

Research Article

Human Papilloma Virus L1 Gene Methylation as a Potential Biomarker for Precancerous Cervical Lesion: a Preliminary Report**Metilasi Gen L1 Human Papilloma Virus sebagai Biomarker Potensial untuk Lesi Serviks Prekanker: Sebuah Laporan Pendahuluan**Suzanna P Mongan¹, Andrijono², Hartono Tjahadi³¹*Division of Gynecology Oncology, Department of Obstetrics and Gynecology
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Prof. Dr. R.D. Kandou General Hospital Manado*²*Division of Gynecology Oncology, Department of Obstetrics and Gynecology*³*Department of Pathology Anatomy**Faculty of Medicine Universitas Indonesia/**Dr. Cipto Mangunkusumo National General Hospital Jakarta***Abstract**

Objective: To determine whether HPV L1 gene methylation can be used in triage of precancerous cervical lesions. The main objective is to determine the genotype of HPV in cervical precancerous lesions and to determine the percentage, the sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio of DNA HPV L1 methylation in precancerous cervical lesions.

Methods: A number of 57 samples of paraffin blocks (FFPE) from precancerous lesions and cervical cancer biopsies in the Department of Pathology Faculty of Medicine-Cipto Mangunkusumo General Hospital that had been re-evaluated by the pathologist, underwent extraction of HPV DNA. The genotypes of HPV DNA were examined using primers GP5 / 6 and specific HPV 16, HPV 18 and HPV 52 probes and analyzed by real time PCR. Sequencing was performed on samples with unknown HPV DNA type that were detected using the specific probes to determine the type of HPV. Bisulfite conversion procedure was then performed for the samples that met the inclusion criteria.

Results: There were 30 samples (52.6%) with CIN 1, 12 samples (21.1%) CIN 2, 9 samples (15.8%) CIN 3 and 6 samples (10.5%) of cervical cancer. Most of the samples were 36-45 years (35.1%). Of the total 57 samples, 55 samples were successfully extracted and determined the DNA genotyping of HPV (96.5%). HPV 16 infections both in the form of single or multiple was found to be 76.36%. The samples were mostly dominated by co-infection of HPV16 and 18 (49.1%) followed by HPV 16 (24.6%) and HPV 18 (14.0%). Based on the sequencing results there were other types of high risk HPV infection found: HPV 33, HPV 35, HPV 58 and also undeterminate risk HPV 53 and low risk HPV 54. After several procedures of optimization for methylation examination of HPV DNA L1 there was thin band found in electrophoresis procedure in 8 of 42 samples (19%) of HPV 16 after bisulfite conversion but once it was purified there weren't any band found so we can not proceed to the stage sequencing. Until now we are still in the stage of optimizing the methylation procedure.

Conclusion: HPV 16 infection were most commonly found in the form of single or multiple. Co-infection of HPV 16 and 18 were found in the majority of the samples. There were no significant correlation between HPV type and the severity of cervical lesions. Until now, the examination of DNA methylation HPV L1 already obtained eight samples of HPV 16 with a thin band on electrophoresis but the result could not be concluded because it is still in the process of optimization.

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Keywords: HPV DNA genotype, L1 gene methylation, precancerous cervical lesions

Abstrak

Tujuan: Untuk mengetahui apakah metilasi HPV DNA dapat digunakan dalam triage lesi prakanker serviks. Tujuan khususnya adalah untuk mengetahui genotipe HPV pada lesi prakanker serviks dan untuk mengetahui persentase, sensitivitas, spesifisitas, nilai duga positif, nilai duga negatif, dan likelihood ratio metilasi HPV DNA L1 pada lesi prakanker serviks.

Metode: Sejumlah 57 sampel paraffin block (FFPE) hasil biopsi lesi prakanker dan kanker serviks di Departemen Patologi Anatomi FKUI-RSCM dilakukan ekstraksi DNA HPV. Dilanjutkan dengan pemeriksaan genotipe DNA HPV menggunakan primer GP5/6 dan probe khusus HPV 16, HPV 18 dan HPV 52 yang kemudian dianalisis dengan real time PCR. Dilakukan sequencing pada sampel dengan DNA HPV yang tidak dikenal dengan probe spesifik untuk mengetahui tipe HPV. Selanjutnya dilakukan prosedur konversi bisulfit untuk sampel yang memenuhi kriteria inklusi.

