

## A comparative study of a Multiplex assay with Conventional in-house PCR assay for HPV detection and subtyping in archived ASCUS samples

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### Abstract

**Objectives:** To evaluate a Multiplex assay in the sub-typing and detection of local prevalence of Human Paillomavirus (HPV). **Materials and methods:** One hundred and thirty six archived ASCUS cytology samples, provided by Biotechnology Limited were included. DNA was extracted using NucliSENS Magnetic system (Biomerieux, France). The assay makes use of the SNIPER HPV (Genetel, Shenzhen) genotyping diagnostic kit to detect 29 common HPV subtypes, including 18 high risk (HR) types. PCR conditions were performed according to method previously described.<sup>1</sup> **Results:** For Multiplex assay, a total of 61/136 cases (44.9%) of HPV subtypes were identified. 40/61 (65.6%) cases were infected by a single HPV subtype whereas 21/61 (34.4%) cases were infected by 2 or more HPV subtypes. Two samples were co-infected containing 4 HR HPV (16, 33, 52, 59 and 16, 51, 57 and 68). A total of 23 HPV subtypes containing 14 HR were detected by Multiplex assay. HPV subtypes 52 was the most prevalent 13/61 (21.3%), followed by subtype 16 (11.5%), subtype 18 (11.5%) subtypes 54 (9.8%) and subtypes 59 (9.8%) respectively. A peak age group of HPV infection was identified in 40-49 (mean= 44.7 years, median = 45 years) which correlated with previous study.<sup>1</sup> **Conclusion:** The in-house conventional PCR assay in the present study is sensitive for HPV DNA detection in general but failed to identify HPV subtype other than 6, 11, 16, 18, 31 and 33. The Multiplex assay is specific for the 29 HPV subtypes but did not cover the existing undefined HPV subtypes in ASCUS+ cytology specimens in Hong Kong population. The prevalence is in the order of subtypes 52, 16, 18, 54, 59, 11, 53, 58, 39, 44, 51, 40, 42, 43, 68, 6, 33, 56, 31, 35 and 45. HPV 52 is a significant local HPV subtype. A peak age-group of HPV infection was identified in 40-49. In the future, the vaccine company could consider the findings for further improvement in vaccine development.

**Key words:** Atypical Squamous Cell of Undetermined Significance (ASCUS), Cervical Cancer (CC), Human Papillomavirus (HPV), Cytology

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## Introduction

Human papillomavirus (HPV) is belongs to family member of Papillomaviridae, which consists of a circular genome size of 8kb with eight open reading frames (ORFs). Viral L1 and L2 gene codes for the outer viral capsid, whereas six early gene codes for E1 E2 E4 E5 E6 E7 proteins.<sup>2</sup> Among them, the main pathological HPV proteins in human are E6 and E7. They are known as onco-proteins which favours neoplasm formation by down-regulating host immune system.<sup>2</sup> HPV can cause a numbers of genital diseases ranging from warts to ano-genital cancers. Currently there are more than 100 types of HPV identified and classified as high risk (HR) type and contrast to the low risk (LR) types of HPV (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81).<sup>3</sup>

Although most are harmless, about 40 types are believed to infect the ano-genital tract. Fifteen of them (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) are known to be associated with high grade squamous intraepithelial lesion (HGSIL) and even cancer.<sup>2,3</sup> The transmission of the virus is by sexual intercourse. Other risk factors include multiple sex partners, prolonged use of oral contraceptive pills and smoking habit, etc.<sup>4</sup> The exact mechanism of carcinogenesis is complex and not well understood but E6 is thought to interrupt the tumor suppressor

p53 protein, Rb gene, cyclin-CDK complex in human resulting in losing the control of cell cycle arrest, apoptosis. E7 induces gene expression which is the perquisite of cell cycle progression and DNA synthesis via E2F proteins, promoting pathological cell growth.<sup>1,5</sup> Most HPV infections have no signs or symptoms. Most infections are transient and can be resolved within two years without treatment. However, some infections persist and may progress to cervical pre-cancer or cancer due to persistent oncogenic infections and integration of viral DNA into host genome.<sup>4</sup> However, it usually takes many years for HPV infection from mild inflammatory changes to cervical cancer stage. If being untreated, patients have a tendency to progress to high grade cervical intraepithelial neoplasia (HGSIL) and eventually cervical cancer (CC). There were approximate 630 million people being infected and type 16 and 18 were the most frequently detected in cervical cancer. They were classified as human carcinogens by the International Agency for Research on Cancer. Among all HPV types, HPV 16 contributed about 50% to 55% of all cases of squamous cell carcinoma and play important role in persistent infections and cell transformation.<sup>6</sup> According to the statistical Report of Hong Kong Cancer Registry 2011, CC ranked the 9th most common incidence (391 cases) and 8th commonest

