

Polymerase chain reaction-based fluorescent Luminex assay to detect the presence of human papillomavirus types

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Because 40% of human papillomavirus (HPV) infections are mixed infections, the accurate identification of high-risk HPV genotypes in mixed infections is important for defining a woman's risk for progression to cervical cancer. Thus, advanced Luminex-based HPV genotyping has been developed to simultaneously detect the presence of multiple HPV types. Here, we describe the development of a Luminex-based HPV genotyping that combines polymerase chain reaction amplification with hybridization to fluorescence-labeled polystyrene bead microarrays (Luminex suspension array technology). New HPV type-specific oligonucleotide probes and YBT L1/GP6-1 primers were used to detect the HPV types in 132 clinical samples. We simultaneously evaluated the usefulness of this technique on clinical samples. We detected 15 specific HPV types (6, 16, 18, 31, 35, 42, 51, 52, 55, 56, 58, 59, 66, 67 and 68) examined with specificity without known cross-reaction to other HPV types. The detection limit for the different HPV types was above 500 plasmids. We compared the performance of the Luminex-based assay to the established HPV DNA microarray chip for polymerase chain reaction products derived from 53 clinical samples. The evaluation showed excellent agreement. The Luminex-based HPV genotyping was a sensitive, reproducible technique for the simultaneous genotyping of all clinically relevant genital HPV types. This assay system may be used to provide critical clinical information for early detection of HPV, especially in cases where the HPV copy numbers are low and the latency period of HPV infection is prolonged. (*Cancer Sci* 2007)

Human papilloma virus (HPV) is the most common sexually transmitted infection in the world. Certain high-risk types can cause cancers of the cervix, vagina, vulva, penis and anus. More than 100 viral types of HPV have been identified, and over 30 types of these are associated with sexually transmitted infections;⁽¹⁾ individuals may be infected with more than one type at a time.⁽²⁾ Because most HPV infections have no visible signs or symptoms,⁽³⁾ the development of detection tools for HPV identification in asymptomatic patients has been very important.⁽⁴⁾

Several HPV DNA detection methods have been described during the last decade, each of which allows the detection of a wide spectrum of HPV types, such as DNA amplification-based methods. Due largely to routine screening using Pap tests, the number of deaths attributed to cervical cancer continues to drop nearly 4% annually. However, the Pap smear has some weaknesses, not least of which is its limited sensitivity for detection of cancer precursors.⁽⁵⁾ Therefore, the false-negative rate of the Pap smear ranges from 20 to 30% when cytology is used alone. In addition, there is a false-positive rate of 5–15%.^(6–8) To carry out

HPV genotyping, a reliable, FDA-approved test to distinguish the specific HPV types will need to be developed and validated. A DNA-based technology, the Hybrid Capture II HPV test, has been used to detect 13 high-risk types of HPV.^(9,10) However, this routine test is not recommended for women under the age of 30 years unless they have atypical or equivocal Pap test results. In addition, despite its high sensitivity, false-negative results are known to occur for histologically confirmed cervical intra-epithelial neoplasia (CIN)2 or CIN3 cases, with its impossibility to perform genotyping.^(11,12) Therefore, the current techniques available for detection of HPV types all have shown limited ability for complete detection; there is no single technique that provides complete detection to date. Therefore, a new and improved HPV assay that is highly sensitive and reproducible is required. Multiplex technology is a new method that is based on fluorescent bead technology, and allows simultaneous detection of nucleic acids against up to 100 different HPV types.^(13,14) Recently, this technology has been used for the genotyping of HPV types using polymerase chain reaction (PCR) products.^(15–17)

In the present study, we developed Luminex HPV genotyping with optimal performance characteristics that combined PCR amplification with Luminex hybridization to liquid bead microarrays. We used new HPV type-specific oligonucleotide probes and YBT L1/GP6-1 primers to detect HPV types in the high-risk group (16, 18, 31, 35, 51, 52, 55, 56, 58, 59, 66, 67 and 68) and the low-risk group (6 and 42). This assay detected the HPV types in 132 patient samples. In addition, we compared the performance of the Luminex-based assay to the established HPV DNA microarray chip for PCR products derived from 53 clinical samples. Also, we simultaneously evaluated its usefulness for clinical investigations.

