Evaluation of a liquid bead array system for high-risk human papillomavirus detection and genotyping in comparison with Hybrid Capture II, DNA chip and sequencing methods

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Since persistent infection with high-risk human papillomavirus (HPV) is a known cause of high-grade cervical intraepithelial neoplasia and cervical cancer, several HPV DNA detection methods have been developed during the last decade. The Hybrid Capture II (HCII) assay, which allows detection of 13 high-risk HPVs, has been well validated; however, it does not provide any genotype-specific information. The oncogenic activity of HPV is dependent on its genotype. The prophylactic effects of HPV vaccines are based on L1 virus-like particles and are limited mainly to infections corresponding to the HPV type used to develop the immunogen. Therefore, accurate detection and genotyping are important for treatment as well as screening. A newly developed HPV genotyping system using a liquid bead array was evaluated with 286 cervical samples and the results were compared to two commercially available methods, i.e. the HCII and HPV DNA chip assays, and sequencing. The sensitivity for detection of high-risk HPV was 85.3 % (HCII), 94.7 % (DNA chip) and 99.0 % (liquid bead array). The liquid bead array showed almost perfect agreement ($\kappa=0.95$) with genotype information confirmed by sequencing, while substantial agreement ($\kappa=0.8$) was observed between DNA chip and sequencing. Furthermore, the liquid bead array had superior detection of 26 HPVs (16 high-risk and 10 low-risk types) and has proven to be as accurate as sequencing in identifying individual HPV types, even in cases with multiple HPV infections.

Received 22 April 2010

Accepted 17 October 2010

INTRODUCTION

Human papillomaviruses (HPVs) are causal agents involved in the development of carcinomas in the cervix uteri as well as pathological changes of the skin including mucosa, particularly warts, condylomas and dysplasias (Remmerbach *et al.*, 2004). Due to the central role of HPV in anogenital carcinogenesis, HPV testing has received intensive scrutiny as an adjunct or replacement of cervical cytology analysis for cervical cancer screening (Coutlée *et al.*, 2009). Currently, there are about 100 identified genotypes of HPV, of which about 40 are genital HPV types that invade the genital organs such as the uterine

cervix, vaginal wall, vulva and penis (Kawana *et al.*, 2009). Genital HPV types are classified into high-risk types associated with cervical cancer and low-risk types known as causative pathogens of condyloma acuminatum. Based on epidemiological studies, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 have been classified as high risk, and types 26, 53 and 66 as potential high risk, whereas types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and 89 have been regarded as low risk (Muñoz *et al.*, 2003).

The HPV prophylactic vaccine against types 16 and 18 has the potential to eliminate up to 70 % of cervical cancers, depending on the coverage and acceptance of the vaccine (Bhatla & Moda, 2009). Two prophylactic HPV vaccines have thus far been developed: Gardasil, a quadrivalent vaccine targeting HPV 6, 11, 16 and 18 (Villa *et al.*, 2006), and Cervrix, a bivalent vaccine that targets HPV 16 and 18

Abbreviations: HCII, Hybrid Capture II; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesion; SCC, squamous cell cervical carcinoma; WNL, within normal limits.

(Harper *et al.*, 2006). However, these vaccines have negligible prophylactic effects on other HPV types (Ochi *et al.*, 2008). Therefore, HPV typing will have to continue to deal with cancers caused by non-vaccine types as well as women already infected prior to vaccination. HPV typing also can be used for determination of the contribution of individual HPV types in infections involving multiple HPV types.

Currently, the most common tests used to detect genital HPV in cervical samples are hybridization assays such as the Hybrid Capture II (HCII) (Digene HPV test; Qiagen) assay, and PCR systems with the degenerate primer sets MY09/11 and GP5+/6+ (Remmerbach *et al.*, 2004; Winder *et al.*, 2009). The HCII assay is based on hybridization in a solution with a mixture of non-isotopic single-stranded full-length RNA probes against 13 high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). This assay has been validated, detects several high-risk types in one reaction, uses a convenient format and avoids potential contamination, a known problem with PCR-based methods (Coutlée *et al.*, 2005). However, it does not provide any genotype-specific information. Since the various high-risk HPVs differ in their oncogenic potential; improved risk stratification requires accurate information on HPV genotype involved in an infection.

