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High Risk Human Papillomavirus Genotype Distribution and its Relevance to Cytology in Lao PDR

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ABSTRACT

Human papillomavirus (HPV) infection is thought to be a primary cause of cervical cancer, especially the high-risk (HR)-HPV genotype group that causes the development of neoplastic lesions. HPV testing is being conducted worldwide to clarify the risk of cervical cancer. Lao People's Democratic Republic (Lao PDR) is one of the developing countries located in Southeast Asia. HPV genotype distribution analysis has not been comprehensively conducted in Lao PDR. In this study, cervical cytology and presence of HR-HPV were examined, using both hybrid capture 2 (HC2) and polymerase chain reaction (PCR) methods, with self-collected cervical samples from 297 healthy asymptomatic women in Oudomxay district, a northern rural district of Lao PDR. The overall rate of abnormal cytology in Oudomxay was 9.8%. HR-HPV positivity rates by HC2 and PCR were 22.9% and 9.1%, respectively. The most prevalent HR-HPV genotype was HPV 58, followed by HPV 16 and 33 in Oudomxay. Considering together with our previous data in Vientiane, the overall HR-HPV positivity rate and frequencies of HPV 16 and 58 increased as cytological morphology deteriorated. The present results suggest that HPV 58 is associated with high-grade cervical lesions, similar to HPV 16, in Lao PDR. *Ryukyu Med. J., 38 (1~4) 25~34, 2019*

Key words: cervical cytology, HPV genotype, Lao PDR

INTRODUCTION

There were an estimated 311,000 deaths from cervical cancer worldwide in 2018, and the vast majority of which are in less developed regions such as Sub-Saharan Africa and South-Eastern Asia[1)]. Human papillomavirus (HPV) is a primary cause of invasive cervical cancer globally[2)]. Some genotypes that tend to induce high-grade lesions are recognized as high-risk (HR)-HPV. An HPV test has been validated and the HPV genotype geographic distribution has been investigated in recent years[3)].

Lao People's Democratic Republic (Lao PDR) is a developing country located in Southeast Asia. The population of Lao PDR is 6,901,000 people and the average life expectancy is 64 years for men and 67 years for women[4)]. Although the leading cause of death is infectious diseases, the number of deaths related to cardiovascular diseases and cancer has recently increased due to the increasing life expectancy[5)]. According to the HPV Information Centre report[6)], age-standardized cervical cancer

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incidence and mortality rates in 2017 were estimated to be 12.5 (per 100,000) and 7.4 (per 100,000), respectively, and cervical cancer is the third most frequent cancer among women in Lao PDR. As of the year 2018, there is no routine cytology protocol in gynecology or laboratory examinations in any hospitals in Lao PDR, excluding some hospitals in the capital city Vientiane. Cervical cytology screening methods require highly trained human resources including pathologists and cytotechnologists, although there are few pathologists and no cytotechnician system in Lao PDR. Furthermore, to the best of our knowledge, there are only a few reports about the HPV genotype in Lao PDR[7], and HPV genotype distribution has not been comprehensively investigated.

We previously reported cervical cytology and HPV status among healthy asymptomatic women in Vientiane[8]. In this study, cervical cytology and HR-HPV genotype distribution were investigated among healthy asymptomatic women in a northern rural district in Lao PDR. HR-HPV genotype in relation to cytological morphology was also discussed together with our previous data from Vientiane[8]. In addition, since our international support project for cervical cancer screening with cytology in Lao PDR had been carried out by a self-collection method[9, 10], the participants' views about collection method were also gathered in this study.

MATERIALS and METHODS

Study participant recruitment

This study was approved by the Ethics Committee for Clinical Research of the University of the Ryukyus (no. 1317) and the National Ethics Committee of the Ministry of Health of Lao PDR (no. 065).

This study was basically similar in design to our previous study[8]. In brief, 300 healthy asymptomatic women were invited to join the study by a community campaign (an advertisement by a provincial hospital) in Oudomxay. All participants provided written informed consent for study participation.

