

Prevalence of High-Risk Human Papillomavirus (HR-HPV) Infection among Women with Normal and Abnormal Cervical Cytology in Myanmar

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This study aimed to determine the prevalence of normal and abnormal cervical cytology in women who attended the cervical cancer screening clinic of the Department of Medical Research in Lower Myanmar, and to determine the proportion of high-risk (HR) human papillomavirus (HPV) infection and HPV genotypes in women with normal and abnormal cervical cytology. A total of 1,771 women were screened from 2010 to 2011. Among them, 762 women (43.0%) had a normal smear, and 866 (48.9%) and 87 (4.9%) were diagnosed with inflammatory smears and atypical squamous cells of undetermined significance (ASCUS), respectively. Diagnoses of low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) numbered 42 (2.3%) and 11 (0.6%) respectively. Three cases of squamous cell carcinoma (SCC) (0.2%) were detected. Cervical swabs were collected from 96 women with abnormal cervical cytology and 20 with normal cytology. HR-HPV DNA testing was performed by polymerase chain reaction (PCR) with *pU1M/pU2R* primers. HR-HPV were identified in 35.5% (22/62) of inflammatory smears, 60% (6/10) of ASCUS, 86.7% (13/15) of LSIL, 50% (3/6) of HSIL, 100% (3/3) of SCC and 5% (1/20) of normal cytology. In PCR-positive cases, HPV genotyping was analyzed by the cleaved amplification polymorphism method. The most prevalent HPV genotypes were HPV-16 (60.4%) followed by HPV-31 (14.6%), HPV-18 (12.5%) and HPV-58 (12.5%). Women with abnormal cervical cytology were 10 times more likely to be HR-HPV positive than those with normal cytology ($p = 0.0001$). This study suggests that the implementation of a cervical cytology screening program and routine vaccination against HPV in preadolescent and adolescent groups are needed to reduce the burden of HPV-associated cervical cancer.

Key words: human papillomavirus, cervical neoplasia, genotyping, Myanmar

Worldwide, cervical cancer is the third-most-common cancer in women and the seventh overall with an estimated 530,000 new cases in 2008. More

than 85% of the global burden occurs in developing countries, where it accounts for 13% of all female cancers. Cervical cancer remains the most common cancer in women in Eastern Africa, South-Central Asia and Melanesia. Overall, the mortality-to-incidence ratio is 52%, and cervical cancer was responsible for 275,000 deaths in 2008, about 88% of which occurred

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in developing countries. According to the WHO South-East Asia region (SEARO) report, there was an estimated 188,000 new cervical cancer cases and 102,000 deaths in 2008 [1]. This is due to the fact that the majority of women in the world do not have access to cervical screening, which can prevent up to 75% of cervical cancers [2].

In Myanmar, over the past 30 years (1976–2006) cervical cancer has comprised 13,181 (23.5%) of the total 56,097 known female cancer cases, and cervical cancer and breast cancer traded places as the most common and second most common female cancers in those years [3].

Worldwide, human papillomavirus (HPV) -16 and -18, the 2 vaccine-preventable types, contribute to over 70% of all cervical cancer cases, between 41% and 67% of high-grade cervical lesions and 16–32% of low-grade cervical lesions. In South-Eastern Asia, the prevalence of HPV-16 and HPV-18 by cytology is 72.6% in cervical cancer, 33.3% in high-grade squamous intraepithelial lesions (HSIL), 14.2% in low-grade squamous intraepithelial lesions (LSIL) and 3.2% in normal cytology [4]. HPV can be identified in virtually all 99.7% of cervical cancer cases and has been established as an etiological agent of invasive cervical cancer [5], and it is the most common sexually transmitted viral infection worldwide. Persistent infection with oncogenic or high-risk HPV (HR-HPV) is necessary for the development of premalignant lesions and/or progression of the disease [6].

The HPV prevalence and genotype distribution are important for estimating the impact of HPV-based cervical cancer screening and HPV vaccination on the incidence of diseases etiologically linked to HPV. The distribution of HPV genotypes varies across different populations and geographical regions [7]. Only limited data have been available from Myanmar on the distribution of HPV genotypes in the general population and in low-grade and high-grade lesions of the cervix and of cervical cancer.