Materials and methods

Clinical samples. All tested samples were extracted from cervical scrapes collected at the Catholic Medical University, Seoul, Korea. A total of 132 samples were tested 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. The evaluation routinely included a complete history and physical examination, complete blood count, pelvic examination, sigmoidoscopy, cytology and intravenous pyelogram. These were cytologically abnormal smears.

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Table 1. Oligonucleotide probes and primers for the human papillomavirus (HPV) genotyping kit

HPV type or probe name	Accession no.	Probe sequence (5'-3')	Length
Type 06	X00203	Amine-GCA TCC GTA ACT ACA TCT TCC A	22-mer
Type 16	K02718	Amine-TGT GCT GCC ATA TCT ACT TCA GA	23-mer
Type 18	X05015	Amine-AGT CTC CTG TAC CTG GGC AA	20-mer
Type 31	J04353	Amine-GTG CTG CAA TTG CAA ACA GT	23-mer
Type 35	M74117	Amine-CTG TGT GTT CTG CTG TGT CTT CT	20-mer
Type 42	M73236	Amine-CCA CTG CAA CAT CTG GTG AT	20-mer
Type 51	M62877	Amine-ATT AGC ACT GCC ACT GCT GC	20-mer
Type 52	X74481	Amine-GCT GAG GTT AAA AAG GAA AGC A	22-mer
Type 55	U31791	Amine-GCT ACA ACT CAG TCT CCA TC	20-mer
Type 56	X74483	Amine-CAG TTA AGT AAA TAT GAT GCA CGA AAA	27-mer
Type 58	D90400	Amine-TGC ACT GAA GTA ACT AAG GAA GG	23-mer
Type 59	X77858	Amine-TTC TGT GTG TGC TTC TAC TAC TTC TTC	27-mer
Type 66	U31794	Amine-TGC AGC TAA AAG CAC ATT AAC T	22-mer
Type 67	D21208	Amine-AGG AAA AAT CAG AGG CTA CAT A	21-mer
Type 68	M73258	Amine-TCA GCT GTA CCA AAT ATT TAT GAT CC	26-mer
GAPDH	BC083511	Amine-AAT CCC ATC ACC ATC TTC CA	20-mer
YBT L1 F		GCM† CAG GGW‡ CAY§ AAY§ AAT GG	20-mer
GP6-1		AAT AAA CTG TAA ATC ATA TTC CTC	24-mer
GAPDH F	BC083511	GAG TCA ACG GAT TTG GTC GT	20-mer
GAPDH R	BC083511	TTG ATT TTG GAG GGA TCT CG	20-mer

†M, A/C; ‡W, A/T; §Y, C/T.

HPV sequences from GenBank. HPV sequences of different isolates and complete genomes with accession numbers were obtained from the HPV database (<http://hpv-web.lanl.gov/stdgen/virus/hpv/>) and used for probe development. We designed probes for specific sequences in the high-risk (types 16, 18, 31, 35, 51, 52, 55, 56, 58, 59, 66, 67 and 68) and low-risk (types 6 and 42) groups of HPV. These probes were designed to anneal at a melting temperature of 60°C, resulting in probe lengths of 20–27 nucleotides. All probes had 5'-amine modifications for bead coupling as well as 15 bp oligo dT sequences attached for hybridization flexibility (Table 1).

Amplification of L1 fragment. According to the alignment results of 24 types of HPV genome sequences, we developed new primer sequences YBT L1 F (5'-GCMCAGGGWCAYAAAYAATGG-3') for the forward primer and GP6-1 (5'-AATAAAGTGTAAATCATATTCCTC-3') for the reverse primer. Amplification of the ~190 bp-long fragment of the viral L1 open reading frame (ORF) was carried out in 50 µL of PCR mixture containing 0.4 µM HPV primer set, 0.1 mM dNTP mix, 7.5 mM Tris HCl (pH 9.0), 2 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂SO₄ and 1.5 U *Taq* polymerase (Biotools, Madrid, Spain). A 5-min denaturation step at 95°C was followed by 40 cycles of amplification with a PCR thermocycler (Master Cycler; Eppendorf, Hamburg Germany). Each cycle included a denaturation step at 95°C for 30 s, an annealing step at 50°C for 30 s, and an elongation step at 72°C for 30 s. The final elongation step was prolonged for a further 7 min. Similar reactions and cycling conditions were used for the YBT L1 F/GP6-1 PCR on 5 µL of specimens from cervical scrapings. For a positive control of the PCR, two primers were used for amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences: GAPDH F, 5'-GAGTCAACGGATTTGGTCGT-3'; and GAPDH R, 5'-TTGATTTTGGAGGCATCTCG-3'.