In contrast to the HCII assay, the PCR-based assay can detect individual HPV types. HPV typing could improve the specificity of HPV DNA detection tests for clinical use, allow management of HPV-infected women according to the risk of high-grade lesions, evaluate type-specific HPV prevalence, assess the type-specific concordance among detection methods and monitor the duration of protection in vaccinated populations (Meijer *et al.*, 2006). In most of the PCR-based HPV detection systems, a broad spectrum of HPV types is amplified by consensus primers, followed by detection with type-specific probes. Four consensus assays target conserved sequence in the HPV L1 gene. The PGMY09/11 (yielding a 450 bp amplicon), GP5+/6+ (yielding a 150 bp amplicon) and SPF1/2 (yielding a 65 bp amplicon) consensus primer sets can amplify a wide spectrum of up to 36, 37 and 43 genital genotypes, respectively (Gravitt *et al.*, 2000; Van Doorn *et al.*, 2002; Hesselink *et al.*, 2006; Gillio-Tos *et al.*, 2006). After amplification with consensus primers, HPV amplicons are genotyped with reverse hybridization on line filters or on microtitre plates. Typing of PCR products can also be carried out by oligonucleotide microarrays that use immobilized DNA probes specific for each HPV type.

Array technologies permit the use of a number of targets. The Luminex xMAP suspension array technology can simultaneously detect several HPV genotypes by using 5.6 µm polystyrene microspheres that are internally dyed with various ratios of two spectrally distinct fluorophors (Jiang *et al.*, 2006; Schmitt *et al.*, 2006). A variety of HPV type-specific probes can be coupled to different bead sets with specific absorption spectra. These sets are combined

to a suspension array and allow up to 100 different probes to be measured simultaneously in a single reaction (multiplexing). This technology can potentially be fully automated, dramatically decreasing the personnel cost component of the assay; this method has been used for the genotyping of 45 HPV types with PGMY09/11 PCR (Wallace *et al.*, 2005), 25 HPV types with type-specific primers for the HPV-specific E6 and E7 genes (Han *et al.*, 2006), 22 HPV types with GP5+/6+ PCR (Schmitt *et al.*, 2006), 15 HPV types with YBT L1/GP-1 PCR (Oh *et al.*, 2007), and 18 HPV types with GP5+/6+ PCR (Geraets *et al.*, 2009). This multiplex HPV genotyping has been evaluated with other commercially available methods, such as the HCII assay, RFLP and the HPV DNA chip, as not able to guarantee absolute accuracy. Evaluation of the performance of an HPV detection system is most accurately performed with sequencing, which gives the most conclusive genotype information despite being the most labour-intensive method.

The goal of this study was to evaluate an HPV genotyping method using liquid bead array technology that allows for the detection of 26 HPVs (16 high-risk HPV types including 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 54, 56, 58, 59, 66 and 68, and 10 low-risk HPV types including 6, 11, 34, 40, 42, 43, 44, 55, 67 and 70) and compare the results with two commercially available methods, the HCII assay and the HPV DNA chip assay, and sequencing for HPV detection and genotyping in clinical samples. All genotyping results detected by HCII, DNA chip and the liquid bead array were confirmed by sequencing, and the analytical sensitivities and specificities of these methods were calculated based on genotype information confirmed by sequencing.

METHODS

Study subjects. A total of 624 clinical samples collected at the Catholic Medical University from February to October 2006 were prospectively included in this study. The specimens were obtained from patients in the Department of Obstetrics and Gynecology in accordance with procedures approved by the Institutional Review Board of the Catholic University of Korea. The disease status was assigned according to the International Federation of Gynecology and Obstetrics.

Sample collection and isolation of DNA. Cervical swabs were obtained with Cervex-Brush (Pappete; Wallach Surgical Devices). The brush was immediately rinsed into a vial of PreservCyt solution (Cytoc). To isolate DNA, 2 ml cell suspension was transferred to a 2 ml reaction tube and washed twice by centrifugation at 14 000 r.p.m. for 2 min, removal of the supernatant and resuspension of the pellet in 1 ml PBS. The final pellet was resuspended in 200 µl PBS and used for DNA isolation with the Qiagen DNA blood minikit (Qiagen) according to the manufacturer's instructions.