Yet, with the advent of preventive HPV vaccines that target HPV-16 and -18, which are responsible for causing about 70% of invasive cervical cancer in the world, such information is crucial to predicting how vaccination and HPV-based screening would affect the prevalence of cervical cancer. This study aimed to determine the prevalence of normal and abnormal cervical cytology in women who attended the cervical

cancer screening clinic of the Department of Medical Research in Lower Myanmar, and to determine the proportion of HR-HPV in women with normal and abnormal cervical cytologies.

Materials and Methods

A total of 1,771 women attending the cervical cancer screening clinic at the Department of Medical Research in Lower Myanmar (DMR-LM) were screened during 2010 to 2011. Among them, 762 had normal cytology and 1,009 had abnormal cytology. Because of limited resources for further study, we randomly selected 96 women with abnormal cervical cytology and 20 with normal cytology for HPV-DNA testing and genotyping. Cervical swabs were taken after informed consent was obtained, and those cervical cells were collected in phosphate buffer saline and stored at -20°C .

DNA extraction. For DNA extraction, the samples were suspended in $300\mu\text{L}$ of proteinase K and incubated at 50°C for 2h, then treated with $100\mu\text{L}$ of 5-M NaCl. After centrifugation, the supernatant was treated with $900\mu\text{L}$ of ethanol. DNA precipitates were collected by centrifugation at 12,000rpm for 10min and washed with $400\mu\text{L}$ of 70% ethanol. DNA was dissolved in $100\mu\text{L}$ of TE.

High-risk human papillomavirus testing. In this study, HR-HPVDNA testing was performed in 96 women with abnormal cervical cytology and 20 with normal cytology; and their ages ranged from 18 to 69 years. HPV-DNA testing was performed using the polymerase chain reaction (PCR) method. Consensus sequence primer pairs within the E6 and E7 open reading frames, *i.e.*, forward primer (*pU-1M*): 5'-TG TCAAAAACCGTTGTGTCC-3' and reverse primer (*pU-2R*): 5'-GAGCTGT CGCTTAATTGCTC-3') (oligo@sigma genosys-PCR, Japan), were used to amplify HR-HPV (HPV-16, -18, -31, -33, -35, -52b, and -58) [8]. The reaction mixture included $0.15\mu\text{L}$ of taq polymerase (Applied Biosystems, Roche, CA, USA), $2\mu\text{L}$ 10X buffer, $3.2\mu\text{L}$ dNTPs, $0.4\mu\text{L}$ of forward and reverse primers, $12.85\mu\text{L}$ distilled water and $1\mu\text{L}$ DNA. They were subjected to 35 cycles of amplification using ASTEC thermal cycler. Each cycle included a denaturation, annealing and extension step. PCR using β -globin primers, *i.e.*, forward primer: 5'-GACACCATGGT GCACCTGAC-3' and reverse

primer: 5'-CCAATAGGCAGAGAGAGTCA-3'— was also performed for the detection of human DNA integrity. Detection of the PCR products was performed by electrophoresis on 6% polyacrylamide gel (PAGE), 200 V, 30 minutes and silver staining.

HPV genotyping. In PCR-positive cases, HPV genotyping was analyzed by the cleaved amplified polymorphic 6-sequence (CAPS) method. HR-HPV genotypes were detected by polyacrylamide gel electrophoresis and silver staining of the digestion of PCR product with restriction enzyme(s), *Ava* II (HPV-16, HPV-18 and HPV-33), *Rsa* I (HPV-31), *Bgl* II (HPV-52b), *Acc* I (HPV-58) and *Ava* I (HPV-35) (Wako, Japan). The enzymatic digestion was performed under the conditions recommended by the manufacturer [8].

Statistical Analysis. Data analysis was done using Microsoft Office Excel 2007 and the Statistical Package for Social Sciences (SPSS-15) *i.e.*, SPSS full-version with free download (<<http://en.softonic.com/s/spss-15-full-version-free-download/>>, accessed October 2012).

Results

A total of 1,771 women were screened for the cervical cancer from 2010 to 2011. Among them, 762 women (43.0%) had normal smears, and 866 (48.9%) and 87 (4.9%) were diagnosed as inflammatory smears

and atypical squamous cells of undetermined significance (ASCUS) respectively. In addition, LSIL and HSIL numbered 42 (2.3%) and 11 (0.6%) respectively. Three cases of cervical cancer (0.2%) were detected (Table 1).