Hasil: Terdapat 30 sampel (52,6%) NIS 1, 12 sampel (21,1%) NIS 2, 9 sampel (15,8%) NIS 3 dan 6 sampel (10,5%) kanker serviks. Kelompok usia terbanyak adalah 36-45 tahun (35,1%). Dari total 57 sampel terdapat 55 sampel yang berhasil diekstraksi dan diperiksa genotyping DNA HPV (96,5%). Infeksi HPV 16 baik dalam bentuk tunggal maupun multipel ditemukan sebesar 76,36%. Genotipe HPV yang terbanyak ditemukan adalah ko-infeksi HPV tipe 16 & 18 (49,1%) diikuti dengan tipe 16 (24,6%) dan tipe 18 (14,0%). Berdasarkan hasil sequencing ditemukan infeksi high risk HPV 33, HPV 35, HPV 58, undeterminate risk HPV 53 dan low risk HPV 54 pada masing-masing 1 sampel. Setelah dilakukan beberapa prosedur tahap aoptimasi untuk pemeriksaan metilasi HPV DNA L1, didapatkan band yang tipis pada 8 dari 42 sampel (19%) HPV 16 hasil konversi bisulfit tetapi setelah dipurifikasi masih belum didapatkan adanya band pada proses elektro foresis sehingga belum dapat dilanjutkan ke tahap sequencing. Saat ini masih dilakukan proses optimasi untuk pemeriksaan metilasi HPV DNA L1.

Kesimpulan: Genotipe DNA HPV yang paling banyak ditemukan baik dalam bentuk tunggal maupun multipel adalah HPV 16. Infeksi multipel (ko-infeksi) HPV 16 dan 18 ditemukan pada sebagian besar sampel penelitian. Tidak didapatkan hubungan bermakna antara tipe HPV dan derajat beratnya lesi serviks. Hingga saat ini pada pemeriksaan metilasi HPV DNA L1 sudah didapatkan 8 sampel HPV 16 dengan band yang tipis pada elektro foresis namun belum dapat disimpulkan karena masih dalam proses optimasi.

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Kata kunci: genotipe DNA HPV, lesi prakanker serviks, metilasi gen L1

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INTRODUCTION

Cervical cancer is still a burden for women around the world. It is the third most common cancer among, with an estimated 528,000 new cases and 35,673 deaths in 2012. Worldwide, cervical cancer mortality rates are substantially lower than the incidence of mortality. The incidence ratio was 50.3%. Most cases are squamous cell carcinoma, followed by adenocarcinoma.^{1,2}

In Indonesia, cervical cancer ranks as the second most common female cancer. There are approximately 20,928 new cases of cervical cancer annually in Indonesia.^{1,2} Cervical cancer is about 75% of gynecological cancer in Indonesia. It is mostly diagnosed at later stages.³ Throughout 2005-2010 at Dr. Cipto Mangunkusumo National General Hospital (RSCM), there were 2,298 cervical cancer cases and 66.4% were diagnosed at an advanced stage, generally stadium IIIB.⁴ In 2013, according to a report INASGO there were 823 new cases in RSCM.⁵

Human papilloma virus (HPV) which derived from the genus Alphapapillomavirus is the most frequent cause of sexually transmitted infection most frequently worldwide with a broad spectrum of benign and malignant neoplasms.⁶ HPV is the cause of 99.7% cervical cancer.⁷

Cervical cancer, precancer lesion and non-neoplastic HPV infection such as *atypical cells of undetermined significance* (ASCUS) and cervical intraepithelial neoplasia (CIN) are diagnosed using cytology examination (Papanicolaou test, Pap test), colposcopy and histology examination of tissue biopsy. Methods of screening with cytology (Pap smear) has sensitivity (51-86%) with a high false negative rate (15-45%).⁸ Cytological examination can not predict which ones are at risk of becoming malignant, and which are not.

