphoma (HL). In addition, EBV is also an etiological agent of oral hairy leukoplakia (OHL).^{6,7,8,9} Contreras, et al. (2014) showed that EBV has also been detected to cause periodontal pockets. Escalona, et al. (2016) also showed an increase in periodontal disease in HIV positive patients by EBV infection. The amount of EBV in saliva and subgingival plaques has been detected to be higher in HIV positive patients than in HIV negative patients.^{10,11,12,13} OHL is a disorder of EBV-induced mucocutaneous epithelial cell and is the first pathological manifestation associated

with EBV infection, characterized by EBV replication in epithelial cells.⁵ The normal oral mucosa consists of a multilevel squamous epithelium which is divided into four distinct differentiated layers, namely the basal layer which is actively mitosis, the spinosum layer contains keratin, the granular layer and the stratum corneum containing metabolic inert cells. Basal cells express keratin K14 and K5, Bcl-2, and EGFR which maintain proliferation. EGFR is located on the basal cell surface and when binding to a ligand will affect mitogenesis and cell migration. When basal cells differentiate, EGFR is no longer detected, keratin K1 and K10 are expressed suprabasal. The expression of the anti-apoptotic molecule Bcl-2 in the basal cell layer decreases during stratification. Differentiation of epithelial cells involves anoicis, a form of apoptosis induced by loss of contact with the extracellular matrix. The granular epithelial layer contains apoptotic cells, and the stratum corneum is characterized by keratin cells of fibrils which form an extracellular barrier.¹⁴

After lytic replication in epithelial cells, the virus spreads to all lymphoid tissues and infects naive B cells through binding of glycoprotein gp350 virus to the CD21 receptor on the B cell's surface. The infected B cells trigger specific primary cytotoxic cell responses that ultimately control EBV infection. To avoid T cells, EBV decreases the expression of antigens that form a stable reservoir of the B cells' memory, where latent virus proteins are no longer expressed and the EBV genome remains in the form of EBV episomal.⁸ EBV encodes more than 85 genes. EBV will induce proliferation of B cells through EBV nuclear antigen (EBNA) expression, and two integral protein membranes, namely latent membrane protein 1 (*LMP1*) and *LMP2A*. Infected cells then enter the germinal center of the lymph nodes, where EBV gene expression is modified to avoid immunity. EBV subsequently forms latent infections in the B cells' memory.¹⁵

Accurate genetic identification is very important for epidemiological studies. Specific, sensitive, and new molecular epidemiological techniques have been developed to identify EBV genes, namely *LMP1* gene-sequence variation patterns.¹⁶ *LMP1* is the main EBV oncoprotein, its expression is needed for EBV latent infection and B cell's transformation.¹⁴ EBV infection in lymphocytes B generally occurs non-lytically and gives expression to membrane proteins and nuclear proteins. EBV encodes *LMP1* which is a multifunctional oncoprotein that is important for the proliferation and transformation of EBV-induced B cells in vitro.⁶ Detection of the *LMP1* gene is an integral membrane protein that has been detected in OHL. *LMP1* modulates the growth and differentiation of various cell types.^{14,17} This research aims at obtaining data on EBV in saliva in HIV patients with PCR microarray examination and detection of *LMP1* gene in EBV positive patients using nested PCR. This

research is important so that a strategy can be made to prevent the emergence of oral lesions related to *LMP1* genotypic expression in HIV patients.

METHODS

The sample of the research was determined using the consecutive sampling method, where 30 HIV-positive adult patients who met the research criteria at a given time period were included in the research. Application for permission to conduct the research is carried out by submitting a proposal to the Ethics Committee of the RSUP Dr. Hasan Sadikin Bandung to get the legitimacy of the research. All patients included signed a written agreement to participate in this research. PCR microarrays are used to detect EBV in saliva, while nested PCR is used to detect *LMP1* in positive EBV patients.

Salivary retrieval is done by the Navazeh method (1993), which is the spitting method. In the spitting method, the patient is instructed to collect saliva in the mouth for 60 seconds then spit it into a tube that has been prepared. This procedure is repeated for 5 minutes.¹⁸

EBV detection is done by multiplexing PCR DNA microarray.^{19,20} Primer for EBV gene sequences forward: CGCATAATGGCGGACCTAG and reverse: CAAACAAGCCCACTCCCC. Probe: 3'-AAAGATAGCAGCAGCGCAGC, 5'AACCATAGACCCGCTTCCTG. Detection of the *LMP1* EBV gene was carried out using nested PCR.²¹ Nested PCR reaction was carried out with a final volume of 25 µL for each stage and the sample DNA used in PCR stage 1 was diluted with a ratio of 1: 3 (DNA: dH2O free RNase). For stage 1 PCR, 12.5 µL GoTaq Green Master Mix 2X (Promega) is added with each 2.5 µL outer primary (Macrogen), FLMP11 (5'TGATTAGCTAAGGCATTCCCA3'), RLMPEco (5 'CCGTACTGCCTCCGGCAGAC 3'), 6.5 ul dH2O free RNase (Promega) and 1 µL sample DNA. The homogeneous mixture was then placed into Analytik Jena's Biometra thermal cycler with the first PCR program which is 1 cycle of 95°C 2 minutes initials, followed by 30 cycles of denaturation 95°C 1 minute, primary attachment 63°C 30 seconds and installation of 72°C nucleotide base (extension) 1 minute and 1 final cycle extension 72°C 5 minutes. In stage 1 negative control is included, where the DNA sample is replaced with dH2O free RNase.