High-risk human papillomavirus testing. HR-HPV DNA testing was performed in 116 women: 96 with abnormal cervical cytology and 20 with normal cytology, whose ages ranged from 18 to 69 years. All samples (100%) were amplified with β -globin primers which were used to ensure human DNA integrity (Fig. 1). Among them, 48 (41.4%) women were positive for HR-HPV PCR revealing a band around 240–260 bp (Figs. 2A, B). HR-HPV was identified in

Table 1 Cytology screening of women attending cervical cancer screening clinic, Department of Medical Research-Lower Myanmar in 2010–2011

Cytology report	No of cases	Percentage (%)
Normal Smear	762	43.0
Inflammatory Smear	866	48.7
ASCUS*	87	4.9
LSIL**	42	2.3
HSIL***	11	0.6
SCC****	3	0.2
Total	1,771	100

*Atypical squamous cells of undetermined significance; **Low grade squamous intraepithelial lesion; ***High grade squamous intraepithelial lesion; ****Squamous cell carcinoma.

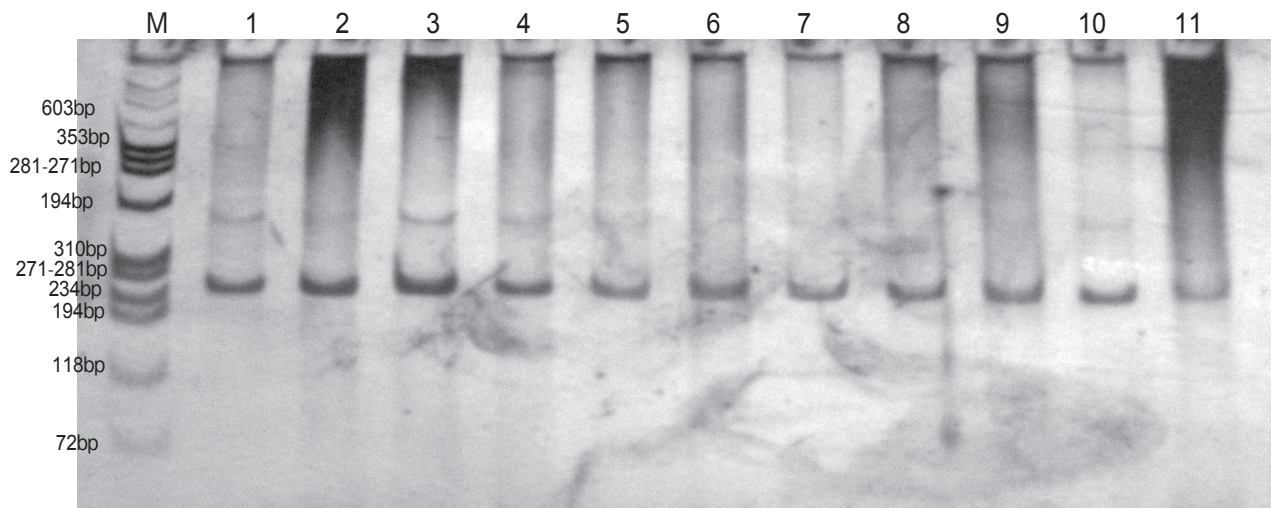


Fig. 1 Amplification of sample DNA using β globin primers M: molecular marker Φ X174/HaeIII digest; lane 1 to 11 HPV-positive human DNA.

48.9% (47/96) of subjects with abnormal cervical cytology and 5% (1/20) of those with normal cytology. Among women with abnormal cervical cytology, HR-HPV was determined in 35.5% (22/62) cases of inflammatory smear, 60% (6/10) cases of ASCUS, 86.7% (13/15) of LSIL, 50% (3/6) of HSIL, and 100% (3/3) of squamous cell carcinoma (SCC) (Table 2). The women with abnormal cervical cytology were 10 times more likely to be HR-HPV-positive than those with normal cytology ($P = 0.0001$).

HPV genotyping. Among 48 HPV-PCR-positive cases using *Ava* II digestion, 29 samples showed 2 fragments, about 155-bp and 80-bp bands, indicating the HPV 16 genotype, and 6 appeared in 170-bp and 90-bp bands, indicating the HPV 18 genotype (Fig. 3A), but 13 samples were undigested with *Ava* II, *Ava* I and *Bgl* II. These were further digested with *Rsa* I and *Acc* I. Seven samples digested using *Rsa* I showed a broad band with 2 overlapping fragments of ~119-bp and 114-bp regions, indicating the HPV-31 genotype (Fig 3B). Six samples using *Acc* I showed a broad band of overlapping ~126-bp and 118-bp regions, indicating the HPV-58 genotype (Fig. 3C).