Cytological examination

The participants were initially instructed on the use of Kato's device (Uterus Cancer Examination Instrument Manufacturer Ltd., Nagoya, Japan) for self-collection of cervical tissue samples. Cervical samples were placed into SurePath Pap test vials and slides were prepared using the liquid-based cytology (LBC) method (SurePath LBC, Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Then, 297 samples that were sufficient for diagnoses were evaluated by two Japanese cytotechnologists, and Laotian and Japanese pathologists. Cytological results were classified as follows based on the Bethesda system[11]: negative for intraepithelial lesions or malignancy (NILM), atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion (ASC-H), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), atypical glandular cells (AGC), squamous cell carcinoma (SCC), and adenocarcinoma (ADC). In addition, it was defined as ≧ASC-US for any cases revealed abnormal cytology with ASC-US, ASC-H, LSIL, HSIL, AGC, SCC and ADC.

HR-HPV analyses

HPV DNA was determined by both the hybrid capture 2 (HC2) and the polymerase chain reaction (PCR) methods using remnant LBC samples after cytological examination[8]. First, HC2 test was conducted in accordance with the standard protocol recommended by the manufacturer (Qiagen GmbH, Hilden, Germany). HC2 detects HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, by hybridization with RNA probes and signal amplification. The threshold for a positive HC2 result is 1 relative light unit / cutoff. Secondly, in the PCR method, DNA in remnant LBC samples were extracted by using the phenol-chloroform protocol and it analyzed with the protocol recommended by the manufacturer (Takara Bio Inc., Shiga, Japan). In brief, HR-HPV DNA was amplified using the consensus primers for HPV E6 and E7 proteins region (Forward 5'-TGTCAAAAACCGTTGTGTCC-3', Reverse 5'-GAGCTGTCGCTTAATTGCTC-3'), for 30 cycles (30 sec at 94℃, 2min at 58℃, 30sec at 72℃) using the Gene Amp PCR System 9700 (Thermo Fisher Scientific, MA, USA). Restriction enzyme digestion patterns of PCR products were used to confirm HR-HPV genotypes as reported previously[8].

HR-HPV genotype distribution was compared with those of previous papers[7, 8] and HR-HPV genotype and its relevance to cytological morphology was also discussed together with our previous data in Vientiane[8], which included additional HPV test results among NILM samples.

Participants' views on using the self-collection device

Questionnaires were used to obtain information about the participants' age, marital status, number of pregnancies and educational background. Participants were also asked about how they felt about the usage of the self-collection device. Then, participants' opinions about the preferred collection method were compared with our previous study in Vientiane[8].

Statistical analysis

All data were analyzed using GraphPad Prism software (version 7, GraphPad Software, San Diego, CA, USA). The chi-square test was used for analyses and statistical significance was defined as $P<0.05$.

RESULTS

Cytological examination

Of the 300 samples, 297 samples were adequate for cytological diagnosis and were analyzed. The participants' age was 37.2±5.8 years (mean±SD) ranging from 24 to 54 years. The baseline characteristics of the participants and the cytological results in Oudomxay were summarized in Table 1. NILM, ASC-US, LSIL and HSIL were observed in 90.2% (268/297), 6.7% (20/297), 2.4% (7/297), and 0.7% (2/297%) of samples, respectively. The overall prevalence of abnormal cytology (≧ASC-US) was 9.8% (29/297). No cases of ASC-H, AGC, SCC and ADC were observed in the present study in Oudomxay. In addition, there was no statistical significance between the appearance of abnormal cytology and each factor, such as age, marital status, number of pregnancies, or educational level. Multiparous women who had got pregnant three or more times tended to have abnormal cytology more frequently than the others although it was not statistically significant ($P=0.0693$).