High Risk-HPV DNA detection has become a powerful criteria to improve the sensitivity of screening for cervical cancer. HR-HPV DNA test has a high sensitivity (88-98%) higher than Pap smear (51-86%), yet with lower specificity (83-94%) compared to Pap smear (92-99%).¹¹ However, since most women who are infected with HPV during their lifetime (> 80%) far exceeded the incidence of cervical cancer (1%) and because the value of HPV DNA testing positive more often indicate a transient infection rather than a development of cervical cancer, the diagnosis of

HPV DNA alone is not enough to distinguish benign infections that require intensive treatment. Although more sensitive than cytology, specificity and positive predictive value (PPV) HR-HPV DNA were low compared with cytology. Combined Pap and HPV testing has a sensitivity and specificity values higher with a very high negative predictive value (99-100%) to strengthen the screening of cervical cancer.⁸ But the positive predictive value for detection of precancerous lesions is still low and can not differentiate HPV infections will regress with who will experience progression to cancer.

Treatment for abnormal screening tests includes colposcopy vs close-surveillance. However, HPV DNA positive specificity is not sufficient to distinguish precancerous lesions associated with HPV (CIN2 +) and the transients are clinically a benign infection. Colposcopy referral rates were found to be higher after skrining.⁹ An unneeded colposcopy examination, can lead to over diagnosis CIN (misclassification) and over treatment. Therefore, a diagnostic test that can differentiate between women who are infected with HR-HPV is transient with risk of progression is necessary.¹⁰⁻¹²

In preventing excessive procedures in patients with abnormal Pap smears who are not at risk for cervical cancer, tests that are sensitive and specific in detecting high-risk patients are required.^{6,13-15}

HPV DNA methylation has emerged as a promising biomarker for diagnosis and prognosis of cervical cancer. Some studies show that women with HPV infection, methylation levels increased slowly with increasing duration of persistence of HPV and more increased with the diagnosis of cervical cancer. There is an increasing trend of HPV DNA methylation, especially in L1 and L2 genes in conjunction with the progressivity disease. DNA methylation changes the transcriptional regulation of HPV because of the repression of the expression of cellular genes and viruses may occur with the addition of a methyl group to the residue cytosin. The occurrence of HPV DNA methylation, especially in the L1 and L2 genes will prevent the transcription L1 and L2 with the result of no expression of L1 and L2 which are the components of HPV capsid. The absence of capsid protein L1, as antigen that can be fully accessible during the early stages of lesions intraepithelial squamous lead to circumstances in which the virus can evade the immune system thus the infection will be

retained causing dysplasia which is not recognized by the immune system, then it will proceed further on the transformation malignant.^{6,13,14,16-19}

This opens up the possibility to predict which woman has the chance of disease progression into cervical cancer. Thus, HPV DNA methylation can be considered as a potential biomarker in the triage of patients with cervical cancer risk.

This study is conducted to determine whether HPV DNA methylation can be used in triage of cervical precancerous lesions or not. The main objective is to determine the genotype of HPV in cervical precancerous lesions and to determine the percentage, the sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio of HPV L1 gene methylation in precancerous cervical lesions. By knowing the correlation of HPV L1 gene methylation in precancerous cervical lesions, it is expected that this examination can be a triage tool to help identify patients with HPV positive who would undergo regression and those at risk of progression into cervical cancer.

METHODS

A cross sectional study design was used to determine the genotype of HPV, assess the sensitivity, specificity, positive predictive value, negative predictive value, and the like hood ratio of HPV DNA L1 methylation in precancerous lesions of the cervix.

The study was conducted at the Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Indonesia - Dr. Cipto Mangunkusumo Hospital, the samples were from the paraffin block bank at the Department of Pathology Anatomy, Faculty of Medicine, Universitas Indonesia - Dr. Cipto Mangunkusumo Hospital and examined in the research and esoteric laboratory Prodia.