The most prevalent HPV genotypes were HPV-16 (60.4%) followed by HPV-31 (14.6%), HPV-18 (12.5%) and HPV-58 (12.5%). HPV genotypes 16, 18, 31 and 58 constituted 11 (50%), 2 (9.1%), 5 (22.7%), and 4 (18.2%) of the 22 cases of inflamma-

tory smear, and 9 (69.2%), 3 (23.1%), 1 (7.7%) and 0% of the 13 LSIL cases, respectively. HPV-16 and -58 were present in 4 (66.7%) and 2 (33.3%) cases of the 6 women with ASCUS. All cases of HSIL were HPV 16. Among women with cervical cancer, 66.7% were genotyped as HPV 16 and 33.3% as HPV 18 (Table 3).

Most patients infected with HR-HPV were aged

Table 2 Proportion of high-risk human papillomavirus (HPV) genotypes in women with cervical cytological abnormalities

Cytology	HPV-PCR		Total
	Positive	Negative	
Normal	1 (5.0%)	19 (95.0%)	20
Inflammatory Smear	22 (35.5%)	40 (64.5%)	62
ASCUS	6 (60.0%)	4 (40.0%)	10
LSIL	13 (86.7%)	2 (13.3%)	15
HSIL	3 (50%)	3 (50%)	6
SCC	3 (100%)	0 (0%)	3
Total Count (% within cytology)	48 (41.4%)	68 (58.6%)	116

