

Original Research Article

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Random Amplified Polymorphic DNA (RAPD) Analysis of *Human papilloma virus* Isolated from Cervical Cancer Patient

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ABSTRACT

Cervical cancer prevalence is increasing every year. The major cause of this cervical cancer is the infection of high-risk *Human papillomavirus* (HPV) types. HPV variation is related to its ethnicity and geographical source. The objective of this study is to analyze HPV genetic diversity from the two Sumatran regions, Pekanbaru and Padang. Twenty DNA samples from cervical cancer patients were used in this study. HPV detection was carried out using MY09/11 primer. Positive HPV samples were amplified with OPO-6, OPO-18 and OPO-19 primer, and the dendrogram was constructed with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. HPV was detected in fourteen DNA samples. PCR amplification by OPO primer showed multiple bands. The dendrogram results of the three primers, DNA samples divided into two groups X and Y. Based on the dendrogram, HPV classified on genotypes and source origin. RAPD data describe HPV variance, but still need further analysis for comprehensive HPV diversity.

Keywords

Genetic relationship, Diversity, PCR, Dendrogram, HPV 16, HPV 18

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Introduction

The most common cancer among women in the world is breast cancer and followed by

cervical cancer. There were an estimated 570,000 new cervical cancer cases and 311,0000 deaths worldwide in 2018 (Bray *et al.*, 2018). Almost 90% of cervical cancer

deaths occurred in developing parts of the world and 144.400 (54%) of cervical cancer cases occurred in Asia (Torre, *et al.*, 2015). In Indonesia, cervical cancer is the second most frequent cancer disease among female between 15 and 44 years old. In 2012-2013 cervical cancer ranks first in the most caused of women death with 98.692 cases. Current estimates in 2017 indicate that every year 20.928 of 93.15 million women population are diagnosed with cervical cancer with the mortality rate of 9.498 (ICO, 2017).

The main cause of cervical cancer is *Human papillomavirus* infection. HPV is sexually transmitted by a person who already infected with this virus (Dunne and Markowitz, 2006). Epidemiology study shows that the main agent in cervical cancer is HPV is type 16, 18, 31, and 45 which includes the High-Risk types of HPV. Epidemiology also proved that HPV type 16 and 18 are the major course of cervical cancer (Serrano *et al.*, 2018).

Our previous study has been reported research with identification and isolation of HPVs from cervical cancer patients in Dr M. Djamil Padang Hospital and Achmad Arifin Pekanbaru hospital. HPV type 16, 18, 45, and 52 were found with percentages of 28.5%, 40.4%, 7.1%, and 2.3% in the 42 samples (54%) of 72 samples that obtained from Formalin-fixed paraffin-embedded (FFPE), patient cervical smears, and patients cervical cancer fresh tissues.

The study shows that HPV type 18 is the most dominant type compared to the other three types of the virus (Marlina *et al.*, 2016). The HPV studies then continued about its protein that causes the highest risk against cervical cancer which are E5, E6, and E7.

A study by Sopiанти *et al.*, (2016) showed that 9 out of 15 samples (60%) are detected as E5 gene HPV type 18 and there is no molecular

variation in its sequence with 100% homologous equations.

The variation HPV genetic has a minor correlation with geographical source and ethnic (Heinzel *et al.*, 1995). Variant and HPV subtypes are having an important role as a guide to understand HPV evolution and to know its genomic intermediate which related to the difference of each type. DNA sequence variation in epidemiology study is used as a marker to track the virus deployment in population and to see the relation of its genomic and phenotypic diversity, as the biologically or pathologically difference for example. Research about DNA is also given a chance to know about the relation of genomic diversity with disease severity (Wuriningtyas, *et al.*, 2014).

One of the approaches to know about genomic diversity is by using *Random Amplified Polymorphic DNA* (RAPD) method, which is a modification of the *Polymerase Chain Reaction* (PCR) method. RAPD method is more simple than the conventional or nested PCR method and there is no need for sequencing step (Welsh and McClelland, 1990). The objective of this study is to see the level of HPV genomic diversity between samples from two Sumatran regions, Padang and Pekanbaru, by constructing its dendrogram.