The samples were obtained from the paraffin blocks of tissue biopsies from patients with precancerous lesions and cervical cancer of the Department of Pathology Anatomy. Slides of precancerous lesions and cervical cancer to be sampled underwent re-evaluation by the Pathologist. Samples were taken from formalin fixed paraffin embedded tissue (FFPE) biopsy of cervical precancerous and cervical cancer lesions from Department of Pathology Anatomy. For

each sample taken 5 slides of FFPE were obtained. Samples along with the examination request form were then sent to research and esoteric laboratory Prodia. HPV DNA was extracted from formalin-fixed tissue Paraffin Embedded (FFPE) biopsy of the cervix. Genotyping of HPV DNA types (HPV 16, 18 and 52) was performed before proceeding to the examination DNA methylation in genes L1 with Bisulfite sequencing.

RESULTS

The study was conducted at the Division of Gynecology Oncology, Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Indonesia - RSCM in January-September 2016 and in the Laboratory of Clinical Research & Esoteric Prodia. Samples were taken from the paraffin block biopsies of precancerous lesions and cervical cancer in the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia-RSCM.

Samples were re-evaluated by the Pathologist to ensure the validity of the sample met the criteria of precancerous lesions and cervical cancer. For uniformity of precancerous cervical lesions samples taken were sampled with pure CIN 1, CIN 2, and CIN 3 lesions. Histopathologic type of cervical cancer samples was squamous cell carcinoma of the cervix. There were 57 sample of precancerous lesions and cervical cancer included in this study.

Characteristics of subjects

The mean age of study subjects was 44 years old. There were 30 samples (52.6%) with CIN 1 lesion, 12 samples (21.1%) CIN 2, 9 samples (15.8%) CIN 3, and 6 samples (10.5%) of cervical cancer.

In type 1 CIN lesions, the largest age group as 26-35 years old (10%). In CIN 2, the age group 26-35 years old and 36-45 years old respectively occupies 41.7%. For CIN 3 age group 36-45 years old and 46-55 years old respectively gained 33.3%. In cervical cancer was found most in the age group of 36-45 years old (50%). There was no significant relationship between the age groups with the degree of lesion ($p = 0.525$).

Genotyping HPV

Of the total 57 samples there were 55 samples successfully extracted and detected its HPV DNA

genotype (96.5%). Based on Table 1, most of the samples were co-infected with HPV type 16 and 18 in 28 samples (49.1%) followed by type 16 in 14 samples (24.6%) and type 18 in 8 samples (14.0%).

There were 5 samples that are not detected by the HPV 16, 18 and 52 probe, sequencing was then performed to determine the type of HPV. Based on the results of sequencing there were 1 sample (1.8%) HPV 33; 1 sample (1.8%) HPV 35; 1 sample (1.8%) HPV 58; 2 samples were not included in the group of high risk HPV: HPV 53 (1.8%) with undeterminate risk and HPV 54 (1.8%) with a low risk potential. HPV type 52 was not found in this study. There are two samples that cannot be amplified in CIN 2 and CIN 3. HPV 16 infections both in the form of single or multiple discovered by 76.36% of the total sample were infected with HPV.

In CIN 1 (56.7%) and in CIN 3 (75%), coinfection of HPV 16 and 18 were mostly found In CIN 2, HPV 16 (54.5%) was the most. In cervical cancer, single HPV infection of 18 and co-infection of HPV 16 and 18 were highest (33.3%).

In CIN 1 there is one sample with HPV 35 infection (3.3%) and one sample of single HPV low risk HPV 54 (3.3%) and 1 sample undetermined risk HPV 53 (3.3%) infection.

In CIN 3 there were two samples with single infection HPV; HPV 16 (12.5%) and HPV 58 (12.5%). There was no significant relationship between the types of HPV with the degree of lesion ($p = 0.181$).

A total of 55 samples were successfully amplified and detected its HPV typing, but the inclusion criteria for methylation are those with HPV 16, HPV 18 either as a single infection or multiple infection (co-infection of HPV 16 and 18) and HPV 52. There was no HPV 52 found in this study. Therefore, 50 samples were then included for the examination of L1 gene HPV DNA methylation.