for abbreviation, see Table 1

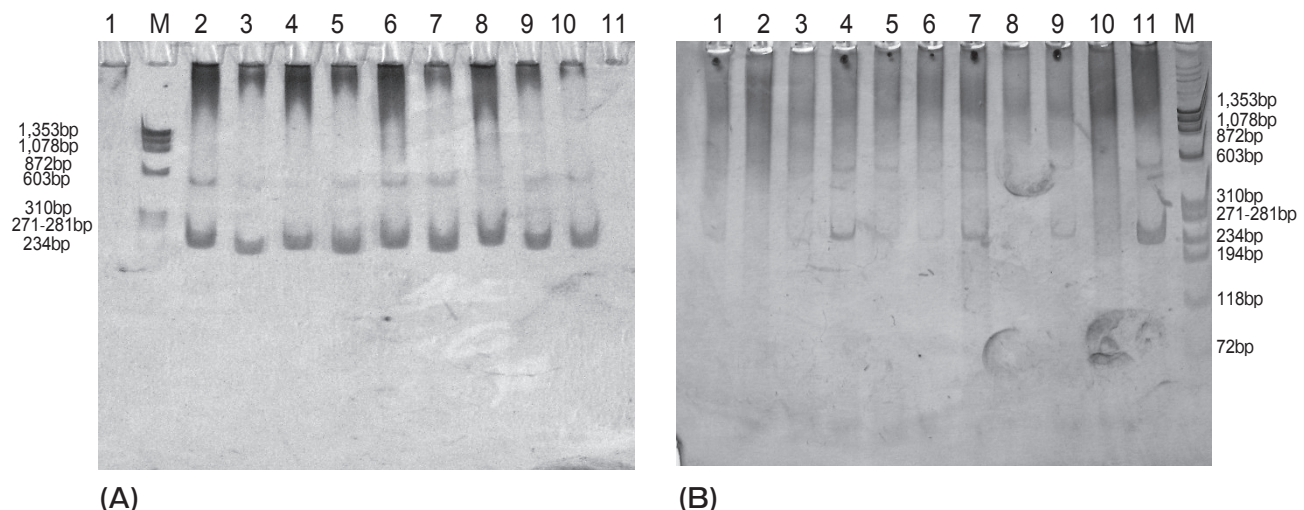
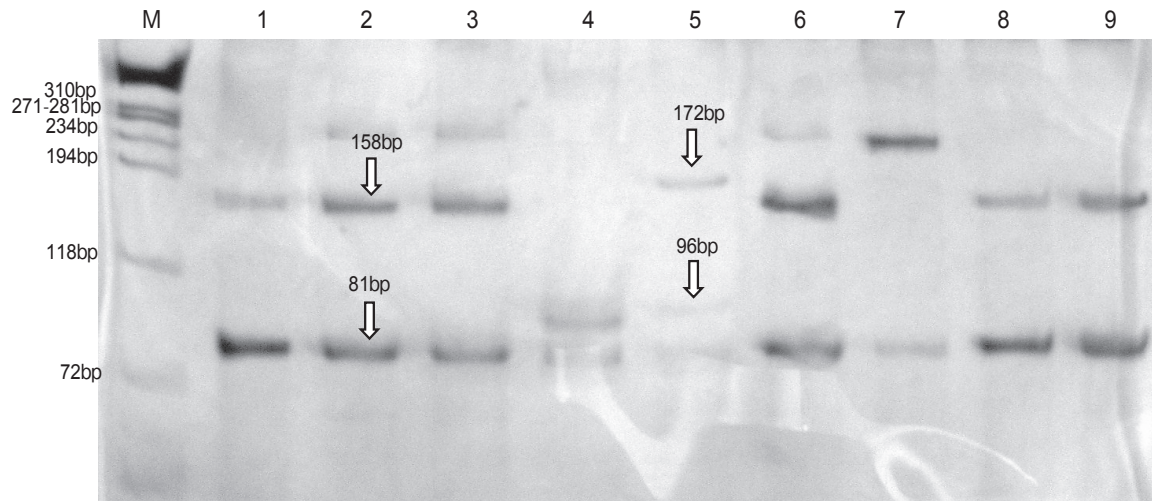
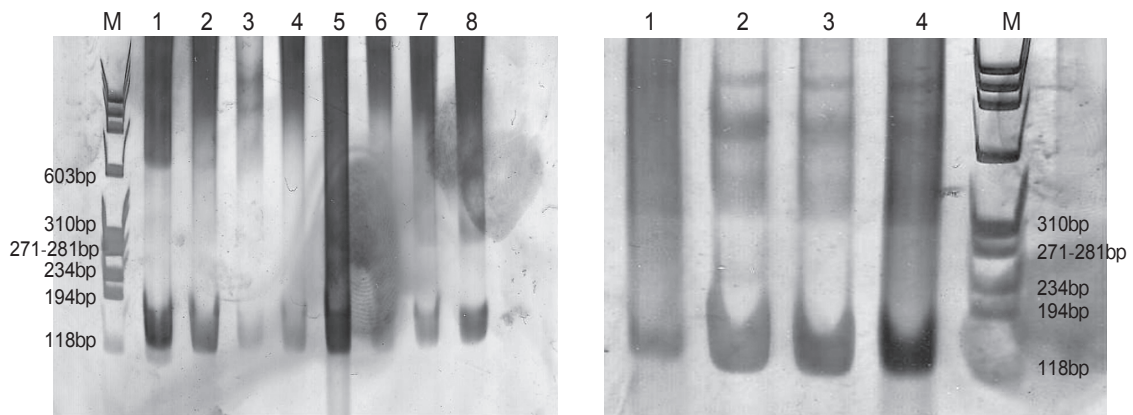


Fig. 2 Amplification of HPV using *pU1M/pU2R* primers showing lane M, molecular marker: Φ X174/*Hae*III digest; (A) lane 1-negative control, lane 2-positive control, lanes 3 to 10-positive HPV DNA, lane 11-negative HPV DNA (B) lane 10-negative control, lane 11-positive control, lanes-1, 3, 4, 5, 6, 7, 9-positive HPV DNA; lane 2, 8-negative HPV-DNA.



(A)



(B)

(C)

Fig. 3 HPV PCR products digested with restriction enzymes (A) *Ava* II showing lane 1-positive control of HPV 16, lane 2, 3, 6, 8, 9-HPV 16, lane 4, 5-HPV 18, lane 7-not completely digested; (B) *Rsa* I showing lane 1-positive control of HPV 31, Lanes 2 to 8-HPV 31; (C) *Acc* I showing lane 4-positive control of HPV 58, lanes 1, 2, 3-HPV 58; lanes M in all figures-molecular marker: Φ X174/*Hae*III digest.

