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Type-Specific Detection of 30 Oncogenic Human Papillomaviruses by Genotyping both E6 and L1 Genes

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Human papillomavirus (HPV) is the principal cause of invasive cervical cancer and benign genital lesions. There are currently 30 HPV types linked to cervical cancer. HPV infection also leads to other types of cancer. We developed a 61-plex analysis of these 30 HPV types by examining two genes, E6 and L1, using MassARRAY matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (PCR-MS). Two hundred samples from homosexual males (HM) were screened by PCR-MS and MY09/MY11 primer set-mediated PCR (MY-PCR) followed by sequencing. One hundred thirty-five formalin-fixed, paraffin-embedded (FFPE) cervical cancer samples were also analyzed by PCR-MS, and results were compared to those of the commercially available GenoArray (GA) assay. One or more HPV types were identified in 64.5% (129/200) of the samples from HM. Comprising all 30 HPV types, PCR-MS detected 51.9% (67/129) of samples with multiple HPV types, whereas MY-PCR detected only one single HPV type in these samples. All PCR-MS results were confirmed by MY-PCR. In the cervical cancer samples, PCR-MS and GA detected 97% (131/135) and 90.4% (122/135) of HPV-positive samples, respectively. PCR-MS and GA results were fully concordant for 122 positive and 4 negative samples. The sequencing results for the 9 samples that tested negative by GA were completely concordant with the positive PCR-MS results. Multiple HPV types were identified in 25.2% (34/135) and 55.6% (75/135) of the cervical cancer samples by GA and PCR-MS, respectively, and results were confirmed by sequencing. The new assay allows the genotyping of >1,000 samples per day. It provides a good alternative to current methods, especially for large-scale investigations of multiple HPV infections and degraded FFPE samples.

The papillomaviruses (PVs), which are now officially recognized by the International Committee on Taxonomy of Viruses as the *Papillomaviridae*, comprise a group of nonenveloped, epitheliotropic DNA viruses that induce benign lesions of the skin and mucous membranes (1). PVs are divided into 12 genera based on the L1 open reading frame (ORF). Human papillomaviruses (HPVs) are clustered among five of these genera: alpha, beta, gamma, mu, and nu (2). HPVs pose a significant human health problem since they are among the most common sexually transmitted agents worldwide. In a cohort study, Giuliano et al. found previously that the incidence of new genital HPV infection was 38.4 per 1,000 person months in men (3).

HPVs are among the most important infectious agents in cancer causation, responsible for 5.2% of all cancers worldwide (4). HPVs comprise more than 100 genotypes defined on the basis of DNA homology. Infection by certain types of HPV is recognized as a causal and necessary factor for cervical cancer (5–7). The largest clinical report of invasive cervical cancer was conducted in 2003 by the International Agency for Research on Cancer (IARC). The pooled analysis of 11 case-control studies from 9 countries evaluated 30 HPV types linked to cervical cancer, including 15 HPV types classified as high-risk types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), 3 classified as probable high-risk types (types 26, 53, and 66), and 12 classified as low-risk types (types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) (8). High-risk types are associated with preneoplastic lesions and carcinomas, while low-risk types are typically associated with benign lesions. Therefore, accurate HPV genotyping is essential for adequate patient classification into low-risk or high-risk groups and for determining whether HPV infection is persistent or transient. An HPV subset is also clearly implicated in the development of human anal, vaginal, vulvar, penile, and oropharyngeal malignancies (2, 9–11). Moreover, anal HPV infections, especially high-risk types, were suggested previously to be independently associated with HIV acquisition (12).