HPV Methylation

Bisulfite conversion procedure was performed according to existing protocols followed by methylation PCR procedures to amplify the HPV DNA bisulfite conversion results. The primers used were special primer for HPV16 FP, RP HPV16, HPV18 and HPV18 FP RP, Integrated DNA Technology.²⁰⁻²² The next procedure was the detection by gel electrophoresis. After several stages of optimization procedures, there were thin band in 8 of 42 samples (19%) of HPV 16 after bisulfite conversion. Six of them were CIN 1, 1 sample CIN 2 and 1 sample of cervical cancer. However, the samples were polluted with dimers, so the procedure was followed by purification of the samples. After PCR was performed on the purified samples there were no band found on the electrophoresis examination, thus we can not proceed to sequencing. Optimization of HPV 16 and HPV 18 samples are still in progress up currently.

Table 1. HPV Genotyping based on the Histopathology

Genotype	Histopathology				
	CIN 1	CIN 2	CIN 3	Cervical Cancer	Total
HPV 16	6 (20%)	6 (54.5%)	1 (12.5%)	1 (16.7%)	14 (25.5%)
HPV 18	4 (13.3%)	2 (18.2%)	0 (0.0%)	2 (33.3%)	8 (14.5%)
HPV 16 & 18	17 (56.7%)	3 (27.3%)	6 (75.0%)	2 (33.3%)	28 (50.9%)
HPV 33	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	1 (1.8%)
HPV 35	1 (3.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.8%)
HPV 58	0 (0.0%)	0 (0.0%)	1 (12.5%)	0 (0.0%)	1 (1.8%)
HPV 53	1 (3.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.8%)
HPV 54	1 (3.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.8%)
Total	30 (100%)	11 (100%)	8 (100%)	6 (100%)	55 (100%)

DISCUSSION

In this study, we obtained 57 samples of paraffin blocks of precancerous lesions and cervical cancer. After extraction of HPV DNA, 55 samples (96.5%) were successfully amplified and performed genotyping. This is a success of its own because the method of detection of HPV in tissue samples is more difficult to do than sitology specimens. This due to formalin fixation which can cause DNA damage, including cross-linking and fragmentasi.²³

HPV DNA genotyping was done by using primers GP5 + / GP6 + with specific probes HPV 16, HPV 18, and HPV 52. The three HPV types were chosen based on the three types of HPV with the highest prevalence in Indonesia on previous studies.²⁴⁻²⁷

In a study involving 11 case control studies from nine countries conducted by Munoz N., et al HPV infection was detected in 90.7% of cases using the primers MY09 / MY011. In this study we used the primers GP5 + / 6 +, and HPV DNA was detected in 96.6% of patients.²⁸

In this study, HPV 16 infection was found the most, 76.36% of the total 55 samples, either in the form of single or multiple infections. Most of the samples were infected with multiple HPV infection 16 and 18 49.1%, followed by a single infection of HPV 16 (24%) and single infection with HPV 18 (14%).

In a study in Jakarta by de Boer et al, it is reported that HPV 16 (35%) and HPV 18 (28%) were the most common types of cervical cancer.²⁶ This is somewhat different from the study in Indonesia conducted Bosch et al in 1995 with the highest prevalence of HPV is HPV 18 (48.9%) in 45 samples followed by HPV 16 as much as 31.9%.²⁹

One interesting thing in this study is there was no of HPV 52 on the overall samples examined. This is different to that obtained in previous studies in Indonesia.

The study conducted by the JNI Vet et al in the three regions in Indonesia (Jakarta, Tasikmalaya and Bali) of 2686 samples most were infected with HPV 52 (23.2%), HPV 16 (18%), HPV 18 (16.1%) and HPV 39 (11.8%). Multiple infection was found in 20.7% of samples.²⁵

A study by Schellekens et al in Jakarta on 74 cervical cancer specimens obtained three types of HPV mostly infected with HPV 16 (44%), HPV 18 (39%) and HPV 52 (14%). Multiple HPV infections

were present in 14.1% of the total HPV positive samples.²⁴ While the study conducted in Singapore by Sahiratmadja et al in 96 cases of cervical cancer 90% samples were infected with multiple HPV 16 infections both with HPV 18, HPV 45 and HPV 52. Only three samples were infected with single HPV 16.²⁷