HCII assay. HPV detection by HCII was performed with the HCII assay system according to the manufacturer's (Digene Diagnostic) protocol. The isolated DNA from cervical samples was denatured at 65 °C for 45 min and hybridized under high stringency conditions with a mixture of RNA probes that detect 13 different carcinogenic HPV types (Table 1). The resultant DNA–RNA hybrids were captured

on the surface of microtitre plate wells that were coated with anti-DNA–RNA hybrid antibody. The immobilized hybrids were then reacted with an alkaline phosphatase-conjugated antihybrid mAb. Light intensity was measured with a luminometer. The positive cut-off value was set at 1 pg HPV DNA ml⁻¹ in each specimen.

HPV genotyping by DNA chip. An HPV DNA chip (MyGene) was used for HPV genotyping. This HPV DNA chip contained 24 type-specific probes that consist of 16 high-risk types and 8 low-risk types (Table 1). Based on the manufacturer's instructions, the isolated DNA was first amplified with a set of consensus primers GPd5+/6+ that were directed to a highly conserved region of L1 gene (GPd5+, 5'-TTTCTTACHGKTGTDGATACAC-3'; GPd6+, 5'-GAAAHATAA-AYTGYAADTCATAYTC-3'; k=g/t; h=t/a/c; d=a/t/g; y=t/c). β -Globin was amplified as the internal control in the PCR. The PCR products of all samples were detected by electrophoresis in a 2.5% agarose gel; the product size of HPV DNA was 150 bp.

The amplified DNA was labelled with Cy5-dUTP (MEN Life Science Products). The labelled product was denatured for 5 min at 95 °C then mixed with a hybridization solution (MyGene) followed by placement of the sample onto the DNA chip. Hybridization was carried out at 43 °C for 90 min and was followed by washing with 3 × SSPE (0.54 M NaCl, 30 mM NaH₂PO₄, 3 mM Na₂EDTA pH 7.4) for 5 min and 1 × SSPE for 5 min, and drying at room temperature. The hybridized HPV DNA was visualized using a DNA chip scanner (ScanArray Lite; GSI Lumonics).

Table 1. Distribution of HPV genotypes recognized by HCII, HPV DNA chip and liquid bead array methods

Oncogenic potential	HPV genotype	Genotyping method		
		HCII	DNA chip	Liquid bead array
High risk	16	+	+	+
	18	+	+	+
	31	+	+	+
	33	+	+	+
	35	+	+	+
	39	+	+	+
	45	+	+	+
	51	+	+	+
	52	+	+	+
	53		+	+
	54		+	+
	56	+	+	+
	58	+	+	+
	59	+	+	+
	66		+	+
	68	+	+	+
Low risk	6		+	+
	11		+	+
	34		+	+
	40		+	+
	42		+	+
	43		+	+
	44		+	+
	55			+
	67			+
	70		+	+

HPV genotyping by a liquid bead array system. The bead-based array uses type-specific oligonucleotide probes for 26 different HPV types including 16 high-risk types and 10 low-risk types, and is based on the DNA sequences of the viral L1 gene, which were obtained from the HPV database (<http://hpv-web.lanl.gov/>) (Tables 1 and 2). These type-specific probes had 5'-amine modification for bead coupling as well as 15 bp oligo-dT sequences attached for hybridization flexibility. The modified probes were coupled to carboxylated beads (Luminex) by a carbodiimide base coupling procedure. The preparation of beads coupled with oligonucleotide was previously reported (Oh *et al.*, 2007). Each bead carrying the type-specific oligonucleotide was finally mixed in equal proportion; the total concentration of oligonucleotide was 0.1 µM. This mixture is referred to as the bead mix.

Amplification of the L1 gene from 200 ng genomic DNA isolated from cervical samples was carried out with the PGMY09/11 primer set. PCR conditions were as published for PGMY09/11 by Gravitt *et al.* (2000). For a positive PCR control, two primers were used for amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences: GAPDH F, 5'-GAGTCAACGGATTGGTC-GT-3' (forward primer) and GAPDH R, 5'-TTGATTTTGGAGGC-ATCTCG-3' (reverse primer).

