Pap smear cytology and identification of Human Papillomavirus (HPV) type 16 and 18 in multiparity women at Aviati Clinic Padang Bulan Medan

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Abstract. Cervical cancer is the second most frequent cancer in woman in developing countries and one of the most crucial health problems in the world. Human Papillomavirus (HPV) is an agent for sexually transmitted disease which is an act of cervical cancer, especially high-risk of HPV type 16 and 18. In this study, we investigated the Pap smear cytology and identification of HPV types 16 and 18 in multiparity women at Aviati Clinic Padang Bulan, Medan. Samples are cervical swabs of 50 multiparity women who met the inclusion criteria (childbirth ≥ three times) was included in the study. Pap smear examination was conducted using Papanicolaou staining and identification of HPV types 16 and 18 using the Polymerase Chain Reactive (PCR) methods. Pap smear cytology showed 80% Negative for intraepithelial lesion or malignancy (NILM) with inflammation and 20% NILM. The result of PCR amplification showed that there weren’t specific band DNA was found at band 414bp and 216bp. That means there weren’t cervical swabs sample had DNA of HPV type 16 and 18.

1. Introduction
Cancer is one of the leading causes of death in worldwide. Based on data Globocan, International Agency for Research on Cancer (IARC), know that in 2012 there are 14,067,894 new cases of cancer and 8,201,575 deaths caused in worldwide. World Health Organization (WHO), in 2010 stated that cervical cancer is the second most cancer in women in the world after breast cancer. Found about 500,000 new cases with 250,000 deaths annually. The IARC has estimated that in 2050 the population of women aged 15 and above with cervical cancer in worldwide reaches three billion.[1]

In Indonesia, according to Riskesdas 2013, the prevalence of cancer is 1.4 per 1000 population, and number 7th in the cause of death (5.7%) from all caused by death.[2] The estimated incidence of cervical cancer is 17 per 100,000 women.[3] The highest types of cancer in hospitalized patients throughout Indonesia in 2010 were breast (28.7%) and cervical cancer (12.8%), respectively.[2]

Based on routine data of Sub Directorate Cancer of Non-Communicable Diseases, Directorate General of Disease Control and Environmental Health, Ministry of Health, until 2013, early detection program of cervical and breast cancer was conducted in 717 health centers from 9422 Puskesmas (primary health care) in 32 provinces. There are 405 trainers assigned to provide the training for early detection providers in every province in Indonesia. The trainers consist of a gynecologist, oncologist, general practitioners, and midwives. Of all provinces, only Aceh has not had an early detection trainer,
while West Nusa Tenggara has the most trainers, which are 36 people. In North Sumatera, Medan, the estimated number of cervical cancer is 4694 cases with six trainers.[2]

Similar to other countries, factors that increase the risk of cervical cancer include multiparity, young age at first intercourse (less than 18 years) and multiple sexual partners. The previous study in Jakarta showed that women having more than one sexual partner (OR: 5.83; 95% confidence interval (CI): 2.98–11.36) and multiparity (OR: 2.7; 95% CI: 1.55–4.72) were at an increased risk for cervical cancer and women with an older age (≥20 years) at initiating sexual intercourse (OR = 0.48; 95% CI: 0.28–0.85) were at decreased risk.[4]

Over 90% of cervical cancers are caused by Human Papillomavirus (HPV) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82.[5] HPV type 16 and 18 have the major role in causing cervical cancer.[6] HPV18 is more commonly found in cervical adenocarcinoma than other virus types, while HPV16 is more common in squamous cell carcinomas.[7,8] In Indonesia, HPV51 is the most common HPV type although not identified as one of the five most prevalent types in cervical cancer cases, implying its less relative importance for the disease. HPV18 is the most frequent type in cervical cancer cases, but it is not highly prevalent in women with normal cytology in Indonesia.[9,10] HPV16 remains the most frequent type in Thailand and Vietnam, and the second most frequent type for Indonesia and the Philippines.[4]