Materials and Methods

Isolation of *Human papillomavirus* DNA

HPV DNA which uses in this study carried out as our previous study (Marlina, 2016). Cervical biopsy from Arifin Ahmad Hospital Pekanbaru and M. Djamil Hospital, Padang was isolated using DNA Extraction Kit gSYNCTM. Isolation of DNA was done according to the protocol of the kit being used.

HPV identification and genotyping

Identification HPV had done by using consensus primer MY09/11 primer (forward 5'-CGT CCM ARR GGA WAC TGA TC-3' and reverse 5'-GCM CAG GGW CAT AAY AAT GG-3') with 450bp of product length (Erhart *et al.*, 2016), while HPV genotyping had done using specific primer for HPV 16 (forward 5'GTC AAA AGC TGT GTC CT-3' and reverse primer 5' CCA TCC ATT ACA TCC CGT AC-3') and HPV 18 (forward primer 5'TCG TTT TCT TCC TCT GAG TCG CCT-3' and reverse primer 5'CCG AGG ACG ACA GGA ACG ACT-3') (Marlina, 2016).

PCR cocktail were prepared for 25 μ L volume, which consist of 2 μ L isolate sample, 12,5 μ L 10X PCR buffer (100 mmol l-1 Tris-HCl, 35 mmol l-1 MgCl₂, 750 mmol l-1 KCl, pH 8,8), 2,5 μ L dNTPs (Promega, Madison, USA), 1 μ L forward primer, 1 μ L reverse primer, 0,2 μ L Taq DNA polymerase (Promega, Madison, USA), 17,8 μ L sterile distilled water. The PCRs were conducted using the Mastercycle Personal® (Eppendorf, Germany) with PCR cycling conditions: hot start 94⁰C for 2 min, 40 cycles of denaturation 94⁰C for 30 sec, annealing 50⁰C for 1 min, extension 72⁰C for 1 min, and final extension 72⁰C for 10 min.

The PCR products were electrophoresed using a 1.5% agarose gel in 1 \times TBE buffer, stained with ethidium bromide (0.5 g/ml) and photographed used gel documentation system transilluminator UV (Alpha Innotech®). A low DNA mass ladder was used as a base-pair molecular weight pattern (DNA ladder 100 bp Thermo scientific®)

RAPD-PCR parameters

Three arbitrary primers from Operon Technologies Inc. (Alameda, CA, USA) were

used for amplification of random DNA markers to reveal the genetic diversity among *Human papillomavirus* populations. Details of the primers used in the present study are given in Table 1. The DNA was amplified through RAPD according to Williams *et al.*, (1990). Approximately 2 μ L of genomic DNA of HPV was used. The RAPD reaction was performed in a Mastercycle Personal® (Eppendorf, Germany) for 1 cycle at 96⁰C for 5 min, followed by 40 cycles of 94⁰C for 1 min, 36⁰C for 1 min and 72⁰C for 2 min, respectively. The final amplification cycle included 7 min extension at 72⁰C.

Separations for band fragments as the result of amplification were made with horizontal electrophoresis with agarose gel 1.5% in Tris-Boric acid EDTA (TBE) solution at 68 volts until the DNA migration reached up to 2/3 of the length of the gel. The gel then stained with ethidium bromide (EtBr). Gene ruler 1 kb ladder (Thermo scientific®) was used as a standard to determine the size of DNA fragments.

RAPD data analysis

Gel documentation acquired was analysed using BioNumerics version 6.6 software (Applied Maths, Kortrijk, Belgium), for cluster analysis via Unweighted Pair Group Mathematical Averaging (UPGMA) and Dice Coefficient. Dendrograms generated from different DNA fingerprinting methods were evaluated as single and as composite dendrograms, to find the most meaningful clustering.

Results and Discussion

A total of 20 DNA samples was carried from our previous study was used for RAPD Analysis (Marlina *et al.*, 2016). The most common molecular diagnosis for HPV is using MY09/11 or GP5+/6+ primer. In this

study, we use MY09/11 primer for detection of HPV existence in our samples. HPV DNA was identified in 14 samples (70%). The positive result determined based on the 450 bp band appear on the agarose gel, shown in Figure 1. The MA.114.05, MA.092.15, and MA.093. 17 samples were negative because of no band on the gel.