The PCR products of all samples were detected by electrophoresis in a 1.5% agarose gel; the product size of HPV DNA was 450 bp. After the PCR, a single strand PCR was carried out for labelling of the complementary sequences of the probe with biotin-14-dCTP (Invitrogen). The PGMY09/11 reverse primer set was used for the PCR product labelling. PCR components in 20 µl PCR mixture were 0.05 µM PGMY09, 50 µM dATP, dGTP, dTTP mixture, 20 µM biotin-14-dCTP, 75 mM Tris/HCl (pH 9.0), 20 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂SO₄, 1 U *Taq* polymerase (Biotools) and 5 µl amplified L1 fragment. A 5 min denaturation step at 94 °C was followed by 35 cycles of amplification: denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min and elongation at 72 °C for 2 min.

Hybridization buffer (21 µl 2 × EZway Hybrisol; Koma) and 1 µl HPV bead mix containing 6.75 × 10⁴ beads (2.5 × 10³ for each HPV type and control) were added to a labelling tube containing the PCR products. The hybridization was conducted in the same way as previously reported (Oh *et al.*, 2007). After hybridization, samples were immediately transferred to a filter plate and washed three times with washing buffer (0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, pH 8.0) at room temperature. The beads were resuspended for 15 min in 100 µl streptavidin-R-phycoerythrin (Strep-PE; Molecular Probes) and analysed for internal bead colour and R-phycoerythrin receptor fluorescence in the Luminex 100 IS system. The median reporter fluorescence intensity of at least 100 beads was calculated for each bead set in the sample.

Confirmation of HPV genotyping for the DNA chip and liquid bead array methods compared to DNA sequencing.

The genotyping results for 286 samples that were HPV positive by both liquid bead array and DNA chip were confirmed by sequencing with type-specific primers. The DNA fragment amplified within the L1 HPV gene by consensus primers was used for sequencing. PCR products amplified were isolated from agarose gels using a gel extraction kit (Qiagen). A total of 20 µl of amplimer solution was eluted and 3 µl eluent at 10 ng µl⁻¹ was used for direct sequencing with the type-specific primer based on the HPV genotype detected by DNA chip and bead-based array for the amplified PCR product. Sequencing reactions of double-stranded PCR products were performed with a dye terminator cycle sequencing kit (ABI Prism dRhodamine terminator cycle sequencing ready reaction kit; PE Applied Biosystems). The DNA sequences obtained were compared to GenBank sequences using the BLAST program from the National Center for Biotechnology Information website.

Cut-off definition and statistical analysis. Reactions of probes with PCR products derived from PGMY09/11 PCR on 200 ng HPV-negative

Table 2. Type-specific oligonucleotide probes for 26 HPV genotypes

The high-risk HPVs are indicated in bold.

HPV type	GenBank accession no.	Probe sequence (5'→3')
6	X00203	ATC CGT AAC TAC ATC TTC CAC ATA CAC CAA
11	M14119	ATC TGT GTC TAA ATC TGC TAC ATA CAC TAA
16	K02718	GTC ATT ATG TGC TGC CAT ATC TAC TTC AGA AAC
18	X05015	TGC TTC TAC ACA GTC TCC TGT A
31	J04353	TGT TTG TGC TGC AAT TGC AAA CAG TGA TAC
33	M12732	TGC ACA CAA GTA ACT AGT GAC AG
34	X74476	TAC ACA ATC CAC AAG TAC AAC TGC ACC ATA
35	M74117	GTG CTG CTG TGT CTT CTA GTG A
39	M62849	TCT ACC TCT ATA GAG TCT TCC ATA CCT TCT
40	X74478	TGC CAC ACA GTC CCC CAC ACC AA
42	M73236	GCC ACT GCA ACA TCT GGT GAT A
43	AJ620205	TCT ACT GAC CCT ACT GTG CCC AGT A
44	U31788	GCC ACT ACA CAG TCC CCT CCG TCT A
45	X74479	ACA CAA AAT CCT GTG CCA AGT A
51	M62877	CAC TGC TGC GGT TTC CCC AA
52	X74481	GCT GAG GTT AAA AAG GAA AGC A
53	DQ486475	TTT CTG CAA CCA CAC AGT CTA TGT CCA
54	U37488	TAC AGC ATC CAC GCA GGA TAG CTT
55	U31791	TGC TGC TAC AAC TCA GTC TCC ATC TAC A
56	X74483	ACT GCT ACA GAA CAG TAA GTA AA T ATG AT
58	D90400	TAT GCA CTG AAG TAA CTA AGG AAG GT
59	X77858	CTA CTT CTT CTA TTC CTA ATG TAT ACA CAC
66	U31794	TGC AGC TAA AAGC ACA TTA ACT AA
67	D21208	TCT GAG GAA AAA TCA GAG GCT
68	AJ831568	CTG AAT CAG CTG TAC CAA ATA TTT AT
70	U22461	TG CAC CGA AAC GGC CAT ACC T

genomic DNA was considered as the background values. In this study, the HPV-negative genomic DNA was extracted from human cervical cancer cell line C33A. The cut-off was defined for each HPV probe individually as the mean of the background plus three times the SDs of 19 replicates (data not shown).