Most of the samples in this study are co-infected with HPV 16 and 18. This is in line with the cohort by Siddiqi A., et al in Pakistan with samples FFPE tissue biopsies using the primers GP5 + / GP6 +, which are also used in this study, and they found HPV positive in 94, 81% of the samples. Infection with HPV 16 in the form of single or co-infection was found in 64.94% of the samples and HPV 18 was found in 66.23% of the samples. Single infection with HPV 16 was found in 24.68% of the sample and a single infection of HPV in 25.97% of the samples. In this study, 40.26% of samples found positive co-infection with HPV 16 and HPV 18. This is significantly higher than the incidence of co-infection with HPV 16/18 found previously in Pakistan (<2%), in Saudi Arabia (6.7%) and Indonesia (4.1%).^{24,30-32}

In this study, we obtained more multiple HPV infection (co-infection with HPV 16 and 18) compared to with single infection. However, one thing to note, in this study we only use three probes specifically for HPV 16, 18 and 52 so co-infection of HPV 16 or 18 other HPV types could possibly present but can not be detected by the probes.

In this study, co-infection of HPV 16 and 17 were mostly found in CIN 1 (56.7%) and CIN 3 (75%). In CIN 2 lesions, HPV 16 (54.5%) was mostly found. In cervical cancer, single HPV 18 infection and co-infection of HPV 16 and 18 were the most found (33.3%). In addition to HPV 16 and 18, in CIN 1 lesion there is one sample with HPV 35 infection (3.3%) and 2 samples were HPV low risk single HPV 53 (3.3%) and HPV 54 (3.3%). Single HPV 58 infection was 12.5% found in CIN 3.

This study was almost in line with the cohort study conducted by Siddiqi A., et al in Pakistan. In the samples of cervical cancer co-infection of HPV 16 and 18 was 34.88%, while a single infection by HPV 16 and HPV 18 were 27.91% and 30.23%, respectively. In high degree lesions (CIN 2 and 3) single infection of HPV 16 and HPV 18 respectively was 18.18%, while the co-infection of HPV 16 and 18 was found 54.54%.³⁰

Differences in the prevalence of HPV can be caused by several factors, including the sensitivity of PCR (primary election: MY09 / MY11 versus GP 5 + / 6 +), the quality of the specimen and specimen storage. Thus, the extent of variability that exists is difficult to compare the prevalence of HPV among research.³³

HPV DNA methylation is a promising biomarker for diagnosis and prognosis of cervical cancer. After HPV DNA genotyping, 50 samples with either single or multiple infection (co-infection) of HPV 16 and 18 were preceded to the examination methylation. Samples with co-infection of HPV 16 and 18 were examined in a both the group of HPV single infection using primers specific for HPV 16 and also with the single HPV 18 group using primers specific for HPV 18.

The bisulfite conversion procedures uses the EZ DNA methylation Kit reagen, Cat No. D5001 Lot.ZRC186674 (Zymo Research Corp., CA, USA). After the bisulfite conversion procedure, PCR procedure was then performed. Primers used for this procedure were HPV16 FP, RP HPV16, HPV18 and HPV18 FP RP, Integrated DNA Technology.²⁰⁻²²

After several stages of optimization, we obtained a thin band on 8 samples (19%) HPV 16 but were polluted with dimers. Purification of the samples was then performed. On the electrophoresis reading after the PCR examination of the purified samples there were no band visible. This is likely due to small amount of DNA yield. It can also occur because the samples have been through the stages of bisulfite treatment that alter the components cytosinthiamine, which has a lower binding affinity. HPV detection methods on the tissue samples that have been processed with paraffin are more difficult to examine compared to the cytology specimens. This can be caused by formalin fixation can cause DNA damage, including cross-linking and fragmentation.²³

As in the case of genotyping procedures that needed several stage of optimization, HPV DNA methylation procedures also needs some optimization. We plan to add the volume of templates and make modifications to the protocol procedures as a part of optimization to achieve valid results.

Until now, the optimization process for HPV DNA methylation is still carried out and therefore we can not take final conclusion.

CONCLUSION

HPV 16 infection were most commonly found in the form of single or multiple. Co-infection of HPV 16 and 18 were found in the majority of the samples. There were no significant correlation between HPV type and the severity of cervical lesions. We already obtained eight samples of HPV 16 with a thin band on electrophoresis, however the result could not be concluded because the optimization is still ongoing.

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