cancer-causing death of women (151 cases) in Hong Kong.<sup>7</sup> Parkin DM (2006) found out that HPV is the second most common type of infectious cancer-agent in human, just followed by *Helicobacter pylori*.<sup>8</sup> Another recent report by World Health Organization, cervical cancer is the second most common malignancy in women worldwide, In 2013, there were an estimated 530,000 of new cases, of which over 85% occurred in developing countries, causing around 270 000 death.<sup>9</sup> This indicates cervical cancer contributes significantly in mortality rate in developing countries and there is a strong association between HPV and cervical cancer.

The aim of the present study is to evaluate multiplex HPV assay in the detection of local prevalence of HPV subtypes. Moreover, its sensitivity and specificity is compared with conventional PCR assay, using archived liquid based samples with ASCUS cytology.

## Materials and Methods

### *Study samples*

One hundred and thirty six archived Thinprep samples, provided by Biotechnology Limited were included. They were all diagnosed with ASCUS cytologic findings. Patient age were between 30 to 92 years old (means = 44.7 years, median 45 years). The time frame of the sample collection was between 2009 and 2010. Since the study uses the same batches of archived samples and is just an extension of

the former study<sup>1</sup>, ethical approval was obtained and already approved by the Ethical committee of HKU SPACE and Edinburgh Napier University. The results of former study did not affect the clinical management of the referring doctors.

### *DNA extraction/purification*

HPV DNA was extracted from thinprep samples using NucliSens Magnetic extraction system (Biomerieux, France) (Koidl C, 2008) according to manufacturer's instruction. The method is suitable for DNA purification in liquid-based cytology media for in vitro diagnostic use.

### *PCR reaction and hybridization*

In order to detect all mucosal HPV type, consensus primers are designed to direct the conserved region of the L1 gene. The Multiplex assay makes use of the SNIPER HPV Genotyping Diagnosis Kit (Genetel Shenzhen) for PCR reactions. Twenty-nine HPV genotypes are covered including HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 67, 68, 69, 73 and 82. The reactions are performed according to manufacturer's instructions. Briefly, L1 gene of HPV are amplified with biotinylated primers at 50°C for 5 min, 95°C for 10 min and then followed by 45 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 45 sec and extension at 65°C for 30 sec. The reaction is further amplified at 65°C for 5 min. The final PCR products are added to the membranes, hybridize to HPV type specific bead bound probes, followed by washing and labeling with streptavidin

(similar to sandwich ELISA) and finally the color signals developed, after the addition of substrate, and are scanned using GenoCam-9600 scanner.

#### Data analysis

The archived samples were divided into three age groups in this study: 30-39 years; 40-49 years and  $\geq 50$  years for further analysis. The McNemar statistical test was used to compare the effectiveness of the two

PCR assays at identifying the virus types. All statistical testing was two-tailed. A P-value of  $<0.05$  at 95% confidence level was regarded as statistically significant.

#### Results

##### HPV prevalence

By means of the Multiplex assay, there were 61/136 cases (44.9%) with HPV DNA identified regardless of HR/LR subtypes. A

**Table 1: Prevalence of HPV +ve subtypes of both assays in 136 study samples**

Methods	Total HPV +ve Cases	Prevalence of HPV +ve Subtypes <sup>a</sup>	LR HPV Infection <sup>a</sup>	HR HPV Infection <sup>a</sup>	2 to 3 HPV Co-infection	>4 HPV Co-infection
In House PCR Assay	n = 125 (125/136)	HPV 16 8.8% (11/125) HPV 18 6.4% (8/125) HPV 33 6.4% (8/125) HPV 31 3.2% (4/125) HPV 11 0.8% (1/125) Undefined Subtypes 74.4% (93/125)	0.8% (1/125) <sup>b</sup>	24.8% (31/125) <sup>c</sup>	1.6% (2/125)	
Multiplex Assay	n = 61 (61/136)	HPV 52 21.3% (13/61) HPV 16 11.5% (7/61) HPV 18 11.5% (7/61) HPV 54 9.8% (6/61) HPV 59 9.8% (6/61)	55.7% (34/61) <sup>d</sup>	77.0% (47/61) <sup>e</sup>	31.1% (19/61)	3.3% (2/61)

<sup>a</sup>No of HPV subtypes in multiple infection were counted more than once.