Sensitivity and specificity were defined as the probability of predicting high-risk HPV and the probability of predicting low-risk HPV given true HPV types in samples showing HPV positivity in both DNA chip and liquid bead array, respectively. Here, the true HPV type is considered to be determined by sequencing. For example, assume that the DNA chip predicts 100 high-risk and 186 low-risk samples for the total of 286 samples. If the 100 high-risk samples consist of 44 true high-risk and 56 true low-risk samples, while the 186 low-risk samples are composed of 6 true high-risk and 180 true low-risk samples, the sensitivity and specificity for detection of high-risk HPV in the DNA chip are 88 % $\{100 \times [44/(44+6)]\}$ and 76 % $\{100 \times [180/(56+180)]\}$, respectively.

Agreement between the HPV assays was assessed using the Cohen κ statistic, with values of: 0 to 0.200, indicating poor agreement; 0.21 to 0.40, indicating fair agreement; 0.41 to 0.60, indicating moderate agreement; 0.61 to 0.80, indicating substantial agreement; and 0.81 to 1.00 indicating almost perfect agreement. Statistical analyses were performed with SPSS software version 10.0 (SPSS).

RESULTS

To evaluate the bead array for HPV typing in clinical samples, a total of 624 cervical samples were used. The

HPV genotyping was first carried out by the liquid bead array and the commercial HPV DNA chip with PCR products derived from the consensus primers on 200 ng genomic DNA isolated from the cervical samples (Fig. 1). The GPd5 +/6 + primers were used in the genotyping with the DNA chip according to the manufacturer's guidelines (see Methods for details). Out of 624 samples, the number of HPV-positive samples detected by the liquid bead array and the DNA chip was 303 and 295, respectively. A comparison of HPV genotyping was made for the 286 samples showing HPV-positive results by both genotyping systems.

Comparison of the sensitivity and specificity of HCII, liquid array and DNA chip assays for high-risk HPV detection

The sensitivity and specificity of three HPV detection systems, HCII, liquid bead array and DNA chip, for high-risk HPV detection were calculated based on the genotype information confirmed by sequencing. In each HPV detection system, the HPV high-risk positive samples were defined according to the probe system for the detection of high-risk HPV (HCII, 13 types; liquid bead array, 16 types; DNA chip, 16 types) (Table 1). The highest overall sensitivity (99.0 %) and specificity (88.5 %) were obtained

Fig. 1. HPV testing of clinical samples by three HPV detection systems: HCII, DNA chip and liquid bead array assays. The numbers in parentheses represent the number of samples.

by the liquid bead array system while the lowest overall sensitivity (85.3 %) and specificity (42.9 %) were obtained by the HCII (Table 3). The sensitivity of HCII had significant variation according to the cytological diagnosis, whereas the liquid bead array showed almost constant sensitivity regardless of cytological diagnosis. The lowest specificity (13.3 %) was obtained in low-grade squamous intraepithelial lesions (LSILs) with the HCII assay; there were many false positives caused by cross-reactions. The HCII assay was designed to detect 13 high-risk types

including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. However, two additional high-risk HPVs, HPV 53 and 66, were also detected by HCII. Detection of these two high-risk HPVs by HCII was considered a false positive because they were not included in the probe system of the HCII.

The distribution of HPV types was surveyed for various stages of the cytological diagnosis on the basis of genotype information confirmed by sequencing. The most common

Table 3. Sensitivity and specificity of the three HPV detection systems for detection of high-risk HPVs

Cytology	HCII		DNA chip		Liquid bead array	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
WNL	77.4	68.8	90.1	100.0	98.6	100.0
ASCUS	81.8	50.0	93.9	85.7	100.0	85.7
LSIL	94.6	13.3	100.0	80.0	98.5	79.3
HSIL	90.6	50.0	96.8	66.7	100.0	100.0
SCC	100.0	–	100.0	–	100.0	–
Overall	85.3	42.9	94.7	82.4	99.0	88.5

ASCUS, Atypical squamous cells of undetermined significance; HSIL, high-grade squamous intraepithelial lesion.