HR-HPV analyses

The overall HR-HPV positivity rates as

Table 1 **Summary of the characteristics of the participants in Oudomxay**

		Total	NILM n (%)	ASC-USa n (%)	ASC-US n (%)	LSIL n (%)	HSIL n (%)
		297	268 (90.2)	29 (9.8)	20 (6.7)	7 (2.4)	2 (0.7)
Age (y)	24-29	1	0 (0.0)	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)
	30-34	111	100 (89.3)	11 (9.9)	7 (6.3)	3 (2.7)	1 (0.9)
	35-39	87	79 (90.8)	8 (9.2)	7 (8.0)	1 (1.1)	0 (0.0)
	40-44	59	53 (89.9)	6 (10.2)	4 (6.8)	1 (1.7)	1 (1.7)
	45-54	39	36 (92.3)	3 (7.7)	2 (5.1)	1 (2.6)	0 (0.0)
Marital status	Single	5	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Married	290	261 (90.0)	29 (10.0)	20 (6.9)	7 (2.4)	2 (0.7)
	Widowed	2	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Number of pregnancies	0	23	22 (95.7)	1 (4.3)	1 (4.3)	0 (0.0)	0 (0.0)
	1	25	25 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	2	102	93 (91.2)	9 (8.8)	5 (4.9)	3 (2.9)	1 (1.0)
	3	78	69 (88.5)	9 (11.5)	9 (11.5)	0 (0.0)	0 (0.0)
	≧4	69	59 (85.5)	10 (14.5)	5 (7.2)	4 (5.8)	1 (1.4)
Educational level	No school	9	8 (88.9)	1 (11.1)	1 (11.1)	0 (0.0)	0 (0.0)
	Primary school	47	45 (95.7)	2 (4.3)	1 (2.1)	1 (2.1)	0 (0.0)
	Lower secondary school	71	64 (90.1)	7 (9.9)	6 (8.5)	1 (1.4)	0 (0.0)
	High school	89	81 (91.0)	8 (9.0)	4 (4.5)	3 (3.4)	1 (1.1)
	College	54	48 (88.9)	6 (11.1)	4 (7.4)	1 (1.9)	1 (1.9)
	University	27	22 (81.5)	5 (18.5)	4 (14.8)	1 (3.7)	0 (0.0)

[a] ≧ ASC-US means ASC-US, LSIL and HSIL

determined by HC2 and PCR were 22.9% (68/297) and 9.1% (27/297), respectively (Fig. 1). In addition, all the samples were divided into NILM and ≧ASC-US samples, and then the positivity rates in both HC2 and PCR methods were calculated. With the HC2 method, the HR-HPV positivity rate in NILM samples and ≧ASC-US samples were 18.7% (50/268) and 62.1% (18/29), respectively (Fig. 1A), while the HR-HPV positivity rate in NILM samples and ≧ASC-US samples with the PCR method were 3.4% (9/268) and 62.1% (18/29), respectively (Fig. 1B). When comparing these positivity rates between NILM samples and ≧ASC-US samples, positivity rates were significantly higher in ≧ASC-US samples than in NILM samples as determined by both HC2 and PCR methods ($P<0.0001$ and $P<0.0001$, respectively). Compared to the HC2 method, the HR-HPV positivity rate of NILM samples was lower than that of the PCR method (Fig. 1). The HR-HPV genotype distribution in Lao PDR is summarized together with previously reported data[7, 8] in Table 2. The most prevalent genotype in Oudomxay was HPV 58. In contrast, the most prevalent genotype in Vientiane was HPV 16 as shown by our previous study[8]. Furthermore, the HPV 33/32/58 group was the most prevalent in three regions, Luang Prabang (a northern district close to Oudomxay), Champassack (a southern district), and the capital Vientiane as described in the original study about HR-HPV in Lao PDR[7]. HPV 58 and 16 are noticeable in every region from these three studies. Fig. 2 shows the genotype distribution of NILM and ≧ASC-US samples with HR-HPV in Oudomxay (Fig. 2A) and in Vientiane[8] (Fig. 2B). In Oudomxay, HPV 58 was the most prevalent in NILM samples while HPV16 was the most prevalent in ≧ASC-US samples (Fig. 2A). In Vientiane, HPV 16 was the most prevalent genotype both in NILM and ≧ASC-US samples (Fig. 2B). In both regions, HPV 16 and 58 were noticeable in ≧ASC-US samples. Fig. 3 summarizes the cytological findings of 611 samples analyzed by PCR (297 from Oudomxay, 314 from Vientiane[8]; 1 sample of which cytology was AGC was excluded). The overall HR-HPV positivity rate increased as cytological morphology deteriorated. The proportions of HPV 16 and 58 were particularly increased according to cytological atypia. Among HSIL and SCC samples, ≧70% samples were positive for either HPV 16 or 58.