Compared with our previous study, all of the selected samples was positive HPV by using GP+/5+ primer (Marlina *et al.*, 2016). A study of HPV detection using primers MY09/MY11 and GP5+/GP6+ in also cervical patients by Venceslau *et al.*, (2014) observed a greater number of positive samples using GP5+/GP6+ primer when compared to MY09/ MY11. From 27 samples, 30% were positive for HPV. HPV DNA was found in 15% and 30%, using MY and GP pair of primers, respectively. From these, 15% were detected by both methods, and 30% only by GP pair.

Another study by Shikova *et al.*, (2009) examined 54 cervical specimens obtained from cytologically normal women and women with low- and high-grade lesions of the cervix for the presence of HPV DNA by PCR using consensus and type-specific primers to determine if HPV detection varies when different primer sets are used. Both MY09/MY11 and GP5+/GP6+ consensus primer sets, exhibited approximately equal sensitivity, as defined by the ability to detect HPV DNA. 27.8% and 25.9% of samples were HPV positive in PCR using MY09/MY11 primers and GP5+/GP6+ primers, respectively. GP5+/GP6+ primer failed to detect HPV DNA in 1 sample found HPV positive by MY09/MY11.

In a study by Qu *et al.*, (1997) about the comparison between MY09/MY11 and GP5+/GP6+ in PCR detection of HPV, 280 samples were evaluated in the and HPV DNA

was detected in 45.2% and 42.8% of the samples by using the MY pair and the GP pair sets, respectively. These results indicate that although the MY and GP pairs identified nearly equivalent prevalence of HPV in a set of clinical samples, differences in the detection of specific types and infections with multiple types were found. Differences in the sensitivities and characteristics of the PCR systems for the detection of HPV within clinical samples should be considered when comparing data between studies and/or in designing new studies or clinical trials.

GP5+/6+ and MY09/MY11 primers able to amplify a wide spectrum of HPV genotypes in cervical cancer patients, it is possible that some variant of HPV types in the samples are might not be generally detected. Both primers have each other's strengths. The GP5+/6+ primer set was better at detecting low viral copy numbers, while the MY09/11 primer set was better at detecting co-infections. The use of a single primer set might lead to underestimating HPV infection rates (Camargo *et al.*, 2011).

HPV genotype prevalence in this study is shown in Table 2. HPV 18 was the most common genotype detected 6 (43%), followed by HPV 16 (36%). Multiple infection HPV 16 and HPV 18 observed in MA.060.10 and MA.054.14 sample. A study in Shandong Province, China reported 62.8% samples to have single infections and others get multiple with two or more genotype infection (Jiang *et al.*, 2019). Multiple high-risk HPV infection also was observed in 10% specimen at Apulia, South Italy Region (Coscia *et al.*, 2015).

A study among women with normal and abnormal cervical cytology presenting in a tertiary gynaecology referral clinic in Ethiopia declare the most frequent genotypes identified were HPV16 (44.1%), followed by HPV35

and HPV45 (each 6.2%), HPV31 (4.4%), 3.1%), HPV58 (2.5%) and HPV39 (1.9%) HPV56 (3.7%), HPV18 and HPV59 (each (Wolday *et al.*, 2018).

Table.1 RAPD primer name and sequence

| No | Locus name | Primer Sequence (5'-3') | %GC | Tm (°C) |
|----|------------|-------------------------|-----|---------|
| 1 | OPO-06 | CCACGGGAAG | 70 | 46 |
| 2 | OPO-18 | CTCGCTATCC | 60 | 40 |
| 3 | OPO-19 | GGTGCACGTT | 60 | 46 |