The HPV virion has a circular, double-stranded DNA genome of approximately 7,900 bp with eight overlapping ORFs, comprising early (E) and late (L) genes, and an untranslated long control region. HPV cannot be grown in conventional cell cultures, and there are no reliable serological tools currently available. Therefore, accurate HPV detection relies on the detection of viral nucleic acids. Many molecular methods (such as *in situ* hybridization, Southern blotting, and PCR-based methods) have been developed to detect HPV DNA and identify HPV genotypes (13). Recently, Zhao et al. showed that an HPV DNA test was a highly accurate screening test for cervical intraepithelial neoplasia (CIN) of grade 3 or higher across various locations and age groups in China (14). Many widely used HPV detection methods target only one gene (L1 or E6). A pooled test includes 13 or 14 high-risk types and a few low-risk types. For example, the Hybrid Capture II

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(HC2) system (Qiagen, Valencia, CA) plays a very important role in HPV detection and has become the standard in many countries. However, these methods generally do not provide information on individual genotypes (13, 15, 16).

Type-specific PCR and broad-spectrum PCR methods are widely used in some laboratories; however, they are too labor-intensive for routine high-throughput applications. When multiple HPV types are present in a sample, the effectiveness of the methods decreases significantly. Additionally, these methods usually target the L1 gene and may fail to detect HPV in high-grade cervical lesions and cancers due to the loss of the L1 gene during the process of integration. Therefore, new high-throughput and highly sensitive methods are needed to compensate for the limitations of existing methods (13). Multiplex PCR using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (PCR-MS) is a powerful tool for microbial detection and confirmation (17–20). The assay utilizes a three-step process composed of conventional PCR, primer extension, and MALDI-TOF MS separation of products on a matrix-loaded silicon chip array. The resultant sensitivity is superior to that of real-time fluorescence PCR-based assays, without compromising specificity (21). PCR-MS was previously shown to have high sensitivity and accuracy for detecting oncogenic HPV types (21–23). However, these methods, which targeted E6 or E7, detect only 14 high-risk oncogenic types. To compensate for the limitation of current methods, we developed a high-throughput 61-plex analysis of 30 distinct HPV genotypes for two HPV genes, E6 and L1, coupled with human β-globin (HBB) as an internal control using the Sequenom MassARRAY MALDI-TOF MS system (Sequenom Inc., San Diego, CA). In this paper, we compare the performances of the 61-plex assay, the HPV GenoArray (GA) test (24) (Hybribio Ltd., Hong Kong), and consensus PCR/specific PCR (MY-PCR) (25) followed by nucleotide sequencing of PCR products for type-specific HPV identification.

MATERIALS AND METHODS

Participants and sample preparation. Institutional Review Board approval was provided by the Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Beijing, China. All participants provided written informed consent.

Exploratory study. Plasmids containing the full-length sequence of L1 or E6 of 30 oncogenic HPV types were used for the determination of the analytical sensitivity of the MassARRAY assay. The plasmids were diluted to a series of concentrations of 1,000, 100, 50, 10, and 5 copies per reaction. Each plasmid concentration was tested in triplicate. We performed a cross-sectional study to investigate anal HPV infection among men who have had homosexual sex with men (homosexual men [HM]) in China (26). These samples were collected from HM from sites in two cities (Beijing and Tianjin) between March and July 2010. Two hundred anal swab samples from that study were analyzed. Sample collection and laboratory tests were performed as previously described (26).

Validation study. This study utilized 135 archived formalin-fixed, paraffin-embedded (FFPE) specimens from histologically confirmed cervical cancer cases, 85 obtained from January to June 2009 and 50 obtained from January to June 2012, in Xijing Hospital, Fourth Military Medical University, Xi'an, China. The mean age of the patient at histological diagnosis was 49 years (range, 17 to 77 years). Squamous cell carcinoma and adenocarcinoma were diagnosed in 133 and 2 patients, respectively. The histology slides were reviewed to confirm the diagnoses. DNA was extracted by using an EZNA FFPE DNA isolation kit according to the manufacturer's instructions (Omega Bio-Tek Inc., Norcross, GA). All samples were quantified by using a NanoDrop 1000 instrument (Thermo Scientific, Waltham, MA).