Participants' views on using the self-collection device

Fig. 4 shows the preferred method for collection in each region. Many participants in each region

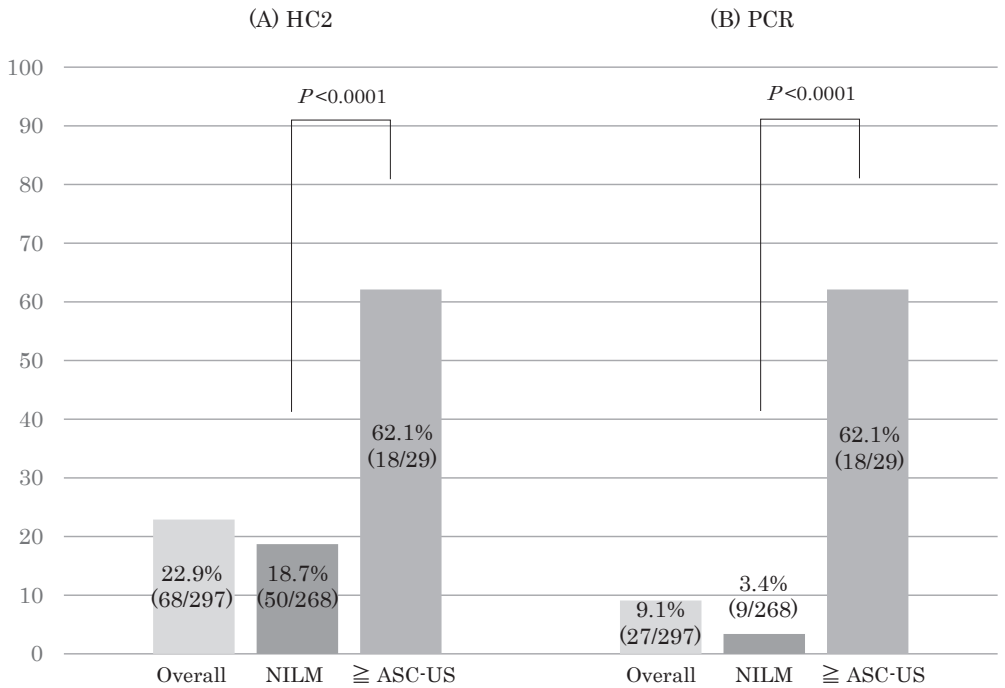

Fig. 1 **The positivity rates of HR-HPV in Oudomxay**
HR-HPV detection was examined by both HC2 (A) and PCR (B) methods (Note the details in Materials and Methods). Overall, including NILM and ≧ ASC-US, means 297 samples examined in the present study.

Table 2 **Summary of HR-HPV distribution in Lao PDR**

	Present study	**Takamatsu et al**[8]	**Phongsavan et al**[7]		
Region	Oudomxay	Vientiane	Vientiane	Luang Prabang [a]	Champassack [b]
	n=297 (%)	n=315 (%)	n=645 (%)	n=886 (%)	n=391 (%)
HPV16	7 (2.4)	54 (17.1)	19 (2.9)	20 (2.3)	4 (1.0)
HPV31	0 (0.0)	11 (3.5)	3 (0.5)	6 (0.7)	0 (0.0)
HPV33	4 (1.3)	4 (1.3)			
HPV52	1 (0.3)	8 (2.5)	29 (4.5) [c]	28 (3.2) [c]	4 (1.0) [c]
HPV58	10 (3.4)	23 (7.3)			
HPV35	1 (0.3)	2 (0.6)	2 (0.3)	0 (0.0)	0 (0.0)
HPV18	1 (0.3)	5 (1.6)			
HPV45	—	—	8 (1.2) [d]	13 (1.5) [d]	1 (0.3) [d]
HPV39	—	—	4 (0.6)	9 (1.0)	0 (0.0)
HPV51	—	—	1 (0.2)	3 (0.3)	0 (0.0)
HPV56	—	-	9 (1.4)	5 (0.6)	4 (1.0)
HPV59	—	—	2 (0.3)	8 (0.9)	0 (0.0)
multiple	3 (1.0)	10 (3.2)	14 (2.2)	15 (1.7)	2 (0.5)
negative	270 (90.9)	198 (62.9)	554 (85.9)	779 (87.9)	376 (96.2)

[a] The northern district of Lao PDR, [b] The southern district of Lao PDR, [c] data detected HPV 33/52/58 together, [d] data detected HPV 18/45 together.