Table 3 Proportion of high-risk human papillomavirus (HPV) genotypes in women with cervical cytological abnormalities

HPV genotypes	Age range (Years)					Total
	20-29	30-39	40-49	50-59	60-69	
HPV 16	1 (3.4%)	10 (34.5%)	13 (44.8%)	3 (10.3%)	2 (6.9%)	29
HPV 18	0 (0%)	0 (0%)	3 (50.0%)	3 (50.0%)	0 (0%)	6
HPV 31	1 (14.3%)	2 (28.6%)	2 (28.6%)	1 (14.3%)	1 (14.3%)	7
HPV 58	1 (16.7%)	2 (33.3%)	1 (16.7%)	1 (16.7%)	1 (16.7%)	6
Total (Count)	3	14	19	8	4	48
(% within genotype)	(6.3%)	(29.2%)	(39.6%)	(16.7%)	(8.3%)	

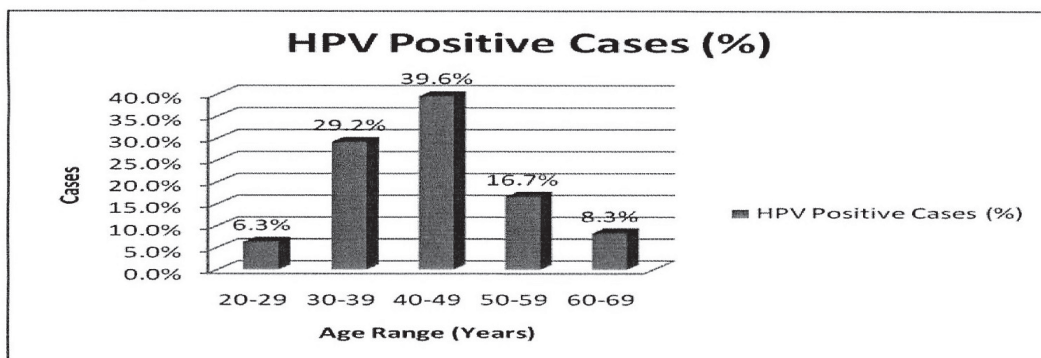
40–49 years (39.6%), followed by 30–39 years (29.2%), 50–59 years (16.7%), 60–69 years (8.3%) and 20–29 years (6.3%) (Fig. 4A). HPV-16 was highest among women 40–49 years (44.8%), followed by 30–39 years (34.5%) (Fig. 4B). As for HPV-18, the detection rate was the same for women 40–49 years and 50–59 years (Fig. 3B). HPV-31 was high in the age ranges of 30–39 years and 49–49 years (Fig. 3C).

HPV-58 was high in the age range of 30–39 years old (33.3%) and was essentially the same in all other age groups (Fig. 3D).

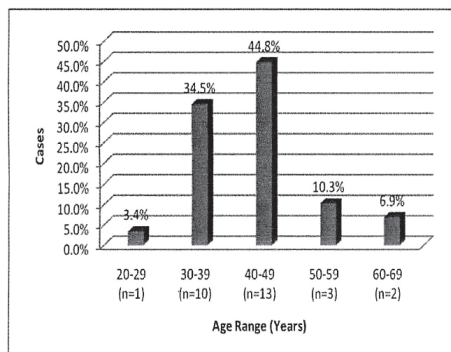
Discussion

The incidence of cervical cancer varies dramatically across the world, largely depending on the avail-