Table 4. Frequency of each HPV type at various cytological stages

The numbers in parentheses represent the percentages.

Cytology	Single infection										Multiple infections	Total
	High risk									Low risk		
	16	18	39	51	52	53	56	58	Others			
										6, 11, 40, 42, 44,70		
WNL	20 (16.0)	12 (9.6)	3 (2.4)	6 (4.8)	6 (4.8)	15 (12.0)	8 (6.4)	11 (8.8)	14 (11.2)	17 (13.6)	13 (10.4)	125 (100)
ASCUS	10 (21.7)	3 (6.5)	2 (4.3)	1 (2.2)	3 (6.5)	5 (10.9)	2 (4.3)	0 (0)	11 (23.9)	3 (6.5)	6 (13.0)	46 (100)
LSIL	14 (21.5)	5 (7.7)	6 (9.2)	1 (1.5)	4 (6.2)	4 (6.2)	4 (6.2)	3 (4.6)	9 (13.8)	4 (6.2)	11 (16.9)	65 (100)
HSIL	13 (35.1)	2 (5.4)	0 (0)	2 (5.4)	0 (0)	0 (0)	1 (2.7)	5 (13.5)	7 (18.9)	3 (8.1)	4 (10.9)	37 (100)
SCC	9 (69.2)	1 (7.7)	0 (0)	0 (0)	1 (7.7)	0 (0)	0 (0)	0 (0)	1 (7.7)	0 (0)	1 (7.7)	13 (100)

ASCUS, Atypical squamous cells of undetermined significance; HSIL, high-grade squamous intraepithelial lesion.

high-risk HPV was HPV 16 regardless of cytological diagnosis. The portion of HPV 16 and 18, in a single infection with high-risk types, increased with the severity of cervical lesion from 33.7% for WNL (within normal limits) to 83.3% for SCC (squamous cell cervical carcinoma) (Table 4). These findings show the association of HPV 16 and 18 with the severity of the cervical lesion. For the SCC samples, the HPV 16 accounted for 69.2% of all HPVs. A total of 63% of all low-risk HPVs and 37% of all multiple infections were observed in the WNL samples. In addition, 96% of all multiple infections included at least one high-risk HPV (data not shown). This finding indicates that the high-risk type of HPV might play an important role in multiple infections.

Evaluation of HPV genotyping with the DNA chip and liquid bead array technology compared to sequencing

In contrast to the HCII assay, DNA chip and liquid bead array technology can be used to identify 24 HPV genotypes; the latter can detect two additional HPVs, HPV 55 and 67 (Table 1). The genotyping performance for 24 common HPVs was used for the comparison. Both genotyping methods use type-specific probes; however, mistyping can occur due to non-specific reactions or cross-reactions. Sequencing provides the most accurate and conclusive genotype information despite being the most labour intensive. Therefore, all genotyping results for 286 samples by DNA chip and the liquid bead array were confirmed by sequencing with type-specific primers.

The overall strength of agreement between DNA chip results and sequencing was considered as substantial agreement ($\kappa=0.8$), while HPV 40, 45 and 54 showed fair agreement ($\kappa=0.33$ – 0.39) (Table 5). Almost perfect agreement was observed between the liquid bead array and sequencing ($\kappa=0.95$). This indicates that the liquid

Table 5. Analysis of the concordance among DNA chip, liquid bead array and sequencing methods

HPV genotype	κ value for HPV genotype		
	Liquid bead array vs sequencing	DNA chip vs sequencing	Liquid bead array vs DNA chip
High risk			
16	0.99	0.94	0.90
18	0.96	0.88	0.90
31	1.0	0.76	0.75
33	1.0	0.77	0.63
35	1.0	0.77	0.80
39	0.96	0.92	0.89
45	1.0	0.33	0.40
51	0.90	0.60	0.60
52	1.0	0.71	0.70
53	0.97	0.66	0.73
54	1.0	0.36	0.40
56	1.0	0.88	0.88
58	0.98	0.79	0.80
59	1.0	0.89	0.72
66	0.89	0.96	0.84
68	0.91	0.95	0.88
Low risk			
6	0.81	0.83	0.81
11	0.91	0.77	0.77
34	–	–	–
40	0.83	0.39	0.34
42	1.0	0.83	0.83
43	–	–	–
44	–	–	–
70	0.92	0.80	0.67
Overall κ	0.95	0.80	0.77
(95% CI)	(0.93–0.97)	(0.78–0.84)	(0.73–0.82)