Labeling of the target sequence. After the PCR reaction, a single-strand PCR was carried out for labeling of the probe complementary sequences with biotin-14-dCTP (Invitrogen, Carlsbad, CA, US). The GP6-1 reverse primer was used for the PCR product labeling. PCR components in 20 µL PCR mixture were 0.5 µM GP6-1, 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, Madrid, Spain) and 2 µL of amplified L1 fragment. A 5-min denaturation

step at 94°C was followed by a 30-cycle amplification step at 72°C for 30 s, an annealing step at 60°C for 30 s, and an elongation step at 72°C for 1 min. Amplified GAPDH was labeled with biotin-14-dCTP by GAPDH R.

Coupling of oligonucleotide probes to beads. The sequences of 5'-amine modified probes (Genotech, Daejeon, Korea) are presented in Table 1. The synthesized probes were coupled to carboxylated beads (Luminex Corp., Austin, TX, USA) through a carbodiimide base coupling procedure. For coupling to the beads, approximately 2.5 million carboxylated beads were suspended in 25 µL of 0.1 M 2-(*N*-morpholino) ethanesulfonic acid at pH 4.5 (MES). Probe oligonucleotides (400 pmol) and 200 µg of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) were added and mixed thoroughly with the beads. Incubation was carried out in the dark under agitation for 30 min and repeat agitation for 20 min with additional 200 µg EDC. After the addition of EDC and a repeat incubation step, the beads were washed once with 0.5 mL of 0.02% Tween 20 and once with 0.5 mL of 0.1% of sodium dodecyl sulfate (SDS) before being stored in 100 µL of TE buffer at 4°C in the dark.

Hybridization assay. Hybridization buffer (21 µL of 2× EZway Hybrisol; Koma Co., Seoul, Korea) and 1 µL of HPV bead mix (~150 000 beads) were added to a labeling tube. The mixture was heated to 95°C for 10 min and kept at 40°C for 30 min for hybridization. After hybridization, samples were transferred to a filter plate immediately, and washed three times with TM hybridization 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 of streptavidin-R-phycoerythrin (Strep-PE; Molecular Probes, Eugene, OR, USA) and analyzed for internal bead color and R-phycoerythrin reporter fluorescence on a Luminex 100 analyzer.

Hybridization reaction on the HPV DNA microarray. We used the HPV PCR-based DNA microarray system as an HPV genotyping method (MyGene Co., Seoul, Korea) for HPV typing. The HPV DNA microarray contains 24 type-specific probes; 16 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 54, 56, 58, 59, 66 and 68) and seven low-risk types (11, 34, 40, 42, 43, 44 and 70). A slide has eight chambers, and each chamber is used for a test. Therefore, a slide tests eight samples at one time. Briefly, DNA was isolated from swab cervical samples using a DNA isolation kit

Table 2. Specificity and cross hybridization test of 15 type-specific probes and reaction-positive probe (glyceraldehyde-3-phosphate dehydrogenase) in multiplex human papillomavirus (HPV) genotyping