<sup>b</sup>LR Subtypes In House PCR Assay include 6 and 11.

<sup>c</sup>HR Subtypes In House PCR Assay include 16, 18, 31 and 33.

<sup>d</sup>LR Subtypes In Multiplex Assay include 6, 11, 40, 42, 43, 44, 54, 55, 57, 67 and 69.

<sup>e</sup>HR Subtypes in Multiplex Assay include 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82.

total of 23 different subtypes including 14 HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68) and 9 LR-HPV (6, 11, 40, 42, 43, 44, 54, 57, 67) were detected. The most prevalent HPV genotypes were in order of 52 (21.3%), HPV 16 (11.5%), HPV 18 (11.5%), HPV 54 (9.8%), HPV 59 (9.8%) and were summarized in Table 1 and Figure 1. The percentages of LR and HR subtypes were 55.7% (34/61) and 77.0% (47/61) respectively. Table 2 showed the types identified for each case for multiplex assay.

#### *Single vs multiple infections*

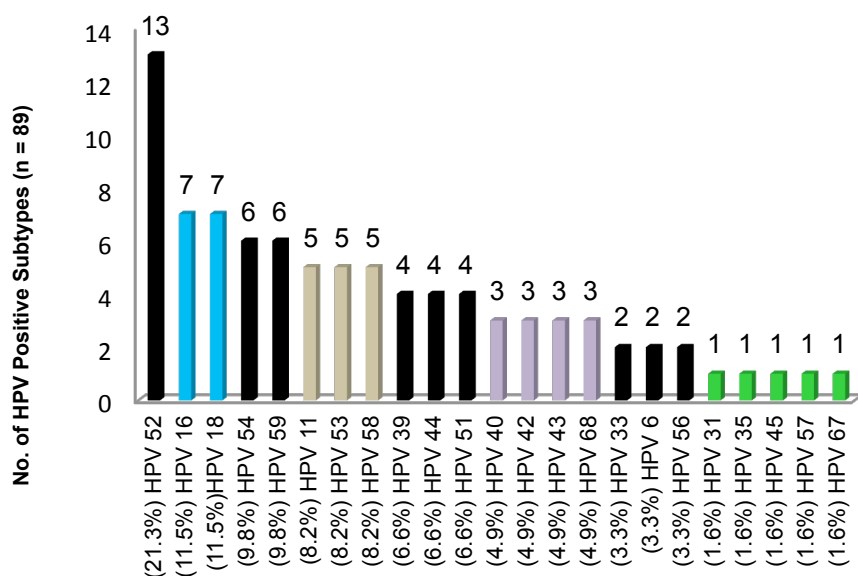
There were (40/61) 65.6% cases were infected by single type whereas multiple infections were detected in (21/61) 34.4 % cases in the ASCUS samples. Seventy pairs of discordant results, including 15 pair discordant results (Multiplex “Not detected” vs conventional-PCR-HPV 6/18/31/33).

They were repeated by a third method, Roche Amplicor Linear array. 14/15 repeated cases by Linear array showed same results with the Multiplex assays and only one case showed concordant result with conventional PCR assay (ref no: 253).

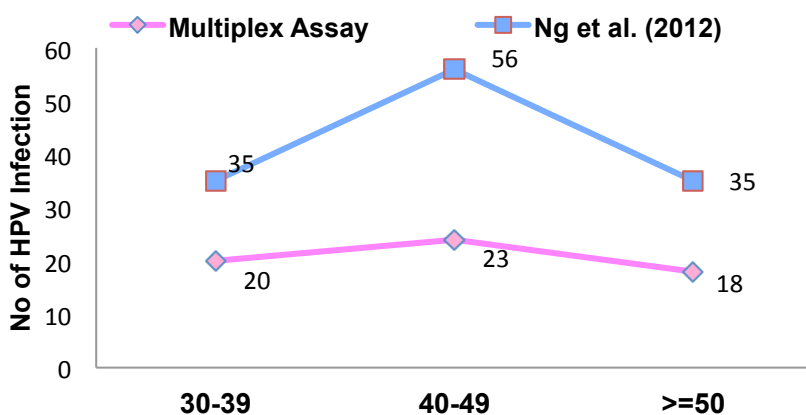
**Table 2 HPV subtypes identified by Multiplex assays in 136 cases with age  $\geq 30$  years**