Table.2 HPV DNA Identification and Genotyping Result

| No. | Sample ID | Source | Origin | HPV Identification | HPV Type |
|-----|-----------|-----------------|-----------|--------------------|----------|
| 1 | MA.109.01 | Cervical Biopsy | Pekanbaru | + | 16 |
| 2 | MA.107.02 | Cervical Biopsy | Pekanbaru | + | 16 |
| 3 | MA.106.03 | Cervical Biopsy | Pekanbaru | + | N/A |
| 4 | MA.105.04 | Cervical Biopsy | Pekanbaru | + | 16 |
| 5 | MA.104.05 | Cervical Biopsy | Padang | - | - |
| 6 | MA.103.06 | Cervical Biopsy | Pekanbaru | + | 18 |
| 7 | MA.102.07 | Cervical Biopsy | Pekanbaru | + | 18 |
| 8 | MA.096.08 | Cervical Biopsy | Pekanbaru | + | 18 |
| 9 | MA.108.09 | Cervical Biopsy | Pekanbaru | - | - |
| 10 | MA.060.10 | Cervical Biopsy | Padang | + | 16, 18 |
| 11 | MA.058.11 | Cervical Biopsy | Padang | + | 16 |
| 12 | MA.056.12 | Cervical Biopsy | Padang | + | 18 |
| 13 | MA.055.13 | Cervical Biopsy | Padang | + | 18 |
| 14 | MA.054.14 | Cervical Biopsy | Padang | + | 16, 18 |
| 15 | MA.092.15 | Cervical Biopsy | Padang | - | - |
| 16 | MA.052.16 | Cervical Biopsy | Padang | + | 18 |
| 17 | MA.093.17 | Cervical Biopsy | Pekanbaru | - | - |
| 18 | MA.091.18 | Cervical Biopsy | Padang | + | 16 |
| 19 | MA.094.19 | Cervical Biopsy | Padang | - | - |
| 20 | MA.095.20 | Cervical Biopsy | Pekanbaru | - | - |

Figure.1 The amplified DNA of HPV using primer MY09/11 (Lane L: 100 bp DNA marker, Lane 1-15 DNA sample (1: MA.109.01, 2: MA.107.02, 3: MA.106.03, 4: MA.105.04, 5: MA.114.05, 6: MA.103.06, 7: MA.084.09, 8: MA.084.09, 9: MA.060.10, 10: MA.058.11, 11: MA.056.12, 12: MA.055.13, 13: MA.092.15, 14: MA.052.16, 15: MA.093.17))

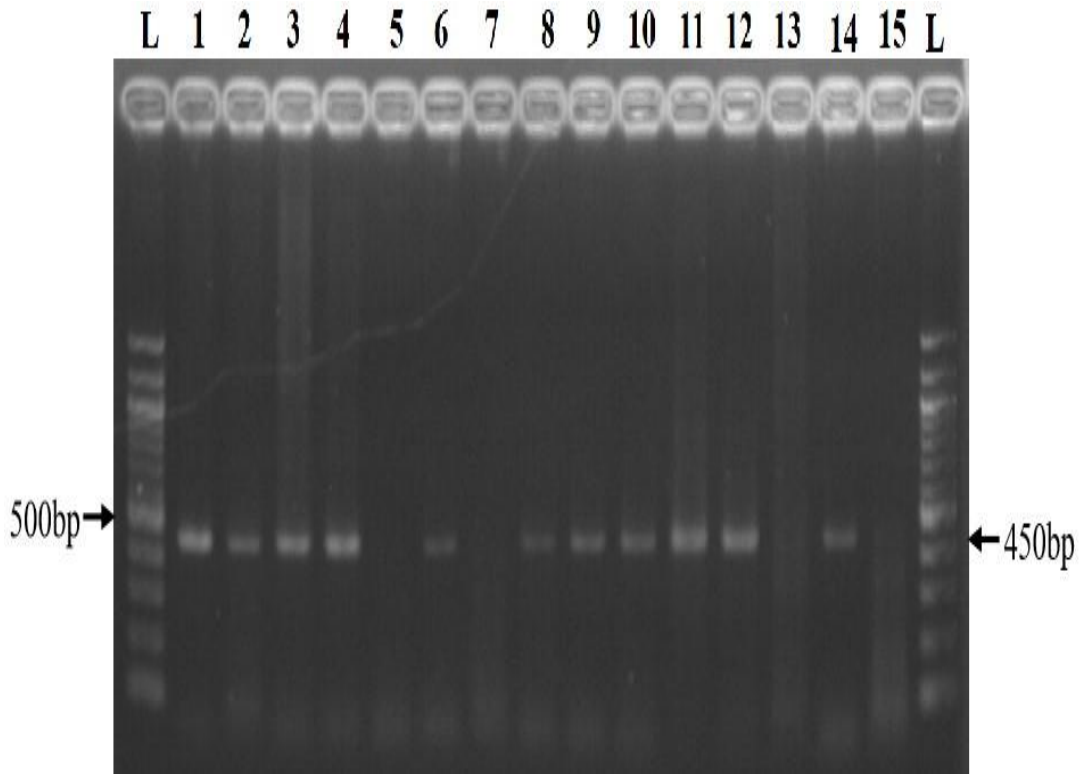


Figure.2 RAPD Fingerprinting of *Human papillomavirus* Strains. The gel image strengthened by BioNumerics version 6.6 software. (A) OPO-06 primer; (B) OPO-18 primer; (C) OPO-19 primer