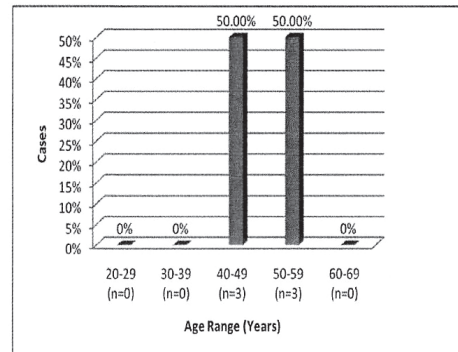
(A) HPV infection



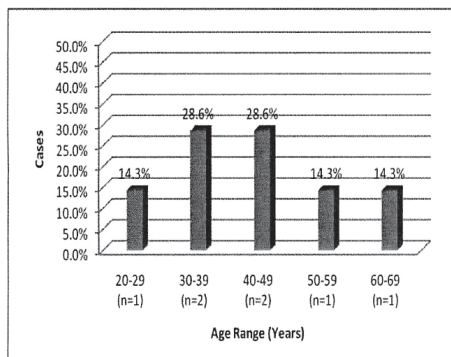
(B) HPV 16



(C) HPV 18



(D) HPV 31



(E) HPV 58

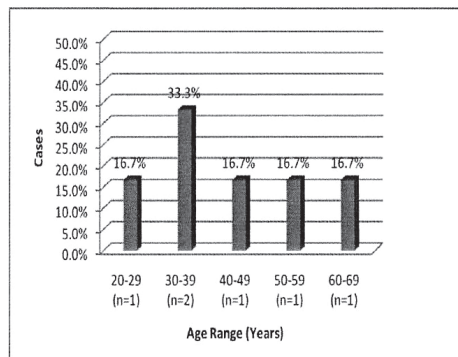


Fig. 4 Proportion of (A) HPV infection, (B) HPV 16, (C) HPV 18, (D) HPV 31, (E) HPV 58 in women with cervical cytological abnormalities by age group.

ability and accessibility of cervical screening programs. Most places in South America and South and West Africa have an age-standardized incidence above 20 per 100,000 women per year; some places in these regions have reached 40 per 100,000 women per year. In contrast, the age-standardized incidence rates are below 10 per 100,000 women per year in North America, Western Europe, Australia and New Zealand. Even within Asia, the age-standardized incidence varies substantially, with 9.6 per 100,000 women per year in East Asia, 15.8 per 100,000 women per year in South-Eastern Asia, 24.6 per 100,000 women per year in South-Central Asia and 4.5 per 100,000 women per year in Western Asia [9].

Human papillomaviruses have a small double-stranded DNA genome about 8kb in length. To date, more than 120 types of HPV have been well characterized, of which about 40 types can infect the genital tract [3]. About 15 types of these genital (mucosal) HPVs are classified as "high-risk" because of their oncogenic or possible oncogenic properties, either demonstrated by *in vitro* biochemical studies or inferred from epidemiological observations [10, 11].

HPV epidemiology can be undertaken in terms of type distribution of cervical HPV infection in women with normal cytology or in those with abnormal cytology. A meta-analysis of relevant studies about the worldwide prevalence and type distribution of cervical HPV DNA in women with normal cytology was recently published. In this manuscript, the overall HPV prevalence was estimated to be 10.4%. However, there were some differences depending on the region of origin. HPV-16 is the most common HPV type, and the 5 most common HPV types in women worldwide were HPV-16, -18, -31, -58 and -52, together comprising 50% of all HPV infections [7].

Zuna RE *et al.* [12] focused on the distribution of HPV genotypes in women with cervical lesions, and found that HPV-16 and -18 were the most frequent HPV types identified in invasive cancers (80%) but that the distribution patterns of HPV types in intraepithelial lesions were highly varied. Cobo F *et al.* [13] published HPV type distributions in females with abnormal cervical cytology, and showed that 75% were positive for HPV DNA and 23.7% were negative. HPV-16 was the most common type followed by HPV-58, -51, -33, -31 and -18.

Recently, Tachezy R *et al.* [14] reported that

HPV-16 was the most prevalent type both in precancerous lesions (45%) and squamous cell carcinomas (59%). HPV-16 and/or -18 was present in 76% of cervical cancer samples, 33% of CIN1, 43% CIN2 and 71% of CIN3. Takehara K *et al.* [16] showed that HPV genotypes were detected in 9.5% of women testing negative for intraepithelial lesion or malignancy (NILM), and 72.2% of ASCUS or other cervical lesions. In this study, HPV genotypes were as follows: HPV-52 at 26.6%, HPV-16 at 25.2%, HPV-58 at 21.8%, and HPV-18 at 7.1%.

As for Myanmar, Mu-Mu-Shwe *et al.* (2009, Myanmar Health Research Congress) reported that the prevalence of HPV in women with premalignant and malignant lesions of the cervix was 77 in 145 (53.1%). HPV was identified in 33.3% of LSIL, 60% of HSIL, 58.7% of SCC and 50% of adenocarcinoma of the cervix. HPV-16 was the predominant genotype, followed by HPV-31 and HPV-18. Thein Myint Thu *et al.* (2009, Myanmar Health Research Congress) reported that HPV DNA was detected in 27 of 131 (20.6%) women tested, including 17.6% of those with a normal smear, 77.1% with an inflammatory smear, 0.8% of LSIL and 4.6% with an unsatisfactory smear.