Simple infection		Multiple infection	
HPV types	Cases (n)	HPV types	Cases (n)
6	1	11 and 54	2
16	3	11 54and 59	1
18	3	11, 44 and 52	1
11	1	16 and 52	1
39	3	16 and 56	1
40	2	16, 51, 57 and 68	1
42	1	16, 33, 52 and 59	1
43	2	18 and 6	1
44	2	18 and 40	1
45	1	18 and 42	2
54	2	31,39 and 54	1
51	3	35 and 56	1
52	6	33 and 58	1
53	2	44 and 68	1
58	3	52 and 43	1
59	4	52 and 58	1
67	1	52 and 53	2
Subtotal:	40	53 and 68	1
		Subtotal:	21
Total : 61			

**Figure 1 Prevalence of HPV subtypes identified by Multiplex assay in the present study**



**Figure 2 Correlation of age-specific prevalence of the present study with previous findings**



When comparing the effectiveness of both methods to identify virus type in all samples, Conventional PCR identified more cases than Multiplex assays for all HPV types studied at significant level. The estimated odd ratio showed that conventional-PCR assay was 64 fold greater chance of

identifying virus present in the ASCUS samples for both HR and LR type investigated.

*Age-specific prevalence HPV (HR and LR) in ASCUS samples*

The data was summarized in Figure 2. A

total of 61 infected patients were identified. 20 samples (32.8%) at 30-39 years; 23 samples (37.7%) at 40-49 years and 18

samples (29.5%) were  $\geq 50$  years. The prevalence of HPV exhibited a peak in 40-49 age group (23/61, 37.7%).

**Table 3 Difference in HPV subtypes not covered by Multiplex assays**

	Primer sets	HR-HPV	LR-HPV
Conventional PCR	GP+ (+ve) SPF(+ve)	26, 35, 39, 45, 51, 56, 58, 59, 66 and 73	30, 40, 42, 43, 55, 62, 67, 69, 74 and 83
	GP+ (+ve) SPF(-ve)		10, 32, 70, 86, 90 and CP8304
	GP+ (-ve) SPF(+ve)	52, 53, 68, 70 and 82	13, 34, 44, 54, 61, 64, 71, 72, 81 and 84
Multiplex assays	Biochips	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82	6, 11, 40, 42, 43, 44, 54, 55, 57, 67 and 69
HPV types not detected by MTG assays		10, 13, 30, 32, 34, 61, 62, 64, 70, 71, 72, 74, 83, 84, 86, 90, CP8304	

## Discussion

Results of Multiplex assay compared with conventional PCR

In the present study, the performance characteristics of the Multiplex assays were evaluated to detect the 29 HPV subtypes. It utilized the protocol of SNIPER HR-HPV assay kit (Genetel Pharmaceuticals Ltd, Shenzhen). The rationale of the assays was based on hybridization of PCR products on chips to detect HPV DNA. A total of 23 subtypes including 14 HR and 9 LR were identified. In contrast to conventional PCR method identified 16, 18, 31 and 33 subtypes. The data indicated a large variety of virus type present in our ASCUS samples,

demonstrating both assays were useful to provide information for HPV infections.

There were 70 discordant cases noticed in both assays and 15 pairs results showed large discrepancy between them. (Multiplex assay showed not detected while Conventional PCR revealed at least one HR subtype 16, 18, 31, 33). All of them were repeated by another Amplicor linear array genotyping (Roche Diagnostics). 14 out of 15 cases showed same results of Multiplex with Roche Linear array and only one compatible result (HPV16) was recorded between the conventional PCR and Roche Linear array (reference no: 253). Our results suggested that conventional PCR was far more sensitive than Multiplex assay in HPV

group sub-typing and the concordance rate between both assays was 52.2%.