95% CI, 95% Confidence interval.

bead array had higher accuracy for HPV genotyping than DNA chip technology. The genotyping results compared between the liquid bead array and DNA chip showed substantial agreement ($\kappa=0.77$). Fair agreement was observed in the comparison between DNA chip and the liquid bead array methods for HPV 40, 45 and 54, similar to the comparison between DNA chip and sequencing methods.

To evaluate the type specificity of each HPV probe for the two genotyping methods, the genotyping results were compared with genotypes confirmed by sequencing. The overall perfect match rate was 81.9 and 95.5 % for the DNA chip and the liquid bead array methods, respectively (Tables 6 and 7). The diagonal number represents the frequency of perfect match while the off-diagonal number presents mismatches. Most of the mismatches were observed in cases with multiple infections. That is, the accuracy of typing tended to decrease with the number of

HPV types present in a sample (data not shown). The mismatch rate between high- and low-risk types was 5.4 and 1.1 % for the DNA chip and the liquid bead array methods, respectively (Tables 6 and 7). The highest mismatch rate was observed for the HPV 40 and 53 by DNA chip technology.

DISCUSSION

Roles for HPV testing in clinical applications include: triage of equivocal cervical cytology results, primary screening as a standalone test or as an adjunct to cytology, and for follow-up of treated high-grade cervical intra-epithelial neoplasia (CIN-2, 3). HPV testing is also essential for studies on the epidemiology and transmission of HPV infections, and the natural history of anogenital precancerous lesions and the efficacy of vaccination rely on the use of HPV DNA detection and typing methods (Coutlée *et al.*,

Table 6. Consistency of HPV typing between DNA chip and sequencing methods

The diagonal value represents the matched frequency of genotype pairs based on genotyping results obtained by DNA chip and sequencing methods; the off-diagonal value represents the unmatched frequency. The region with the genotype pairs of only high-risk HPVs is indicated in bold.

HPV genotype confirmed by sequencing	HPV genotyping by DNA chip																											
	High risk																Low risk											
	16	18	31	33	35	39	45	51	52	53	54	56	58	59	66	68	6	11	34	40	42	43	44	70				
High risk																												
16	87	1								1																		
18		28								1																		
31			1	12	1																							
33			1		7																							
35					1	5			1									1										
39				1		12					1																	
45	1						1																					
51	2		1					7			1		3				1					1						
52			1				2		19	2			3			1			1								2	
53	3	1					1			23	1	1								6				1	1			
54											2																	
56											2	16										1						
58		1							1		1		21															
59	1													4														
66										1						12												
68		1															22											
Low risk																												
6																	10										1	
11			1														1	5										
34																												
40																	1			3	1		1					
42			1																		5							
43																												
44			1								1		1													2		
70										1																	15	

Table 7. Consistency of HPV typing between liquid bead array and sequencing methods

The diagonal value represents the matched frequency of genotype pairs from genotyping results obtained by bead array and sequencing methods; the off-diagonal value represents the unmatched frequency. The region with the genotype pairs of only high-risk HPVs is indicated by bold.

HPV genotype confirmed by sequencing	HPV genotyping by liquid bead array																											
	High risk														Low risk													
	16	18	31	33	35	39	45	51	52	53	54	56	58	59	66	68	6	11	34	40	42	43	44	70				
High risk																												
16	88	1																										
18		30																										
31			13																									
33				10																								
35					7																							
39						12																						
45							3																					
51				1				15					1			1												
52									26																			
53						1				33																		
54											2																	
56												16																
58													23															
59														5														
66															13													
68		1		1							1					10												
Low risk																												
6		1															9		1									
11																	1	5										
34																												
40																				5								
42																					6							
43																												
44																							2			1		
70															2												12	

2009). The results of this study show that about 90 % of all HPV infections were caused by high-risk types of HPVs. This indicates that an HPV detection system should have high sensitivity for the detection of the high-risk types of HPVs. In this regard, HCII is a good method for HPV detection; it is the only HPV DNA test system currently approved by the Food and Drug Administration in the USA. It shows whether any of 13 high-risk types are present, but does not provide type-specific information. Since the various high-risk HPVs differ in their oncogenic potential, the information of HPV genotype is important. HPV typing could be used to evaluate the type-specific HPV prevalence and allow for the management of HPV-infected women according to the risk of high-grade lesions.