Second phase PCR, as much as 12.5 µL GoTaq Green Master Mix 2X (Promega) are added with each 2.5 µL primary inside (inner primer) (Macrogen): FA2 (5 'GCCTATGACATGGTAATGCCTA 3') and RE2 (5 'CTTTCCTCAACTGCCTTGCT 3 '), 5.5 µL dH2O free RNase (Promega) and 2 µL DNA results of PCR

Table 1. Characteristics of the study participants

Variable	Total n=30
Gender	n (%)
Male	21 (70,0)
Female	9 (30,0)
Age (Year)	
Median (Min-Max)	30 (22 – 61)
Age Category	n (%)
21-30 year	14 (46,7)
31-40 year	15 (50,0)
41-50 year	-
51-60 year	-
>60 year	1 (3,3)

Table 2. Distribution of CD4 counts of study participants

Variable	Total n=30
CD4 (sel/mm³)	
Median (Min-Maks)	273 (17 – 790)
Criteria CD4	n (%)
≥ 500	6 (20,0)
350-499	5 (16,7)
200-349	7 (23,3)
< 200	12 (40)

stage 1. The mixture was homogeneous and then placed into Analytik Jena thermal cycler Biometra with PCR program stage 1 which is 1 cycle of initial 95°C 2 minutes, followed 35 cycles of denaturation 95°C 1 minute, primary attachment 68°C 30 seconds and installation of nucleotide bases (extensions) 72°C 1.5 minutes and 1 final cycle extension 72°C 7 minutes. In stage 2, the negative control DNA template was taken from the negative control PCR phase 1.

Electrophoresis results of PCR were carried out using agarose gel (Promega) concentrating 1.5% which was given 2 ul dyes of Etidium Bromide (Sigma). The agarose gel is placed into the electrophoresis tank and given TAE 1X buffer until it is flooded. Each of them has 5 ul negative control, 1 kbp DNA marker (Promega), and the sample is inserted into the well in the gel in sequence. Electrophoresis was carried out with a voltage of 75V for 25 minutes. Electrophoretic gel is placed on top of the UV laminator to visualize DNA bands obtained and recorded with digital cameras.

RESULTS

This research was conducted in HIV patients who had received ART and who had not received ART, with a CD4 range of 17-790 cells/mm³. The research subjects were patients who came to the *Poliklinik Teratai* at R.S. Hasan Sadikin Bandung. The research was conducted

Figure 3. Salivary EBV Distribution of the study participants by means multiplexing PCR DNA microarray

Figure 4 . Distribution *LMP1* gene of Research Subjects

Figure 5. Electrophoresis analysis of *LMP1* gene with nested PCR (1,500 bp) on 1.5% agarose gel. C -: negative control. LMP1 + were on lane 1,2,3,4,7,8,9,10.

Figure 6. Prevalence of OHL in study participants with HIV/AIDS

from January 2019 to February 2019. The samples obtained were 30 subjects, consisting of 13 HIV/AIDS patients who had not received ART and 17 HIV/AIDS patients who had received ART.

From the distribution of research subjects by sex, 21 (70%) men and 9 (30%) women were obtained. From the distribution of research subjects based on age, the highest results obtained in the age group of 31-40 years

were 15 subjects (50%) and the lowest results is in the age group of 40-49 years and there is ≥ 60 years as many as 1 person (3.3%). Distribution of HIV/AIDS patients based on sex and age can be seen in Table 1.

From the distribution of research subjects based on CD4 counts, the highest results were 12 subjects (40%) who had CD4 counts of <200 cells/mm³ and the lowest were 5 subjects (16.7%) who had CD4 counts of 350-499 cells/mm³. Distribution of CD4 counts can be seen in Table 2.

Microarray PCR showed that of the 30 HIV/AIDS patients, there were 26 subjects (87%) with positive EBV and 4 subjects with negative EBV (13%). As many as 13 subjects (38.24%) with EBV positive were receiving ART and 15 subjects (47.05%) without ART. This can be seen in Figure 3.

The subjects of HIV / AIDS patients with positive EBV who had *LMPI* gene expression were 17 patients, as shown in Figure 4. The results of electrophoresis analysis of 1.5% *LMPI* gene agarose gel with nested PCR (1,500 bp) can be seen in Figure 5.

Of the 26 patients with positive EBV, only 5 subjects (16.7%) had oral OHL lesions. OHL distribution of study subjects can be seen in Figure 6.