#### *Discrepancy of results in both assays*

The large discrepancy of results between both may be attributed to the pair of primers used, viral load of the patients or degradation of viral DNA upon storage. Conventional-PCR make use of combination of primers sets GP 5+/6+ and Short PCR Fragments (SPF) 1/2 which target the conserved L1 circular region of the HPV genome. A total of 15 HR and 26 LR subtypes can be identified as shown in table 3. There were at least 17 subtypes not covered by the multiplex assay and might account for the lesser sensitivity for detecting viral DNA.<sup>10</sup> It revealed the use shorter SPF primer (65 bp) was able to discriminate board spectrum of HPV types. Studies reported Multiplex assays uses pool of HPV primers designed to amplify HPVDNA of the L1 gene from 13 HR genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68).<sup>11</sup> Some types such as those listed in table 3 cannot be resolved and “negative” results of Multiplex assay might not be true negative. Moreover, conventional PCR utilizing both primer systems is also amendable for epidemiological screening studies.<sup>10</sup>

Overall, Multiplex assay results were compatible with that of Roche Linear Array genotyping assay. The rationale of was very similar to the multiplex assay in the resent study except the Linear array uses of biotinylated primers PGMY09/11 to amplify a short fragment (65bp) of L1 gene and then

coupled with reverse line blot hybridization.<sup>11</sup> The HR- HPV types identified were very similar (16, 18, 31, 33, 35, 38, 45, 51, 52, 56, 58, 59, 68, 73 and 82) and most common ones can be covered.

Furthermore, the lowest limit of detection (LOD) of Multiplex assay was 1000 copies/ml. The linear dynamic range may not be sensitive to detect HPVDNA if the patients had low copies number or had drug therapy. Moreover, it might be degradation of viral DNA upon prolonged storage on refrigerator and presence of potential PCR inhibitors in liquid-based medium may suggest the inability to amplify viral DNA during the PCR reactions.

#### *Some pros and cons of both assays*

When considering the advantages and disadvantage of both assays, Multiplex assay has lower sensitivities for HPV DNA detection in ASCUS+ cytology specimens when compared with conventional PCR. Based on our findings, it was more specific when identifying more than one virus type in multiple infections (21/61, 34.4%) but is not comprehensive for all subtypes. Conventional PCR with both primer pairs was far more sensitive in viral sub grouping but less specific in identify other viral subtypes other than oncogenic HPV 16, 18, 31, and 33, while other reported as undefined risk type. In terms of throughput, conventional assay involves time-consuming, laborious procedures. Therefore, it might not generate data rapidly and not subject to large throughput screening. Contrast to Multiplex (SNIPER) assay, which make use of the in–

situ hybridization of PCR products, is more rapid, simplified in procedures and amendable to large-scale study. As PCR reaction proceeds, reactions can be proceed in multiples (multiplexing) so that different viral genotyping in different channels.<sup>11</sup> This greatly enhanced the ability in identifying multiple viral types in a single patient.

#### *Age-specific prevalence subtype in ASCUS samples*

In the present study, the prevalence of single HPV exhibited a peak of infections in 40 – 49 age group and spontaneous drop in age group  $\geq 50$  years. The shape pattern of HPV prevalence was correlated with Ng et al study (2012).

This pattern may be explained by newly acquired infections or reactivation of latent infections in the middle age women.

HPVDNA were found in (61/136) 44.9% of cases including (40/61) 65.6% of single infection and (21/61) 34.4% multiple infections by Multiplex assay. Our findings revealed the most prevalent HR type was 52 (21.3%), 16 (11.5%), 18 (11.5%), 54 (9.8%) and 59 (9.8%) etc. The results correlated with the findings of Luk WH (2012) conducted that male was a transmission media of HPV types 16, 33, 52, 58. Such types were isolated in patients' penile swabs. It was suggested that women acquire the infections through sexual activities of the infected partners.<sup>13</sup> Our findings also in line with a retrospective analysis of type-specific HPV by Dickson et al (2013), which revealed the most common HPV type was

type 16, Their two-way HPV type comparisons analyzed types 52, 53, 81, and 83 were more likely to occur in multiple infections with other types, suggesting that there may be both competitive and cooperative interactions between HPV types. Findings of Dickson et al correlated with us since HPV type such as 16 and 52 might acts synergistically with each other's to induce cervical neoplasms.<sup>13</sup>

#### **Conclusion**

The in-house conventional PCR assay is sensitive for HPVDNA detection in general, but failed to identify HPV subtype other than 6, 11, 16, 18, 31 and 33. In contrast, Multiplex assay is specific for the 29 HPV subtypes but did not cover the existing undefined HPV subtypes in ASCUS+ cytology specimens in Hong Kong population. HPV 52 is a significant local HPV subtype. A peak age-group of HPV infection was identified in 40-49. The implementation of new era vaccine program should consider the findings, which is important in designing new polyvalent vaccines not only against subtype 16 and 18, but also against other HR subtypes.

(Part of the results was accepted for poster presentation in 4th Congress of AAMLS in (2-4 October 2013, Singapore)

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