HPV detection and typing with consensus PCR (MY-PCR) and specific PCR. Consensus PCR targeting the HPV L1 region was performed by using consensus PCR primer pair MY09/MY11 (25). Specific PCR primers were designed based on primer pair MY09/MY11 (Table 1). The final 50-μl PCR mixture contained 10 μl sample, 25 μl PCR Master Mix (Promega, Fitchburg, WI), 3 mM $MgCl_2$, and 20 pmol each primer. Amplifications were performed by using an S1000 thermal cycler (Bio-Rad, Hercules, CA), with the following cycling profile: incubation at 94°C for 5 min; 40 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final step at 72°C for 10 min. Amplified DNA was visualized by agarose gel electrophoresis, purified by using a commercial protocol (Qiagen), and sequenced on an ABI3730 automated sequencer (Applied Biosciences, Foster City, CA), using BigDye reagent (version 3.0). Sequences were compared with documented viral sequences in the GenBank database using the BLAST search engine (http://www.ncbi.nlm.nih.gov/BLAST/). All HPV DNA-negative samples were reevaluated for conclusive results.

HPV detection and typing with MassARRAY MALDI-TOF MS. (i) Primer design and primary PCR. Representative HPV strains used for the determination of genotype groups are shown in Table S1 in the supplemental material. Their sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). Optimal primers and extension primers were designed (see Table S2 in the supplemental material for details), using Assay Design v4.0 software according to the user's guide (Sequenom Inc.). Primers were supplied by Newtsingke Biotechnology (Beijing, China) and contained a 5′ 10-base extension (ACGTTGGATG). PCR mixes (5 μl) contained 2 μl of DNA template and final concentrations of 0.1 μM primer mix, 25 mM nucleotide mix (dATP, dCTP, dGTP, and dUTP) (500 μM each deoxynucleoside triphosphate), 4 mM $MgCl_2$, 0.2 U DNA polymerase enzyme (Roche Molecular Systems Inc., Pleasanton, CA), and 0.2 U uracil-DNA glycosylase (ShineGene Molecular Biotechnology, Shanghai, China). The samples were amplified in 384-well plates (Applied Biosciences, Foster City, CA). All primary PCR amplifications were performed by using an S1000 thermal cycler (Bio-Rad), as follows: 45°C for 2 min; a denaturation step at 95°C for 4 min; 45 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final step at 72°C for 5 min. Primary PCR mixes were dephosphorylated with shrimp alkaline phosphatase, according to the manufacturer's protocol (Sequenom, Inc.). SiHa (HPV16-positive), HeLa (HPV18-positive), and HEK293 (HPV-negative) cells and sterile water were used as controls.

(ii) iPLEX reaction. The single-base extension reaction was performed with the iPLEX Pro Reagent kit, using ThermoSequenase enzyme to ensure an accurate reaction, according to the manufacturer's instructions (Sequenom Inc.). The iPLEX reaction mix was added to the dephosphorylated primary PCR mix and included 1 μM each extension primer (see Table S2 in the supplemental material for details), 0.2 μl terminator mix, 0.2 μl iPLEX Pro buffer, and 0.041 μl iPLEX enzyme (Sequenom Inc.), for a final volume of 9 μl. The PCR program was performed according to standard procedures. After desalting by the addition of 6 mg Clean Resin (Sequenom Inc.) to each 384-well plate, we applied approximately 10 nl of each iPLEX product onto a 384-spot SpectroChip II with the MassARRAY Nanodispenser RS 1000 instrument (Sequenom Inc.). Data acquisition was automatically performed by the use of SpectroAcquire (Sequenom Inc.). MS analysis was performed and interpreted with MassARRAY Typer software, version 4.0.3 (Sequenom Inc.)