High incidence of cervical cancer was associated with minimum detection, such as Pap smears, lack of access to health facilities and also limited knowledge about early detection, risk factors, prevention and treatment of cervical precancer lesions.[1] Based on WHO data estimates in 2008, there was only 5 percent of women in developing countries, including Indonesia received this services.[11]

Issues in the community awareness of health in public, especially reproductive health are still low. Dishonorable in the most of women when talking about the reproductive disease is the inferior reason for early detection such as Pap smear and physician consultation. Moreover, the myths and local beliefs about alternative treatments still major in the community also about financial problems. It’s shown many cases found in Puskesmas (primary health care) or hospital has been in an advanced stage. The multiparity women which more than three children have a higher risk of cervical cancer than nonmultiparity.[12] Therefore, this study was conducted to determine Pap smear cytology features and identification of HPV types 16 and 18 with Polymerase Chain Reaction (PCR) method in multiparity women as early detection of cervical cancer. Aviati Clinic, Padang Bulan Medan was chosen because the location is easy to reach by the researcher and is one of the clinics that have high coverage with the average number of visitation 2500 patients/month, we expect easy access for patients or samples.

2. Method

Cervical swabs were obtained from 50 multiparity women at Aviati Clinic, Padang Bulan Medan, from July until August 2017, who met the inclusion criteria were included in the study. Permission and approval were obtained from the Medical Ethics Committee Universitas Sumatera Utara (No.451/TGL/FK/KEPK FK USU-RSUP HAM/2017).

2.1. Pap smear examination

Specimens (cervical swabs) were collected for cytology examination by cytobrush and stained with Papanicolaou. Cytological grade according to the Bethesda System.[13] The cytological results evaluated with photomicrography using digital photomicrographic microscope at Anatomic Pathology Laboratory, Department of Anatomic Pathology, Universitas Sumatera Utara.

2.2. Identification of HPV types 16 and 18 using PCR methods

The isolated DNA samples from cervical swabs were using Presto™ Buccal Swab gDNA extraction kit then amplified using the PCR method. PCR kit components used for HPV gene amplification were DNA template of 6 μl, GoTaq® Green Master Mix (Promega) of 12.5 μl, primer of forwarding HPV
16 and 18: 5'-GGC-TTT-GGT-GCT-ATG-GAC-3' and reverse HPV 16 and 18: 5'-AGT-AGA-TAT-GGC-AGC-ACA-3' and 5'-CAC-GCA-CAC-GCT-TGG-CAG-GT-3', respectively. 1 μL nuclease-free water as much as 7.5 μL and 3 μL DNA with final volume is 25 μL. The amplification process consisted of an initial denaturation stage of 5 min at 95°C, followed by a 1 min denaturation step at 95°C at 35 cycles, annealing stage for 1 min at 55°C, extension stage for 1 min at 72°C, final extension for 10 min at 72°C later cooling 12°C for 10 minutes. 

A qualitative examination of DNA isolation result using Electrophoresis Method. This process begins with using 3% agarose, 3.6 grams of agarose powder, then agarose dissolved in 120 mL TAE 1x in Erlenmeyer, then heated in the microwave for 5 minutes and adding one μL Ethidium bromide. After that, poured on the electrophoresis mold to cool and dry. For markers, a 1.5 μL DNA ladder was used. It was then fed into agarose gel wells. 7μL samples were also included in subsequent wells. Electrophoresis was performed at a voltage of 80 volts for 70 minutes. The gel is photographed using a gel documentation system (Uvitec). The electrophoresis pattern showed one band 414 bp on primer HPV16 and one band 216 bp for HPV 18.

3. Results and discussions

3.1. Pap smear cytology evaluation

Pap smear cytology results showed 80% negative for intraepithelial lesion or malignancy (NILM) with inflammation (included atrophic smear with inflammation and reactive changes) and 20% NILM.