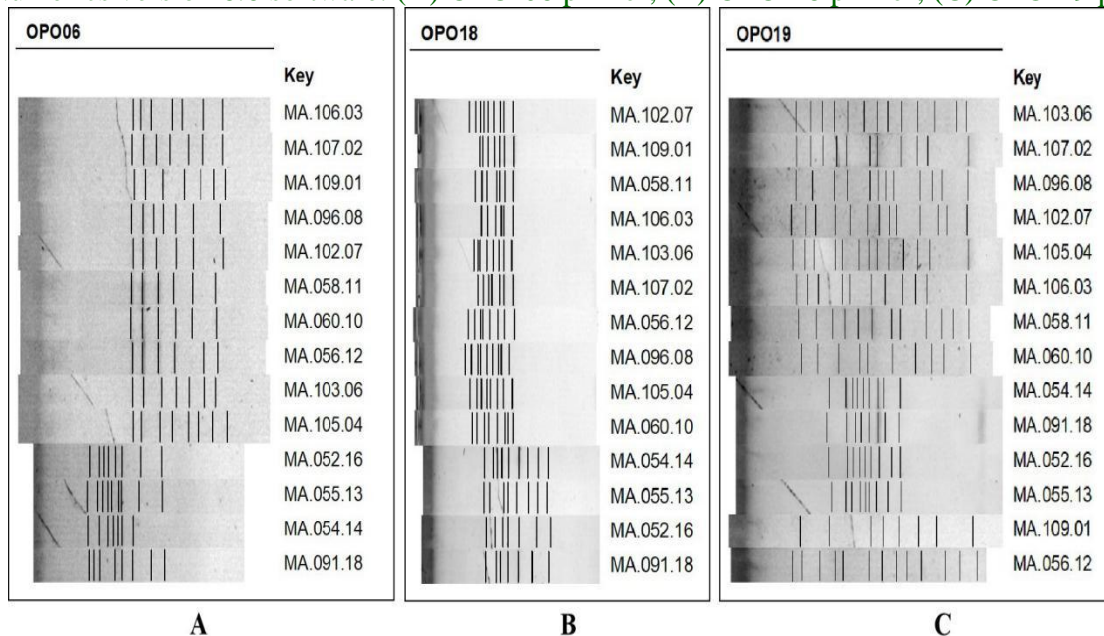
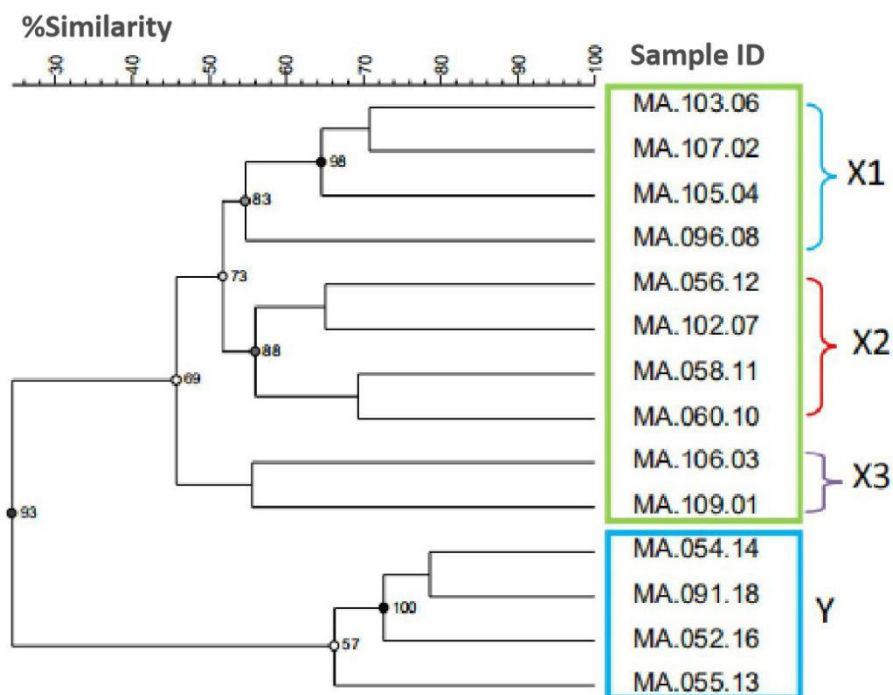