HPV detection and typing with the HPV GenoArray test. The HPV GenoArray test utilizes L1 consensus primers to simultaneously amplify 21 HPV genotypes (genotypes 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68, and CP8304 [81]), followed by flowthrough hybridization with immobilized genotype-specific probes. The assay was performed according to the manufacturer's protocol (24).

TABLE 1 Specific PCR primers used in this study

HPV type	Primer	
	Forward	Reverse
HPV6	AARGCCCAGGGACATAACAA	TCCCAAMGGATACTGATCCA
HPV11	AAGGCTCAGGGACATAACAATG	TCCAAGGGGAAACTGATCTAA
HPV16	GAGCACAGGGCCACAATAAT	TCCTAAAGGAAACTGATCTAGG
HPV18	GCACAGGGTCATAACAATGG	GTCCAAGGGGATATTGATYT
HPV26	TGGTATCTGTTGGGGCAATC	GCCCTAGTGGAAATTGATCC
HPV31	CGTGCTCAGGGACACAATAA	ACCCAGTGGAAACTGATCTAAA
HPV33	CGTGCACAAGGTCATAATAA	TCCCAAAGGAAACTGATCTAAA
HPV35	CGTGCACAAGGCCATAATAA	GCCYAACGGAAAYTGATCT
HPV39	GCCCAGGGYCAYAAYAATGGT	CTGATCAAGTTCCAAACTAAACTTTTC
HPV40	AGGGCCATAACAATGGCATA	TGGAAATTGATCTAATTGGGAAG
HPV42	AAGCACAAGGACACAATAATGG	GGAAATTGATCTAAATCAGTAGAAAAC
HPV43	AAGGCCCAGGGACATAATAA	GCCCTAAGGGAAACTGGGTA
HPV44	GCAGGGCCACAATAATGGTA	CCAAGGGGATATTGATCCAAC
HPV45	CCCAGGGCCATAACAATG	TGATCYAAATCGGAGGAAAA
HPV51	GTGCGCAGGGTCACAATAAT	CCCAATGCAAATTGGTCTAAA
HPV52	GCGCAGGGCCACAATAAT	GGAAACTGATCTAAATCTGCAGAAAAC
HPV53	CGTGCCCAGGGACATAATAA	GCCAAGAGGAAACTGATCCA
HPV54	CCAGGGTCAAAACAATGGTA	AGGGAAACTGGTCAAGGTCA
HPV56	CGTGCCCAAGGCCATAATA	CCCAGTGGAAATTGATCCAG
HPV58	CGTGCACAAGGTCATAACAA	TCGTCCCAAAGGAAACTGAT
HPV59	TGGTATATGTTGGCACAATCAA	ACGTCCCAARGGAWACTGAT
HPV61	CAGGGCCAYAACAATGGTAT	CCCAAAGGAAACTGATCCAA
HPV66	AATGGCATATGCTGGGGTAA	AGGTCTGCAGAAAAGCTGTC
HPV68	AAGGCACARGGACACAACA	GCGTCCTAAWGGRAAYTGGTC
HPV70	AGGCCCAGGGACACAATAAT	GCCCCAAAGGAAACTGATCT
HPV72	GGGTCACAACAATGGCATCT	GCGCCCTAAAGGAAACTGAT
HPV73	AGGCACAGGGACAAAATAATG	TTCCCAAAGGAAACTGGTCT
HPV81	GGGCACAGGGHCATAATAA	GCGACYCARAGGAWACTGAT
HPV82	CAATAATGGCATTTGCTGGA	CCAATGCAARCTGATCYAAAT
HPV89/CP6108	CGTGCACAGGGYCATAATA	CCGCCCAATTGTAATAAAAC

Nucleotide sequence accession numbers. The sequences obtained in this study have been submitted to GenBank under accession numbers JQ902108 to JQ902139.

RESULTS

Result of the exploratory study. The analytical sensitivity of the PCR-MS assay was determined with plasmids carrying the full-length sequence of L1 or E6 of 30 HPV types. The PCR-MS assay was able to detect 10 to 50 copies of the corresponding HPV plasmid per reaction (see Fig. S1 in the supplemental material).