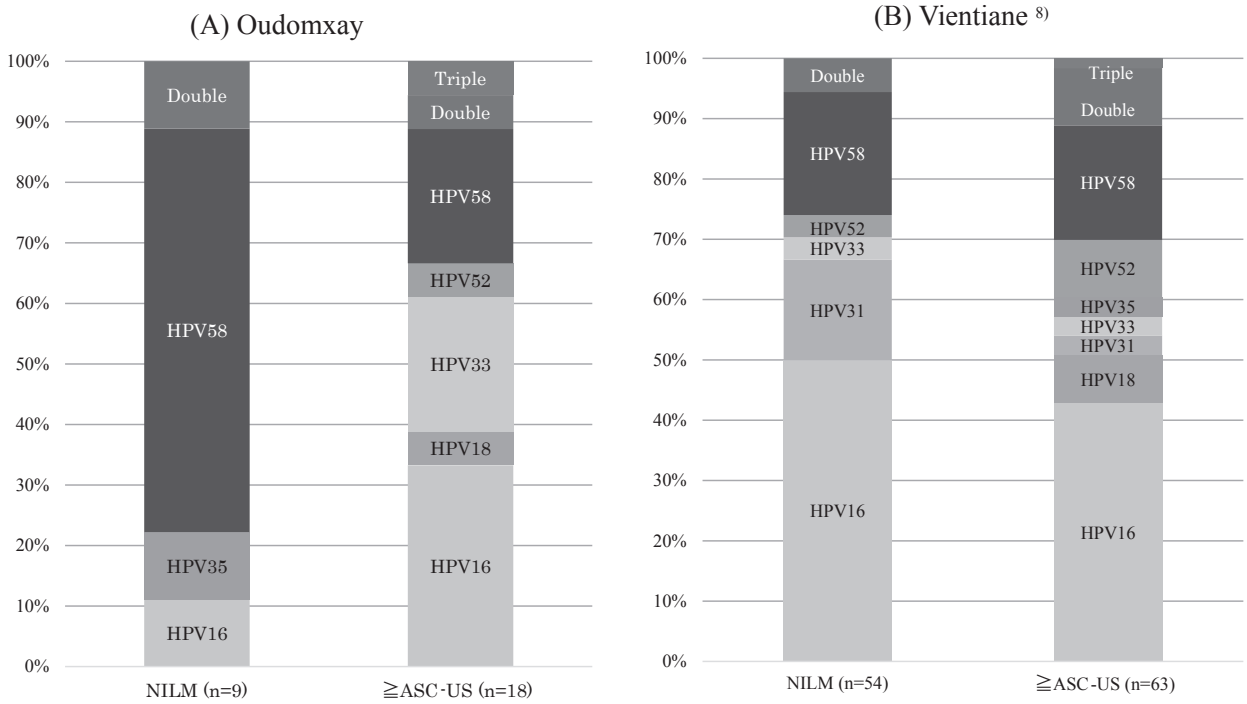

Fig. 2 **The genotype distribution of NILM and ≧ ASC-US samples with HR-HPV**
It shows the HR-HPV genotypes by PCR method, in NILM and >ASC-US samples (A;Oudomxay B;Vientiane8)). Double: HPV16 and 31, 16 and 33, 16 and 35, 16 and 52, 16 and 58, 33 and 58, 31 and 58, 33 and 58, 31 and 52. Triple: HPV 16, 58 and 35, HPV 16, 18 and 33.