![A) Pap smear showed superficial (red) and intermediate (blue) cells. B) Cervicitis, acute inflammatory exudate (numerous neutrophils) (Ecto/endocervical smear, Pap, 200x).](image)

In this study, there weren’t malignancy lesions in Pap smear cytology. The multifactorial to explain this results, the involvement of other risk factors including lifestyle, sexual behaviors, and genetic background. Most of the sample showed first sexual intercourse at the mature age (25-30 years old), shown in Table 2. This study was in line with a study by de Boer et al. which suggests that women with older age (>20 years) at first sexual intercourse (OR = 0.48; 95% CI: 0.28–0.85) were at a decreased risk of cervical cancer. [4]

Most of the multiparity (80%) showed the presence of inflammation process, need to be aware because chronic inflammation can trigger developing of carcinogenesis. Regarding parity, multiparity women (childbirth more than three times) have a higher risk of cervical cancer than non-multiparity women. [12] The dangerous parity was had children more than two children, or the labor distance is too short, it can cause abnormal cells changes in the cervix and can develop into malignancy. Nurhasanah et al. [15] showed the number of children increased the risk of cervical cancer. The mechanisms due to malignancy such as cervical trauma that occurs due to repeated births, hormonal changes in pregnancy, chronic infections and irritation. This study contradicts with the study.
conducted by Izza et al. which indicates that the risk is 3-5 times higher in multiparity women than multiparity women with cervical cancer.[16]

**Table 1. Distribution level of age.**

| Age           | N  | %  |
|---------------|----|----|
| <35 years old | 6  | 12 |
| 35-45 years old | 33 | 66 |
| >45 years old | 11 | 22 |
| **Total**     | **50** | **100** |

In this study, as shown in Table 1, most of the multiparity with 35-45 years old had 33 people (66%), followed with more than 45 years old had 11 people (22%) and less than 35 years old had six people (6%). According to WHO (2006), women 40-45 years old have the highest risk for cervical cancer. When a woman has entered premenopausal, she should have started doing Pap smears as an attempt too early detection of cervical cancer.[6]

**Table 2. Distribution level of married or first sexual intercourse.**

| Age            | N  | %  |
|----------------|----|----|
| <20 years old  | 2  | 4  |
| 20-25 years old| 20 | 40 |
| 26-30 years old| 28 | 56 |
| **Total**      | **50** | **100** |

Most of the multiparity married or first sexual intercourse showed the mature age (26-30 years old) as much as 56%, followed by 20-25 years old as much as 40% while married age <20 years only 4% (Table 2). According to Riskesdas 2015, married or first sexual intercourse at a young age (less than 18 years) is one of the risk factors for cervical cancer.[2]

3.2. *Identification of HPV DNA types 16 and 18*

The result of PCR amplification showed that there weren’t specific band DNA was found at band 414 bp on HPV type 16 and 216 bp on HPV type 18.

![Figure 2](image)

*Figure 2. Visualization of HPV DNA type 16 on sample 1-50 after PCR amplification by electrophoresis method. (K+: positive control showed a band 414 bp; K-: negative control).*
Figure 3. Visualization of HPV DNA types 18 on sample 1-50 after PCR amplification by electrophoresis method. (K+: positive control showed a band 216 bp; K-: negative control).

This study on the fifty samples none showed the bands at 414 bp and 216 bp, this means the samples showed HPV type 16 and 18 negative results. The results of this study contradict the study conducted by Mijit et al. [16] which indicate that the HPV infection rates in Uyghur-Muslim women Xianjiang-China with cervical inflammation, CIN, and cancer were 18.18%, 64.71%, and 100%, respectively. HPV-16, HPV-58, and HPV-39 are the most prevalent genotypes.[17]

This study showed bands more than 216 bp and less than 414 bp. It is thought to be another DNA that has not yet been determined to come from what microorganisms, which have nucleotide arrangements similar to HPV types 16 and 18. The bands are formed more than one and are quite clear, so it was suspected that it is also the result of amplification.

4. Conclusion

Pap smear cytology from 50 cervical swabs of multiparity women showed most of the inflammatory lesions not found malignancy and the PCR amplification showed that there was no cervical swabs sample had DNA of HPV type 16 and 18. The limitation of this study that the number of samples is too small. It is recommended for advanced study, the larger number of samples so that identification of HPV types 16 and 18 in multiparity women will be achieved.

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