Therefore, the accurate identification of high-risk HPV genotypes is critical for defining a women's risk of progression to cervical cancer. Currently, most of the HPV genotyping methods are based on HPV DNA

amplification by PCR with consensus primers. Consensus PCR assays have been developed for amplification in one reaction that includes the majority of known, as well as novel, anogenital HPV genotypes (Coutlée *et al.*, 2009). After amplification with consensus primers such as MY09/11, PGMY09/11 or GP5+/6+, HPV amplicons were genotyped by reverse hybridization on line filters or microtitre plates. Typing assays using membrane-based reverse line blot methods are limited to a maximum of 40 probes per hybridization reaction. This restriction can be overcome with array technologies, such as the solid-phase microarray or liquid bead array. In addition, the array-based HPV detection system can detect the presence of multiple HPV types at the same time.

Luminex xMAP suspension array technology can simultaneously detect several HPV genotypes using 5.6 µm polystyrene microspheres. Suspension arrays have several advantages over microarrays; these include favourable

hybridization kinetics that lead to shorter reaction times and a faster instrument read-out. These features of suspension arrays make large-scale epidemiological and vaccination studies possible. HPV genotyping by the liquid bead array have been reported elsewhere (Wallace *et al.*, 2005; Schmitt *et al.*, 2006; Han *et al.*, 2006; Oh *et al.*, 2007; Geraets *et al.*, 2009); however, the performance was evaluated by commercially available methods, such as the HCII assay, RFLP and HPV DNA chip methods, which could not guarantee absolute accuracy.

A novel HPV genotyping system using liquid bead array technology has been developed and reported in this study. This system allows for the detection of 26 HPVs (16 high-risk HPV types and 10 low-risk HPV types). To evaluate clinical performance of the liquid bead array, the HPV genotyping results of the liquid bead array were compared to two commercially available HPV detection systems, i.e. HCII and HPV DNA chip methods, and the DNA sequencing method. The liquid bead array system demonstrated better performance than the two commercial HPV detection systems, the HCII and DNA chip methods. In addition, the accuracy of genotyping in the liquid bead array was comparable to that of the sequencing method ($\kappa=0.95$) (Table 5). The liquid bead array results also showed high accuracy for multiple infections (data not shown). Multiple infections were found to be common among high-risk populations and are associated with a greater risk of a poorer outcome. In addition, the oncogenic potential varies by HPV type and the HPV vaccine is type specific. Therefore, the accurate detection and typing of multiple infections are important for patient treatment.

The difference in the performance of HPV genotyping tests can significantly affect the results of clinical and epidemiological studies. Mistyping of high- and low-risk types might lead to more significant problems than mistyping among high-risk types. Mistyping between high- and low-risk types was 5.4 and 1.1% for the DNA chip and the liquid bead array methods, respectively. Most of mistyping occurred in samples from multiple infections. The overall frequency of mistyping was 18.1 and 0.5% for DNA chip and the liquid bead array methods, respectively (Tables 6 and 7). The high-level mismatches with DNA chip technology might be due to the sequence similarity among the 24 probes designed against the 150 bp L1 fragment, or suboptimal hybridization conditions. However, the genotyping accuracy of the liquid bead array was comparable to the accuracy of sequencing. These findings show that the liquid bead array was more accurate for HPV typing than DNA chip technology. The better performance of the liquid bead array might be due to the favourable hybridization kinetics, which closely approximate the kinetics of solution-phase hybridization.

The ideal HPV genotyping system should have accuracy comparable to sequencing, and should allow for rapid and simple genotyping. The results of this study suggest that the

liquid bead array might be an excellent system for highly accurate HPV genotyping. In addition, it can be automated or deployed on a high-throughput platform, which is essential for an assay intended for large-scale epidemiological and vaccination studies.

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