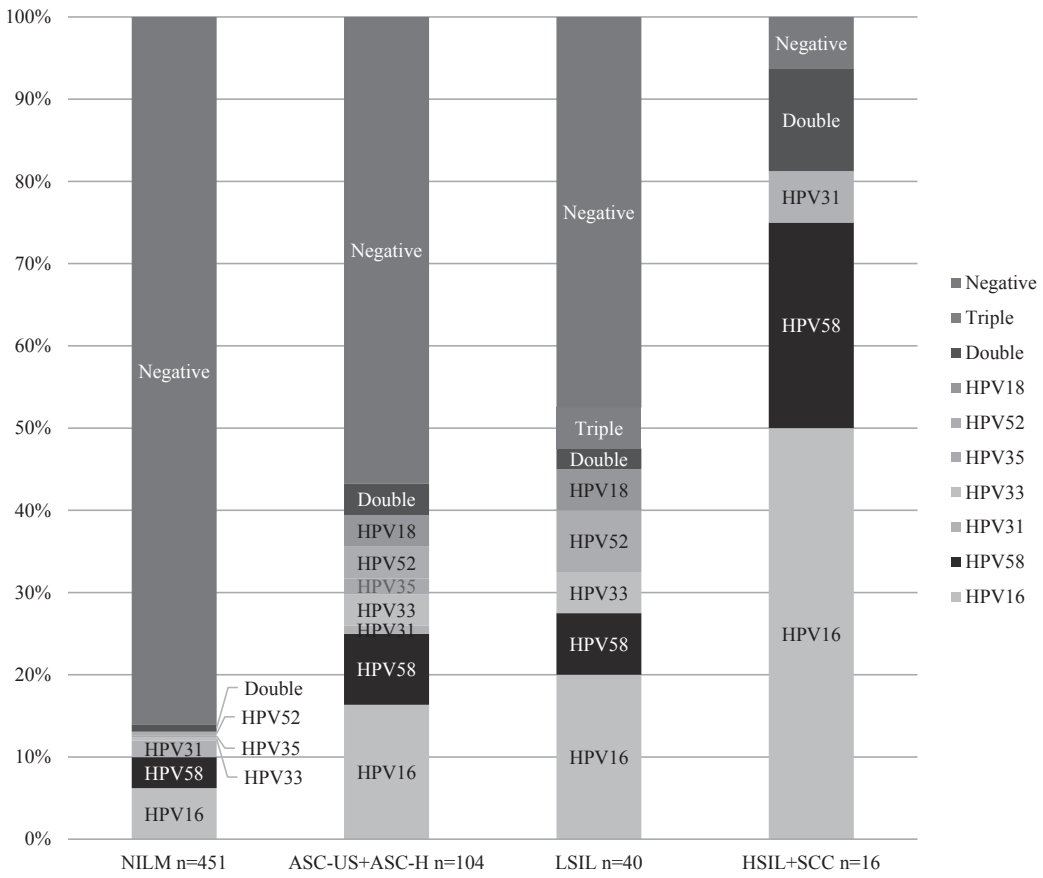

Fig.3 **The relevance of HR-HPV genotypes to cytological findings**
It was summarized cytological findings of all HPV-analyzed samples in the present study including previous data8). Negative: HR-HPV is not detected. Double: HPV16 and 31, 16 and 33, 16 and 35, 16 and 52, 16 and 58, 33 and 58, 31 and 58, 33 and 58, 31 and 52. Triple: HPV 16, 58 and 35, HPV 16, 18 and 33.