In the present study, 47 of 96 (49%) women with abnormal cervical cytology and 1 of 20 (5%) with normal cytology were positive for high-risk human papillomavirus (HR-HPV). HPV was identified in 35.5% of those with an inflammatory smear, 60% of ASCUS, 86.7% of LSIL, 50% of HSIL and 100% of SCC. The prevalence of HPV in the present study was much higher than that in the previous one (86.7% vs. 33.3% in LSIL, 100% vs. 58.7% in SCC samples. The highest percentage of women with positive results for HR-HPV was found in patients with LSIL and cervical cancer. Therefore, HPV DNA testing could be especially useful for triage of low-grade smears to improve the sensitivity of cytology alone and select women at greater risk who require colposcopy.

The most prevalent HPV genotype in this study was HPV-16, one of the vaccine-preventable HPV genotypes (60.4%), which was consistent with other studies mentioned above, followed by HPV-31 (14.6%), HPV-18 (12.5%) and HPV-58 (12.5%). Among cervical cancer cases, 66.7% were genotyped as HPV-16 and 33.3% as HPV-18. Clinical studies of HPV vaccines have demonstrated close to 100% protection against HPV-16 and HPV-18 related infections and

diseases, implying potential cross-protection against HPV-31, -33, -45, -52, and -58 [16, 17].

In this study, most patients infected with HR-HPVs and the HPV-16 genotype were ages 40-49 years, followed by those in the 30-39 age range. As for HPV-18, the detection rate was the same in the 40-49 and 50-59 age ranges. HPV-31 was highest for ages 30-39 and 49-49. HPV-58 was highest in ages 30-39 years. A study by Takehara K *et al.* [15] reported that most patients infected with HPV-16 were 20-29 years, decreasing with age thereafter. HPV prevalence by age revealed higher infection rates in young women aged 15-25 years with a second peak in women 55 years or older.

The analysis described here does not need isotopes and is therefore convenient, especially where facilities to handle radioactive substances are not available. The procedure is simpler than dot blot hybridization and can be performed within a day, although this system is less sensitive than dot blot hybridization using isotopes. Digestion of PCR products with restriction enzymes could identify HPV-specific genotypes efficiently in clinical samples. In the same way, any combination of mixed infection could be identified because restriction fragments from each HPV type are of different sizes. But the decision of HPV genotypes by the PCR-RFLP method alone is sometimes difficult since various nucleotide replacements frequently occur in the sequence of HPV DNA. It is considered that the sequencing method might be a better way to genotype HPV. This restriction enzyme analysis method followed by sequence analysis is particularly useful for identifying potentially new HPV types.

HPV infections occur with a high attack rate soon after sexual initiation. Follow-up studies in several countries of virgins after their sexual debut have shown up to 70% of women becoming HPV DNA positive at least once within 48 months. The cumulative lifetime exposure to HPV has been estimated to be close to 80%; for HPV-16 or -18 it is 20%. Thus, primary prevention with HPV vaccines should focus on the years before sexual initiation, in adolescent and pre-adolescent age groups [18].

In the future, HPV DNA testing in conjunction with cervical cytology testing could be used for cervical cancer screening or in a follow-up program after conservative treatment of cervical lesions since 35.5% of inflammatory smears, 60% of ASCUS and 86.7%

of LSIL were HR-HPV positive. If no intervention is implemented in the near future, a dramatic increase in the numbers of cervical cancer cases is predicted. HPV vaccines offer an efficient way to prevent HPV-related cervical cancers. Results of this study together with those of several other studies help us to estimate the potential benefit that could be achieved by the implementation of routine vaccination for the prevention of HPV-associated cervical cancer in Myanmar.

In conclusion, the most prevalent HPV genotypes in this study were HPV-16, followed by HPV-31, HPV-18 and HPV-58. Among cervical cancer cases, the vaccine-preventable HPV genotypes, *i.e.* HPV-16 and HPV-18, comprised 66.7% and 33.3%, respectively. Women with abnormal cervical cytology were 10 times more likely to be HR-HPV positive than those with normal cytology. This study suggests that the implementation of a routine vaccination program against HPV in preadolescent and adolescent age groups would greatly reduce the burden of HPV-associated cervical cancer in Myanmar.

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