Demographics of the 200 HM participants enrolled in the study were supplied in a previous report (26). HPV was detected by PCR-MS or MY-PCR in 129 samples (64.5%); 71 (35.5%) samples tested negative by the two methods. All 30 HPV types were detected in the study, and all 61 PCR-MS primer-extension probes worked successfully.

All 30 L1 probes were confirmed by MY-PCR sequencing for each type directly, and this sequencing also indirectly confirmed each of 30 E6 probes in all cases in which samples were both E6 and L1 probe positive. Direct confirmation was not possible because there were no common standard primers for the E6 region. Infections with single HPV types were found for 62 of 129 samples (48.1%), and infections with multiple types were found for 67 of 129 samples (51.9%). Throughout the PCR-MS testing, there were no results from the negative controls, which ensured the validity of the results.

For the 62 samples detected as single HPV infections (Table 2), the results for 37 samples (59.7%) determined by MY-PCR were the same as those determined by PCR-MS. These included 19 types (types 6, 11, 16, 18, 33, 35, 39, 40, 43, 45, 51, 52, 56, 59, 61, 66, 81, 82, and 89). Two samples were identified as HPV type 84 (HPV84) by consensus PCR; HPV84 is not one of the 30 types included in the PCR-MS panel.

For the remaining 67 samples, the PCR-MS method detected multiple HPV types, whereas consensus PCR followed by nucleotide sequencing detected only one HPV type (see Table S3 in the supplemental material for details). PCR-MS results, which were scored as HPV positive by L1 probes, were confirmed by specific MY-PCR followed by nucleotide sequencing of PCR products including 11 types (types 26, 31, 42, 44, 53, 54, 58, 68, 70, 72, and 73). All 30 types were detected by PCR-MS of these samples. Two HPV types were detected in 28 samples, while 3 to 11 HPV types were detected in 39 samples.

Results of the validation test. A total of 135 FFPE invasive cervical tumor samples were analyzed by using PCR-MS, and results were compared to those of the validated and commercially available GenoArray (GA) method. PCR-MS and GA detected HPV in 97% (131/135) and 90.4% (122/135) of the samples, respectively. All the 122 positive samples detected by GA were also detected by PCR-MS. The 4 samples scored negative by PCR-MS were also negative by GA (Table 3). The 9 discordant samples which tested positive by PCR-MS and negative by GA included 3 internal control negative (IC-N) samples. To resolve the discrep-

TABLE 2 Comparison of single HPV genotypes obtained by multiplex PCR-MS and consensus PCR

Sample ID	HPV type determined by:	
	Consensus PCR[a]	MassARRAY
S1	66	66
S2	35	35
S3	45	45
S4	18	18
S5	33	33
S6	84	11
S7	43	43
S8	52	52
S9	56	56
S10	56	56
S11	40	40
S12	84	11
S13	51	51
S14	N	54
S15	16	16
S16	N	68
S17	61	61
S18	16	16
S19	56	56
S20	N	6
S21	16	16
S22	56	56
S23	81	81
S24	N	11
S25	56	56
S26	81	81
S27	89	89
S28	81	81
S29	40	40
S30	N	89
S31	N	42
S32	N	11
S33	59	59
S34	N	6
S35	N	68
S36	6	6
S37	N	42
S38	N	6
S39	N	54
S40	82	82
S41	N	72
S42	N	54
S43	56	56
S44	N	52
S45	11	11
S46	N	6
S47	6	6
S48	56	56
S49	16	16
S50	N	11
S51	N	11
S52	40	40
S53	40	40
S54	16	16
S55	N	11
S56	N	6
S57	N	51
S58	N	11
S59	39	39
S60	N	51
S61	33	33
S62	16	16

[a] N, negative.

ancy, these samples were analyzed by sequencing. Type-specific MY sequencing correctly identified the 3 IC-N samples as HPV82, HPV16/31, and HPV68 and the other 6 discordant samples as HPV16, HPV18, HPV58, HPV 16/18, HPV16/18/31, and HPV18/31/42, which were completely concordant with the PCR-MS results. Multiple HPV types were identified in 25.2% (34/135) and 55.6% (75/135) of the samples by GA and PCR-MS, respectively.