DISCUSSION

Based on the characteristics of the research subjects, this research consisted of 70.58% males and 29.42% females, with the highest age range of 30-39 years at 53.3%. The cumulative number of HIV infections reported up to June 2018 was 301,959 people and most were found in the age group of 20-24 years and 25-49 years. The highest immunosuppression rates from CD4 counts in this research were found in the range of <200 cells/mm³ as many as 12 patients. CD4 cell count below 200 cells/mm³ is one of the predisposing factors for the emergence of oral lesions in HIV/AIDS patients. People with OHL generally have a moderate to severe level of immunity suppression with an average CD4 count of about 235 cells/mm³.²² The number of patients with positive EBV in this research was 87%. These results are close to the percentage in previous studies that showed the serological prevalence of EBV in adult humans worldwide, estimated at around 95%.^{5,6} According to Wolf, et al. (1984) EBV is present in nasopharyngeal epithelial cells and salivary glands. This shows the persistent location of the virus with virus particles released at saliva.¹⁶

EBV expression of *LMPI* and integral membrane protein have been detected in OHL. *LMPI* will alter cell function by inducing cellular gene expression and in epithelial cells by inducing EGFR expression.

LMPI also induces the expression of antiapoptotic A20 molecules by activating NF- κ B in epithelial cells and lymphocytes. In normal epithelial cells, EGFR expression is localized in the basal layer, whereas in OHL high EGFR expression levels are detected in suprabasal cells in the stratum spinosum. In normal tissue, basal cells lose their proliferative ability when migrating upwards into spinosum cells. Epidermal growth factor receptor (EGFR) activates the pathway of mitogen-activated protein kinase (MAPK) and provides a stimulus for cell growth. Suprabasal EGFR detection on OHL shows that spinosum cells retain the ability to proliferate resulting in thickening of the spinosum cell layer which is one of the characteristics of OHL.^{14,17}

Some studies show that OHL is determined by the expression of several genetic viruses that cause infection and subsequent cell transformation, which contribute to pathogenesis. The spinosa OHL cell layer shows latent and lytic virus proteins that play an important role in the development of lesions.²³ Previously, it has been shown that *EBERs*, a sign of latent infection, are not expressed in OHL. The characteristics of latent infection of *EBNA2* and *LMPI* have been detected in OHL in the stratum spinosum.²⁴ The description of OHL microscopic characteristics is epithelial hyperplasia, acanthosis, hyperkeratosis, and balloon cells in the stratum spinosum, but with little or no inflammatory cell infiltration in lamina propria. Hypha candida is generally absent, but if present indicates a secondary fungal infection.²⁵

OHL is a relatively common condition in HIV positive patients associated with EBV infection. Accurate diagnosis is important because it can be a marker of immunodeficiency and early indicators of HIV infection that are undiagnosed.^{22,23} Factors that play a role in the emergence of OHL lesions by EBV are immune system dysfunction and the absence of Langerhans cells that have been observed in OHL. EBV has been clinically observed in the epithelium of HIV seropositive patients who can precede the onset of OHL. OHL oral lesions in this research are the same as in previous studies. It is in the form of white plaque, asymptomatic, choppy, painless, not scraped, and located on the lateral tongue, both unilateral or bilateral, with a wavy or smooth surface.⁵ OHL is often associated with HIV infection, commonly occurring in 50% of untreated HIV patients, especially on CD4 less than $0.3 \times 10^9 / L$ (normal $0.3-1.4 \times 10^9 / L$).²⁶ There is a positive correlation between OHL prevalence and a decrease in the number of CD4 T cells (12%).²⁷

The previous research conducted by González, et al. (2010) showed the same results, namely the expression of a high *LMPI* gene (84.6%) in patients with clinical features of OHL, and research conducted by Walling, et al. (2004) showed the *LMPI* gene detected in 12 tongue specimens with OHL (100%).^{23,28} According to Dawson, et al. (2003), *LMPI* is the main EBV protein involved

in molecular induction of cell surface adhesion and antigen activation, regulation of anti-apoptotic proteins (Bcl-2, A20), and stimulation of cytokine production (interleukin-6 and interleukin-8). *LMP1* is expressed during EBV lytic infection, and oral OHL lesions in patients have high levels of *LMP1* expression.¹⁴

This research showed that 3 patients had CD4 of <200 cells/mm³ and 2 patients had CD4 of 200-349 cells/mm³. This is consistent with the research conducted by Bravo, et al. (2006). OHL is often associated with HIV infection and a high CD4 cell count indicates a decrease in the number of clinically opportunistic oral lesions. Generally, OHL occurs in 50% of untreated HIV patients, especially with CD4 less than 0.3×10⁹/L (normally 0.3–1.4×10⁹/L).⁽²⁶⁾ Based on the average (median), CD4 count of HIV-AIDS patients with oral OHL lesions of 136 cells/mm³ is lower than patients who did not have oral OHL lesions, which is 260 cells/mm³. Research conducted by Kokila, et al. (2014) showed that patients with OHL generally have moderate to severe immunity suppression, with an average CD4 count of 235 cells/mm³.²⁹

CONCLUSION

The high prevalence of EBV in saliva and LMP1 gene are the risk factors for OHL. Early screening for EBV infection in patients with HIV/AIDS is important to prevent or reduce the risk of diseases caused by EBV viruses such as OHL.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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