Figure.3 Representative dendrogram showing similarity of 14 *Human papillomavirus* strains based on the combination RAPD-PCR gel documentation using UPGMA method



Different result reported by Wang (2018) study in invasive cervical cancer, cervical intraepithelial neoplasia and asymptomatic women in Southeast China. This study reveals HPV 18 is the most prevalent genotype among in the positive cases.

In another study conducted in Ghana, HPV genotype distribution not only discovered high-risk HPV but also low-risk HPV. There is eleven genotype detect in this study, 9 high-risk HPV; HPV-16 (8%), HPV-35 (5%), HPV-33/39/-68 (3%), HPV-52/51/59 (2%) and HPV-18 (1%) and 2 Low-risk types; HPV-42(3%), and HPV-43 (1%) (Adams *et al.*, 2019). HPV genotypes distribution differs between regions. This difference causes the need for different prevention and therapeutic efforts for each geographic area.

PCR-RAPD amplification of the samples in this study with OPO-6 primers obtained 6-8 fragments, with OPO-18 primers obtained 6-9 fragments, and OPO-19 produces more

amplification results than other primers namely 8-13 bands (shown in Figure 2). The size of the bands produced by each primer varies from 250-1500 bp. HPV genetic variation observed from the difference in the number of DNA fragments amplified in each primer.

The result of HPV samples dendrogram using OPO-6, OPO-18, and OPO-19 primer data shown in Figure 3. Based on the dendrogram, samples were divided into two large groups of X and Y cluster. In the dendrogram which can be seen in cluster X were divided into three subgroups named X1, X2, and X3. It can be seen from the dendrogram that cluster X1, X2 came from Pekanbaru and cluster Y came from Padang, while at cluster X3 came from Padang and Pekanbaru. These suggest that the genetic diversity of HPV is related to geographical regions. According to Heinzl, *et al.*, (1995) that there is a minor correlation between HPV variants with geographic and ethnic origin but it is uncertain whether the

spread of viral variants occurred during the evolutionary process or related to human ethnic mobility worldwide.

HPV distribution in the world is different. HPV-16 variations can be classified based on phylogenetic and distributed across five continents and in different ethnic groups. These variants include European, Asian, Asian-American, which also North American variants, type 1 African and type 2 African (Terai *et al.*, 2005). For HPV-18, different variants have been defined as Asian-American, African, and European variants (Chen *et al.*, 2009).

De Boer, *et al.*, (2004) used the samples of HPV-16 patients with cervical cancer from the Indonesian population, Suriname, and the Netherlands. In Indonesia, the variant occurs at the L1, E7, and E6 regions, and this variant called as Java or Javanese variant because it came from Javanese ethnic. This Javanese variant was found to be positive in the three Suriname samples, this could be due to the migration of the Javanese population to Surinam. In a study conducted by Fadhillah, *et al.*, (2015) showed that HPV type 16, isolated from cervical cancer patients from Bandung which located in one subgroup with HPV from Asia and East Asia, and no Javanese variants were found in the isolates. Thus, ethnic differences, as well as the presence of different genotypes of HPV genes in the Padang and Pekanbaru regions, resulted in differences of HPV genetic sequences.

The dendrogram showed that RAPD cannot be used to distinguish the type of *Human papillomavirus*. As in cluster X1, there are two HPV types 18 and two HPV type 16. RAPD used the entire genome for its analysis while HPV is classified as a molecular variant if it has more than 98% similarity at the L1 sequence of prototype genes (Bernard *et al.*, 2010) and the identification of new HPV

genotypes should have a complete isolation genome, indicating 10% of the diversity of nucleotide sequences in L1 genes isolated from all known genotypes (Villers *et al.*, 2004).

Based on the results of this study, it can be concluded that HPV variation can be influenced by geographical regions. Furthermore, it is desirable to research the molecular variation of HPV using another method, so that it can be used to control HPV infection.

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