High-risk HPV type 16 was predominant. It was identified in 112/122 (91.8%) and 119/131 (90.8%) of the samples by GA and PCR-MS, respectively. HPV genotypes were detected in 158 and 233 samples by GA and PCR-MS, respectively. Seventy-five samples with genotypes detected by PCR-MS were subjected to MY-PCR sequencing, and 87% (65/75) of the results were confirmed. The other 10 samples positive for HPV types were shown by E6 and L1 peaks in multiple-infection samples, but they could not be identified by sequencing due to the high level of background from other high-level HPV genotypes (Table 3).

A total of 15 HPV types were seen in the cervical cancer samples, including 11 high-risk types (HPV types 16, 18, 31, 39, 45, 52, 56, 58, 59, 68, and 82), 1 probable high-risk type (HPV type 66), and 3 low-risk types (HPV types 11, 42, and 89). Besides HPV16, other common types were HPV types 18 (59 samples; 45%), 31 (16; 12.2%), 45 (10; 7.6%), 58 (8; 6.1%), 52 (4; 3.1%), 68 (4; 3.1%), 59 (3; 2.3%), 56 (2; 1.5%), 82 (2; 1.5%), 39 (2; 1.5%), 66 (1; 0.8%), 42 (1; 0.8%), 11 (1; 0.8%), and 89 (1; 0.8%), considering both single and multiple infections (Table 4).

The high percentage of HPV16-positive samples seen for 135 cervical cancer samples was detected by two methods. A total of 50 of 135 samples were collected and extracted after 10 months from the first batch of 85 samples from the same hospital and tested at least 2 weeks after the first test. Thus, the high percentage of HPV16-positive samples may be caused by the specific region and population.

The GA test was approved for ThinPrep samples only. It may not be suitable for the testing of degraded FFPE samples, so we used specific MY-PCR sequencing for additional confirmation. MY-PCR identified 87% genotype samples as positive and confirmed the results by PCR-MS. Our results from FFPE cervical cancer specimens showed that dual gene detection for each specific HPV type greatly assists in the analysis of degraded FFPE DNA cancer samples with multiple HPV types. It even worked for GA internal control negative samples.

DISCUSSION

A new high-accuracy HPV genotyping method was developed based on three principles: (i) targeting short regions (60 to 120 bp) of dual genes to distinguish each HPV genotype, (ii) preventing PCR carryover contamination and multiple-probe interactions, and (iii) adopting high-sensitivity two-round detection using multiplex PCR with type-specific primers followed by a single-base extension of different specific probes detected by MALDI-TOF MS. In this study, a three-well assay comprising a 61-plex analysis of 30 HPV types was developed by targeting two genes, E6 and L1, using β-globin as a DNA extraction quality control. All 61 probes worked successfully in an initial study of anal swab samples from 200 homosexual men.

Dual-gene detection avoids the false-negative results of current methods, which target only one gene. This improved approach will deepen our understanding of HPV infection and provide more comprehensive data for epidemiological studies.

