RESEARCH ARTICLE

Detection of Human Papillomavirus DNA in Routine Cervical Scraping Samples: Use for a National Cervical Cancer Screening Program in a Developing Nation

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Abstract

Background: Human papillomavirus is a well-established cause of the development of a variety of epithelial lesions in the cervix. However, as yet, incorporation of HPV testing into cervical cancer screening either as an adjunct or stand alone test is limited due to its cost. We therefore here ascertained the presence and type specificity of human papilloma virus (HPV) DNA in routine cervical scrapings. Materials and Methods: Cervical scrapings were collected from women attending clinics for routine Pap smear screening. HPV-DNA was detected by PCR using MY09/11 and GP5+/GP6+ primer sets and genotyping was accomplished by cycle-sequencing. Results: A total of 635 women were recruited into the study with mean±SD age of 43±10.5 years. Of these 92.6% (588/635) were reported as within normal limits (WNL) on cytology. The presence of HPV infection detected by nested MY/GP+-PCR was 4.4% (28/635). The overall prevalence of high-risk HPV (HR-HPV) in abnormal Pap smears was 53.8% (7/13). HPVs were also seen in 3.1% (18/588) of smears reported as WNL by cytology and 5.9% (2/34) in smears unsatisfactory for evaluation. Conclusions: The overall percentage of HPV positivity in routine cervical screening samples is comparable with abnormal findings in cytology. Conventional Pap smear 'missed' a few samples. Since HPV testing is expensive, our results may provide valuable information for strategising implementation of effective cervical cancer screening in a country with limited resources like Malaysia. If Pap smear coverage could be improved, HPV testing could be used as an adjunct method on cases with ambiguous diagnoses.

Keywords: HPV-DNA detection - cytology - PCR - cervical cancer screening - developing country

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Introduction

Incidence of cervical cancer in developing countries is common where its accounts for 15% of female cancers with a risk of 1.5% at age below 65 years old (Parkin and Bray, 2006). As for developed countries, cervical cancer accounts for only 3.6% of new cancers, with a cumulative risk of 0.8% (<65 years) (Parkin and Bray, 2006). Cancer of the cervix was the third most common cancer among women and fifth most common cancer in the entire general population of Malaysia (NCR, 2011). There are about 9 million women eligible to be screened [age 22-60 years] for cervical cancer and the presence of abnormal smears in routine cervical scrapings cytology accounts to 4-7%. Cervical cancer incidence rate increased after 30 years old and peaks at ages 65-69 years. Currently, though there is no national screening program available, around 30-40% coverage is achieved by opportunistic screening.

HPV infection is now a well-established causal agent in the development of a variety of epithelial lesions, which range in severity from benign warts to invasive cervical cancer (Carter et al., 2011). More than 90% of invasive cervical cancer specimens contain HPV-DNA with approximately 5% may be unrelated to HPV infection (Janicek and Averette, 2001; Bhatla et al., 2008). HPV prevalence among countries may vary considerably across studies. This is due to the insufficient degree of geographical coverage and sample size, diversity in the techniques used for HPV detection and different methods used for estimating HPV type-specific prevalence (Bosch et al., 2008; Giuliano et al., 2008). The adjusted global prevalence of HPV infection was 10.4% (Burchell et al., 2006). The overall HPV-DNA prevalence in invasive cervical cancer is 96%, based on pooled analysis of 12 studies (Clifford et al., 2006b). The prevalence of HPV types that cause cervical cancer are; HPV-16 (53.5%), -18 (17.2%), -45 (6.7%), -31 (2.9%), -33 (2.6%), and other high-risk types; -52, -58, -35, -59, -56, -39, -51, -73, -68 and -66 (15-20%) (Clifford et al., 2006a).

Complete data is not yet available on HPV burden in

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general population of Malaysia. However, according to the South-Eastern Asia HPV prevalence, about 6.2% of women are likely to harbour cervical HPV infection at any given time (Castlellsagué et al., 2007). A report issued by HPV Information Centre (WHO/ ICO, 2007) showed that the HPV prevalence in Malaysia in cervical cancer cases (n=23) was 95.7% (95%CI: 78.1-99.9). Domingo and co-workers (2008) reported that, HPV-16 and -18 were the two most common HPV types in Malaysia (excluding East Malaysia) contributing 73.9% and 65.2% of total HPV prevalence, respectively. Whilst, HPV-31 contributes 13% followed by HPV-33 which accounts for 4.3% from a total of 23 cases of cervical cancer (Domingo et al., 2008).

The purpose of our study were: a) to ascertain the presence of HPV infection in routine cervical samples from women of North-Eastern region of West Malaysia; b) to determine the feasibility of detecting the presence of HPV-DNA in routine cervical sampling taken as for conventional Pap smear; c) to ascertain the number of 'missed cases' in which the cytology diagnosis is Within Normal Limits (WNL) or Unsatisfactory for Evaluation (USFE) while the cervical samples contain high risk HPV sub-types.