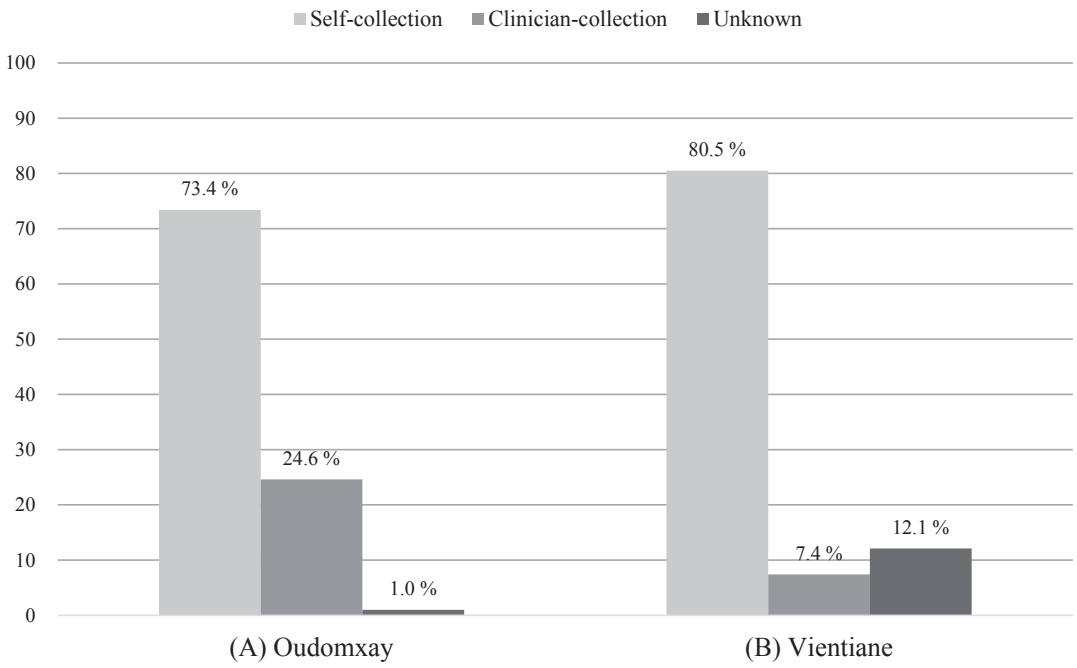

Fig.4 **The preferred collection method in each region**
Participants' views about collection method were summarized in Oudomxay (A) and in Vientiane (B).

tended to favor the self-collection method. The number of people who favored clinician-collection was higher in Oudomxay, compared to that in the capital Vientiane of previous study[8]. In the questionnaire, there were several views about the self-collection method. The most common reason supporting the self-collection method was simplicity, followed by unnecessity of going to hospital, and no feeling of shame against gynecologist. In contrast, most people who favored the clinician-collection method felt anxiety about suitableness of samples collected by themselves.

DISCUSSION

Cervical cytology and HR-HPV genotype distribution were examined using samples from 297 healthy women in Oudomxay, a rural district of Lao PDR. HR-HPV is recognized as the primary cause of cervical cancer, and HPV testing is being conducted worldwide. In this study, both HC2 and PCR methods were used for HR-HPV detection. While the HC2 test is useful to detect HR-HPV easily and has been commonly applied worldwide, it is unable to determine HPV genotype. HR-HPV genotype analysis has recently become important because some genotypes are more virulent than others; HPV 16, 18, 31, 33, 35, 52, and 58 are more likely to be associated with high-grade lesion than other HR-HPV genotypes[12]. In this study, these more virulent HR-HPV genotypes were identified by PCR and their distribution in Oudomxay was clarified. The overall positivity rates of HR-HPV by HC2 and PCR were 22.9% and 9.1%, respectively. HR-HPV positivity rates were significantly higher in ≧ASC-US samples than in NILM samples with both HC2 and PCR (Fig. 1). Within the NILM samples, the positivity rate was much lower when determined by PCR than HC2 (Fig. 1). As shown in the previous paper[12], the 7 more virulent HR-HPV genotypes (HPV 16, 18, 31, 33, 35, 52, and 58) that were detected by PCR seemed to be related to the development of abnormal cells rather than the other 6 genotypes (HPV 39, 45, 51, 56, 59, and 68) which can be detected only by HC2.