The genotype-specific assay design which we used eliminated false-positive results caused by probe interactions and nonspecific binding, which frequently interfere with ordinary multiplex PCRs. In the exploratory study, 29 samples were determined to have infection by a single HPV type by dual probes, and these

TABLE 3 Comparison of the results of PCR-MS and GA genotype analyses followed by sequencing

Comparison	HPV type	No. of positive samples/total no. of samples tested by GA	% of samples positive by GA	No. of positive samples/total no. of samples tested by PCR-MS	% of samples positive by PCR-MS	Compensation test for different cases: No. of positive samples/total no. of samples tested by sequencing	Compensation test for different cases: No. of positive samples by GA + sequencing vs PCR-MS/total no. of samples tested	Compensation test for different cases: Detection rate (%)
1	11	1/122	0.8	1/131	0.8		1/1	100
2	16[a]	112/122	91.8	119/131	90.8	7/7	119/119	100
3	18[a]	25/122	20.5	59/131	45.0	33/34	58/59	98.3
4	31[a]	7/122	5.7	16/131	12.2	7/9	14/16	87.5
5	39[a]	2/122	1.6	2/131	1.5		2/2	100
6	42	0/122	0	1/131	0.8	1/1	1/1	100
7	45[a]	4/122	3.3	10/131	7.6	3/6	7/10	70.0
8	52[a]	2/122	1.6	4/131	3.1	0/2	2/4	50.0
9	56[a]	0/122	0	2/131	1.5	2/2	2/2	100
10	58[a]	3/122	2.5	8/131	6.1	3/5	6/8	75.0
11	59[a]	0/122	0	3/131	2.3	3/3	3/3	100
12	66[b]	1/122	0.8	1/131	0.8		1/1	100
13	68[a]	1/122	0.8	4/131	3.1	3/3	4/4	100
14	82[a,c]			2/131	1.5	2/2	2/2	100
15	89[c]			1/131	0.8	1/1	1/1	100
Total		158		233		65/75	158 + 65/233	95.7

[a] High-risk type.
[b] Pobable high-risk type.
[c] Not included in the GA assay.

TABLE 4 Distribution of HPV types in cervical cancer samples

Infection type	No (%) of samples
Negative	4 (3.0)
Single infection	
HPV16	45
HPV18	5
HPV31	1
HPV45	1
HPV58	2
HPV68	1
HPV82	1
Total single infections	56 (41.5)
Multiple infections	
HPV16/18	36
HPV16/31	12
HPV16/45	3
HPV16/52	4
HPV16/58	1
HPV16/82	1
HPV16/18/31	2
HPV16/18/45	5
HPV16/18/58	2
HPV16/18/59	1
HPV16/18/66	1
HPV16/18/68	1
HPV18/31/42	1
HPV11/16/18/56	1
HPV16/18/58/59	1
HPV16/18/56/58/59	1
HPV16/18/39/58/68/89	1
HPV16/18/33/39/45/68	1
Total multiple infections	75 (55.6)
Total	135

results were confirmed by MY-PCR sequencing. Most of the L1 probes adopted in the PCR-MS assay were excluded from the MY region, which is generally used and might cause widespread contamination in HPV studies. Therefore, the new assay should be a useful alternative for samples or laboratories with suspected MY-PCR amplicon contamination.

Uracil-DNA glycosylase was used in our assay to control carryover contamination, since the primary source of exogenous templates leading to false-positive amplification is products from previous PCR amplifications. Negative controls were negative throughout the study.

Using our assay, multiple infections can be detected simultaneously. The percentage of infections by multiple HPV types among cervical cancer patients reported previously ranged from 3.7 to 58% and varied across countries and in relation to the HPV detection assays used (27, 28). However, PCR with consensus primers or general primers may underrepresent the true prevalence of multiple-HPV infections (22, 28, 29). About 57% of our cervical cancer patients were infected by multiple HPV types. Patients with multiple infections are at risk for treatment failure (30), which further highlights the clinical significance of our new method. The new method does not use fluorescent dye, which will help to improve the stability and repeatability of the experimental results. PCR-MS has the capability to identify more than 40 single-nucleotide polymorphisms (SNPs) in a single reaction (17). We currently use three wells to cover 61 SNPs. This leaves plenty of room to expand the range of SNPs covered by our method, to increase further its power as a tool for epidemiological research and clinical utility. The method has a high level of flexibility in adding or changing probes as necessary.