Materials and Methods

Cervical samples

The subjects were women volunteers who attended three main hospitals in North-Eastern region of West Malaysia [Hospital Kota Bharu (HKB), Hospital Universiti Sains Malaysia (HUSM) and Hospital Kuala Terengganu (HKT)] for routine cervical cancer screening. Conventional Pap smear was collected and immediately smeared on slide, alcohol-fixed, stained with Papanicolaou stain and read by cytologists according to the Bethesda System 2001 (TBS, 2001). Immediately afterwards, the same cervical broom used was cut off and inserted into liquid solution (preservative solution) for HPV study. This study was approved by the local institutional human ethics review board.

HPV-DNA detection and typing

Genomic DNA was extracted from preservative solution using QIAamp DNA mini kit (QIAGEN, Hiden, Germany) according to the manufacturer's instructions.

The HPV-DNA detection was carried out by two-tube nested PCR. The first PCR (using MY primer) assay was carried out containing 5U Taq DNA Polymerase (0.05U), 10X Buffer, 200mM dNTP, 1.5mM $MgCl_2$ (Fermentas) and 0.5 mM of each of primer. The nested-PCR (using GP+ Primer) master mix used was as above except 1 μl of first nested-PCR was added as a template and 3.0mM $MgCl_2$. The cycle conditions for first PCR were as follows: denaturation of the template DNA for 1 cycle of 95°C for 3 min, amplification of the target DNA for 30 cycles of 95°C for 30s, 53.1°C for 30 s and 72°C for 30s; and a final extension for 1 cycle of 72°C for 7 min. The cycle condition for nested PCR was similar as above except the annealing temperature was 42.3°C. PCR amplification was carried out in a DNA thermal-cycler (Eppendorf AG, Hamburg, Germany). The amplified DNA was visualized on ethidium bromide stained 1.5% agarose gel after electrophoresis. Positive controls and negative water blanks were included in each run for quality control. HPV testing and smear interpretation were blinded.

HPV positive samples were subjected to cycle-sequencing using BigDye terminator v3.1 cycle sequencing mix (Applied Biosystems). It was analyzed using BioEdit Sequence Alignment Editor version 7.0.5.3. Sequences were submitted to the NCBI nucleotide–nucleotide BLAST (blastn) website (online alignment analysis) (http://www.ncbi.nlm.nih.gov/BLAST/) to arrive at specific genotyping.

Classification

The HPV types were classified into three categories; high-risk oncogenic HPV (HR-HPV:-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, -82), low-risk oncogenic HPV (LR-HPV:-6, -11, -40, -43, -44, -54, and -70) and undefined HPV risk (either HR or LR).

Results

Participant characteristics

A total of 635 women were recruited into the study with mean±SD age of 43±10.5years. They were stratified into four age groups, as follow: under 35 years (24.4%), between 35-44 years (30.9%), between 45-54 years (34.3%) and older than 55 years (10.4%). The ethnic grouping of the women studied were predominantly Malays (85%), followed by Chinese (10.4%), Indian (0.6%) and other ethnics (3.9%) comparable to the ethnic proportion of these three states in Malaysia.

HPV positivity and cytology diagnosis

HPV-DNA was detected in 24 of 635 cervical samples in the first PCR. Of 635 cervical samples tested, 25 samples showed visible band in nested PCR. A total of 28 samples were considered HPV positive by both PCR (Table 1). Overall, pathologic findings were observed in 11 (1.7%) cases: 8 (72.7%) with low-grade squamous intraepithelial lesions (LSIL) and 3 (27.3%) with invasive cervical cancer. Two (0.3%) cases of Atypical Squamous/ Glandular Cell of Undetermined Significance (ASCUS/ AGUS) were identified. Smear within normal limit (WNL) was detected in 588 (92.6%) samples with 34 (5.4%) cases of 'unsatisfactory smear for evaluation (USFE)

Table 1. HPV Positivity in Different Lesions Diagnosed by Conventional Pap smears

Cytology (No. of Cases)	No.	%
WNL (588)	18	3
AS/AG-CUS (2)	1	50
LSIL (8)	3	37.5
ICC (3)	3	100
USFE (34)	2	6
Total (635)	28	4.4

*WNL: Within Normal Limit; AS/AG-CUS: Atypical Squamous/Grandular Cell of Undetermined Significance; LSIL: Low-grade Squamous Intraepithelial Lesion; ICC: Invasive Cervical Cancer; and USFE: Unsatisfactory Smear for Evaluation

Table 2. Performance of the PCR in Detecting HPVs in Normal and Abnormal Cases of Conventional Pap smear