There have been few reports concerning to HR-HPV genotypes in Lao PDR. The most prevalent HR-HPV genotype in Oudomxay was HPV 58 as shown in the present study. Similar to these data, the most prevalent genotype was reported as HPV33/52/58 group in three regions in the first report about HR-HPV in Lao PDR[7], although the most prevalent genotype in Vientiane was HPV 16 as shown by our previous study[8] (Table 2). Taking into account the cytology, HPV 58 was the most prevalent genotype in NILM samples in Oudomxay (Fig. 2A), while HPV 16 was the most prevalent genotype in NILM samples in Vientiane (Fig. 2B). Thus, it is suggested that Oudomxay and Vientiane differ in the predominant genotype in healthy women without atypical cells. Baloch et al. stated that women living in different regions harbor different HR-HPV genotypes, and their source of income or access to health facilities is related to these geographical variations in HPV distribution[13]. HPV 16 is the most prevalent genotype in almost all regions of the world, but the second most prevalent genotype varied with location[14]. For example, it has been reported that HPV 58 is more common in Eastern and Southeastern Asia than in other regions[15]. Especially in East Asia, HPV 58 accounts for a large proportion of HPV in cervical intraepithelial neoplasia (CIN) samples and is thought to have an important role in cervical cancer[16]. Although the most prevalent genotype among NILM samples was different, HPV 16 and 58 were commonly detected in ≧ASC-US both in Oudomxay and Vientiane [8](Fig. 2 and 3).

The overall HR-HPV positivity rate in the present study increased as cytological morphology deteriorated. Interestingly, proportions of HPV 16 and 58 profoundly increased. Since there are many reports about virulence of HPV 16 and 18[17-19], these two genotypes are considered to be responsible for the development and progression of cervical lesions. Therefore, a bivalent HPV 16/18 vaccine has been used worldwide[20]. In fact, the present data support the notion that HPV 16 is related to HSIL/SCC in Lao PDR. However, HPV 58 was also highly detected in HSIL/SCC in the present study, similar to HPV 16 (Fig. 3). As mentioned above, some genotypes are more likely than others to progress to high-grade lesions. According to this concept[12], HR-HPV is divided into a more critical group and an intermediate risk group, and HPV 58 is grouped in the former. Another study showed that HPV 16 was the most predominant, followed by HPV 58, in cases with ≧CIN2 in China[21]. Furthermore, Song et al. found a strong association between HPV 58 and the development of HSIL in women who are HPV-positive

and cytology-negative[22]. In the present study, HPV 58 which has a high probability of progressing to HSIL was frequently detected in NILM samples in Oudomxay. Therefore, regular follow-up is needed for early detection of precancerous lesions. However, adoption of these methods is presently challenging in Lao PDR.

As part of an international collaboration at our institution, we have helped Laotian co-researchers, who had studied in our university, and conducted cervical cytology and HPV genotype testing using self-collection device in Lao PDR. It has been reported that the self-collection method can collect adequate samples and is as accurate as the clinician-collection method[23, 24], although many gynecologists have felt it was unsatisfactory for cytology. In this study, many participants in each region tended to favor the self-collection method to the clinician-collection method (Fig. 4). As mentioned in the results, the self-collection method is simple, unnecessary to go to hospital, and it does not cause to feel shame against gynecologist. The present result suggests that the self-collection method could be accepted by many women in Lao PDR. However, there were some people who favored the clinician-collection to the self-collection in rural region compared with those in capital city (Fig. 4). People who favor the clinician-collection seemed to lack confidence in using the device by themselves. It may be because cervical cytology is not well known in Oudomxay, gynecologists are more respected in rural regions, and the local people seem to rely on doctors. Currently, HR-HPV testing is being conducted using the self-collection method[25]. It is useful for women living in under-resourced regions lacking sufficient medical services to improve screening coverage and acceptability. Furthermore, HPV testing and co-testing with cytology identified more histologically proven HSIL cases than either cytology or HR-HPV testing alone[26]. Thus, it is clear that in addition to HPV testing, cytological diagnosis is important for an accurate cervical cancer screening system.

CONCLUSION

Cervical cytology and HR-HPV genotype distribution were investigated using samples from healthy asymptomatic women in a northern rural district of Lao PDR and their relevance to cytology was discussed. The overall rate of abnormal cytology was 9.8%. The positivity rates of HR-HPV as determined by HC2 and PCR were 22.9 % and 9.1%, respectively. The most prevalent HR-HPV genotype was HPV 58, followed by HPV 16 and 33. Considering together with our previous data in Vientiane, the overall HR-HPV positivity rate and the frequencies of HPV 16 and 58 increased as cytological morphology deteriorated. The present results also suggest that HPV 58 is associated with high-grade cervical lesions, similar to HPV 16, in Lao PDR.

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