The new method demonstrates high throughput and cost-effectiveness. The new assay allows the genotyping of >1,000 samples per day. At the current conservative level of multiplexing,

7,800 assays can be performed in a single 384-well microplate, allowing the rapid acquisition of valuable information. The HPV typing turnaround time for a 384-well microplate is less than 10 h. The routine provision of these HPV tests can have a substantial impact on health care and has been shown to be highly cost-effective.

Because HPV infections can lead to many diseases, especially some cancers, HPV detection has very important clinical significance. Epidemiological data suggest that persistent infections with carcinogenic HPV cause virtually all cervical cancers and substantial fractions of other anogenital and oropharyngeal cancers (8, 31). Several studies have pointed out that the detection of the same carcinogenic HPV type over time is particularly important for cervical carcinogenesis, and HPV persistence is a clinical marker and endpoint (32–35). Ronco et al. showed previously that HPV-based screening is more effective than cytology in preventing invasive cervical cancer (36). Accurate HPV genotyping is key to the identification of persistent infections. The new method can detect 30 HPV genotypes simultaneously, opening up new possibilities for detection and the study of epidemiological or pathological features. Expanding the survey types can help us gain more accurate information for disease prevention and therapy as well as provide data for second-generation vaccine research. Currently used screening methods failed to identify HPV infection in many samples. Research on the relationship between HPV and cervical cancer suggests that it is necessary to investigate HPV infections among relevant populations. Rijkaart et al. indicated previously that the implementation of HPV DNA testing in cervical screening will aid in the early detection of clinically relevant CIN of grade 2 or higher (37).

Following HPV infection, prognosis is significantly linked to the HPV type. Epidemiological studies can determine the type of HPV infection, which is informative for the development of new HPV screening methods and vaccines. HPV prevalence is dependent on the geographical region (38, 39). Compelling evidence shows that HPV16 and HPV18 are the most oncogenic types (8, 40). Prophylactic HPV vaccines composed of HPV type-specific L1 proteins are now available. Effective monitoring and surveillance of those individuals vaccinated and of the general population will be required. Furthermore, accurate data on HPV type distribution is essential for estimating vaccine impacts and the development of screening programs. Worldwide, it is estimated that about 291 million women with normal cytology have HPV DNA (38). Therefore, it is necessary to develop high-throughput and highly sensitive methods suitable for epidemiological research. Our new method should serve as a powerful tool for epidemiological research and clinical use.

There are two limitations of our method. First, the 30 HPV types chosen were based on a report by the IARC on cervical cancer in 9 countries in 2003 (13). It may not be suitable for other cohorts such as infected homosexual men. Our assay should be updated as MY-PCR sequencing results for other cohorts emerge. Second, the volume of sample used in the primary PCR was 2 μl. When the HPV load is very low, the PCR-MS method may fail to detect it. In this situation, a larger primary PCR volume should be used in the first PCR run for some samples that are suspected to be positive that initially test negative.

Consensus PCR is widely used as an HPV typing method (13). In this study, consensus PCR detected all single-HPV infections. It also identified HPV84 infection in two samples. However, previous research has shown that consensus PCR followed by sequencing is ineffective in detecting multiple-HPV infections (22, 28, 29). In our study, there were 34 samples with multiple-HPV infections detected by the MassARRAY method. Consensus PCR failed to detect infections in any of these samples, which highlights its limitations. Specific PCR followed by sequencing is efficient for HPV typing. In this study, this method detected all multiple-HPV infections; however, it is not suitable for large-scale screening projects.

In conclusion, our new assay is a reliable and highly accurate method. It provides a good alternative to current methods, especially for large-scale investigations of multiple-HPV infections and degraded FFPE samples. It will greatly improve the application and evaluation of HPV vaccines and the study of the etiology of human cancers.

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