Detection method		Conventional Pap smear		Sens (%)	Spec (%)	PPV (%)	NPV (%)	FN rate (%)	FP rate (%)	Agreement (%)	Kappa (%)	χ^2 test
		Normal	Abnormal									
PCR	Neg	602	5	61.5	96.8	28.6	99.2	38.5	3.2	96.1	0.37	p<0*
	Pos	20	8									

*Test statistically significant (p value<0.05). Neg., Negative; Pos., Positive; Sens., Sensitivity; Spec., Specificity; PPV, Positive Predictive Value; NPV, Negative Predictive Value; FN, False Negative; FP, False Positive; and χ2 test, Chi-Square Test. [Normal means the cytology diagnosis is WNL and Abnormal is when cytology diagnosis AS/AG-CUS, LGSIL or higher]

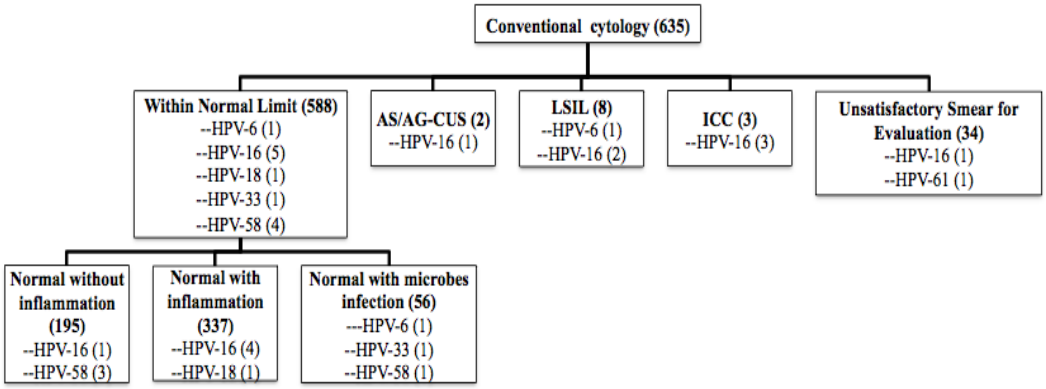

Figure 1. HPV Genotype Distribution within Cytological Groups. *Legends: AS/AG-CUS, Atypical Squamous/Grandular Cell of Undetermined Significance; LSIL, Low-grade Squamous Intraepithelial Lesion; HPV, Human papillomavirus; ICC, Invasive Cervical Cancer; and USFE, Unsatisfactory Smear for Evaluation

(Table 1). Included in the category of "unsatisfactory smear for evaluation (USFE)" were broken slides, scanty squamous cells (if cells are less than 10% of the smear), cells obscured by blood, thick smears, poor fixation, air-drying artefact or lack of endocervical cells/transformation zone component.

HPV types-distribution in positive samples

Seven PCR positive samples were weakly positive after gel electrophoresis and failed in sequencing . Of 28 nested-PCR positive samples, 21 exhibited 6 different genotypes; 85.7%(18/21) were HR-HPVs and 14.3%(3/21) were LR-HPVs. HPV infection was found in 2.1%(4/195) and 1.5%(5/337) among women with normal without and with inflammation on cytology respectively. Among women with microbes infection seen in cytology, 5.4%(3/56) was HPV positive. HPVs was also found in 5.9%(2/34) of smears unsatisfactory for evaluation. Additionally, 37.5%(3/8) and 50% (1/2) were HPVs positive respectively among women with Low-grade Squamous Intraepithelial Lesion and Atypical Squamous/Glandular Cell of Undetermined Significance. All Invasive Cervical Cancer cases were positive for HPVs(100%). HPV-16 was by far the most prevalent genotype 57.1%(12/21) of all HPVs, representing 1.9%(12/635) of total samples, followed by HPV-58 (19.0%; 4/21); and HPV-6 (9.5%; 2/21). There were equal incidences of HPV-18, 33 and 61(4.8%; 1/21). Type-distribution of HPVs in different cytological diagnosis of conventional Pap smear is shown in Figure 1. Thirteen out of 635 cases (2.0%) were found to be abnormal during classification of the conventional Pap smear into normal [cytology diagnosis is WNL] and abnormal cases [Cytology diagnosis is AS/AG-CUS, LSIL and higher]. Of this, 61.5% (8/13) was HPVs positive (Table 2).

Discussion

This is a first large scale HPV study on women population conducted on women populations of the North-Eastern region of West Malaysia, who are predominatly Malays unlike the central and west coast region of the country. Indian women had the highest incidence rate for cervical cancer followed by Chinese and Malay (NCR, 2011) these states. We noted that among the women with normal cytology, 3.0% harboured HPV-DNA, of which 91.7% were HR-HPV. Conventional Pap smear 'missed' to detect high-risk type of HPVs in a small proportion of samples. Our present HPV burden in normal population was comparable with various countries in Europe (1.4-9.2%) (Clifford et al., 2006b). However, it was lower than those generally observed in France (20.2%) (Casalegno et al., 2011), USA (27%) (Evans et al., 2006) and worldwide (10.5%) (Clifford, et al, 2006a).

There were only two cases of ASCUS/AGUS in this study and HPVs (HPV-16) was noted in the AGUS sample not in the ASCUS sample. HPV in ASCUS samples had been reported worldwide in variable proportions; 33% by Boulanger et al. (2004); 42% by Clifford et al. (2006a) and 89.5% by Evans et al. (2006). This could mean that we are more stringent in making diagnoses of ASCUS and AGUS. Lower HPV prevalence in ASCUS/AGUS had previously been reported in United States (4.1%) (Stoler et al., 2011). In our study, 37.5% of cases diagnosed as LSIL in cytology had HPVs. The presence of HPVs in such lesions seen by other researchers is also variable; ranging from 50% to above 90% (Clifford, et al, 2006a; Evans, et al, 2006). As in other studies, we noted high-risk HPV in all cases (albeit a small number) of invasive cervical cancer confirming an established fact that there is a strong relationship of HPVs and cervical cancer.

However, in Hanoi, Vu et al. (2012) reported only 91.3% HPVs infection of their cervical cancer cases. Such low number we believe could be due to technical issue rather that the real prevalence of HPV in cervical cancer.

Our HPV type distributions in various lesions, based on the nested PCR plus sequencing were heterogeneous and in agreement with previous studies elsewhere in Asia and Europe (Speich et al., 2004). HPV-16 is known to be the most prevalent genotype in Southeast Asia and the rest of the world, regardless of the cytological status except in Indonesia and the Philippines (Clifford, et al, 2006a; Domingo, et al, 2008). Our HPV16 prevalence (57.1% of all HPVs) is lower than the values reported by Domingo and co-workers (2008); 73.9%. Interestingly, we found that HPV58 is the second (19.0%) most prevalent types followed by HPV6 (9.5%) and HPV18, 33 and 61 (4.8% each).

Domingo and co-workers (2008) reported that the second most prevalent types of HPV in Malaysia were HPV18 (65.2%) followed by HPV31 (13.0%) and HPV33 (4.3%). The biased result might be due to the samples they studied. Our samples came from women who attended gynae clinics for screening whilst Domingo's study involved samples of cervical cancer. Furthermore they did not include populations from the East Coast of West Malaysia. Due to the low number of cases of high- and low-grade lesions obtained in samples recruited, we were unable to determine the real HPV type specific prevalence and type-distribution in respective lesion. In 2009, one study from Malaysia reported the prevalence of HPV in abnormal cervical lesions as 94.7% (Sharifah et al., 2009). In our study all the cervical cancer cases were HPV positive.

These findings highlight the presence of various high-risk HPV types in different cervical lesions. It may estimate the theoretical fraction of cervical cancer which could be prevented. Until recently, cytology-based screening programmes (using Pap smears) were the main tool to prevent cervical cancer. Well-organized programmes established in developed countries are able to detect and identify precancerous lesions at the early stages as they can easily be treated thus preventing up to 80% of cervical cancers. The programmes incorporate HPV testing in their routine cervical cancer screening. However, many countries which have high prevalence of cervical cancer are developing nations thus including HPV testing in the screening program not feasible. Having said that if pap smear coverage could be increased, the incidence of cervical cancer could be alleviated. A cost of one pap test is around one-tenth of HPV test.

The limitation of our study is in the number of cases recruited. The number was limited by the cost of HPV detection based on PCR and DNA sequencing. Nonetheless, the results of this study will provide the clinicians and pathologists with relevant information about HPV infection HPV types among populations of the North eastern region of Malaysia. The results may also give implication whether current HPV vaccine available is useful or otherwise.

In conclusion, our study showed significant findings which could be used in strategising and implementing an effective cervical cancer screening program in a developing nation such as Malaysia. This is the first study done to determine the burden of HPV infection in routine conventional Pap smear. The technique we used for HPV detection was robust and allowed inter-lab comparison. We had shown the possibility that some cases diagnosed as within normal on cytology also contained HR-HPV. These cohorts of subject were likely to be grouped together with subjects who were diagnosed WNL but negative for HR-HPV thus might miss the chances of getting optimal follow-up. This study also demonstrates the use of robust PCR technique on residual material obtained from the brush of cervical samples used for routine conventional smears is capable in detecting the presence of HR-HPV even when the viral load is low. Such technique could be used as adjunct in routine practice.

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