HPV type	HPV type-specific probe														
	6	16	18	31	35	42	51	52	55	56	58	59	66	67	68
6	6803	0	0	0	9	10	0	11	0	0	0	32	0	1	8
16	1	5120	0	0	4	0	12	1	14	0	0	33	0	0	0
18	0	0	6977	0	0	3	3	0	5	0	0	139	0	1	3
31	0	0	0	3163	0	4	0	0	0	0	0	44	0	5	6
35	0	0	0	0	3504	2	0	0	0	0	0	1	0	0	3
42	0	0	0	0	0	4324	5	0	0	0	0	0	0	6	2
51	0	0	0	0	0	12	4652	2	0	0	0	12	0	0	9
52	0	0	0	0	0	14	4	543	0	0	0	0	0	3	10
55	162	0	0	0	0	1	0	0	3466	0	0	0	0	3	1
56	0	35	0	0	0	6	0	0	0	3532	0	5	0	0	2
58	8	0	0	0	0	8	0	0	0	0	2305	0	0	9	1
59	0	0	0	50	7	1	0	4	3	0	0	2876	0	0	10
66	53	0	0	0	0	0	0	0	0	10	0	0	1789	0	3
67	0	0	0	0	0	0	0	0	0	0	0	0	0	4119	0
68	0	0	0	0	0	0	2	3	0	0	0	0	0	0	2579
Threshold	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200

(MyGene); the target L1 regions of HPV DNA were amplified and labeled by a single dye, indocarbocyanine-dUTP (NEN Life Science Products, Boston, MA, USA), using consensus GPd5+/GP6d+ primers. The samples were mixed with a hybridization solution (MyGene) then applied to the DNA microarray. Hybridization was carried out at 43°C for 90 min and was followed by washing with 3× SSPE (0.18 M NaCl, 10 mM NaH₂PO₄ and 1 mM EDTA; pH 7.7) for 5 min and 1× SSPE for 5 min, and drying at room temperature. Hybridized HPV DNA was visualized using a DNA Chip scanner (Scanarray Lite; GSI Lumonics, Ottawa, ON, Canada).

Cut-off signal intensity. Reaction of probes with negative PCR products from other HPV types was considered as background values. After subtraction, bead signal intensity was read with mean fluorescence intensity (MFI) over 200.

Results

Amplification and labeling of L1 fragment. We developed a HPV genotyping detection protocol using the Luminex system. The assay system consisted of three procedures: (1) amplification of the L1 fragment with YBT L1 F and GP6-1 reverse primers; (2) labeling the amplified target sequence with the GP6-1 reverse primer; and (3) hybridization to the beads in parallel and then reading the phycoerythrine dye and bead number with the Luminex 100.

When we amplified a region of the viral coat protein L1 ORF for HPV detection, the GAPDH sequence was also amplified as a PCR positive control. The sizes of the PCR products from the HPV and GAPDH were approximately 190 and 150 bp, respectively. If the GAPDH signal was detected but the HPV sequence-coupled beads was not detected, it suggested that the sample was not infected by HPV. The MFI of GAPDH was clearly higher than any non-specific hybridization signal and generally lower than specific hybridization with any HPV type. The copy number of the GAPDH gene, in the sample, was usually higher than that of the HPV. Therefore, it was necessary to adjust the GAPDH primer concentration for the proper amplification of a positive control, and to provide a balance between the HPV signals and the GAPDH signal. Without adjustment of the primer concentration, the amplification of the HPV signal was at times hindered by the GAPDH amplification.

Hybridization and signal detection. PCR amplicons containing biotin-14-dCTP were denatured at 95°C for 5 min and hybridized to the beads at 40°C for 30 min.

The 15 different amine-modified probes were coupled to carboxylated beads for HPV typing and one probe for GAPDH. The L1 sequences of 15 types of HPV (6, 16, 18, 31, 35, 42, 51, 52, 55, 56, 58, 59, 66, 67 and 68) were cloned in our laboratory. These clones (10⁵ copies) were tested for sensitivity and cross hybridization (Table 2). From the 15 examined HPV types, 15 probes reacted with absolute specificity. Even though there was some minor cross hybridization between HPV types, all of them were lower than the cut-off value (MFI 200).

To test the suitability of Luminex-based HPV genotyping, we tested a total of 132 clinical samples extracted from cervical scrapes (Table 3). The probe sequence for the specific HPV type resulted in at least a 10-times higher MFI value than the non-specific probe sequence. For example, the MFI value of the HPV 52 probe was 256, but the highest non-specific MFI value for the HPV 58 probe was 19 from patient sample number 223. The ratio of specific over non-specific was 13 for sample number 223. The MFI value of GAPDH for this sample was 246, higher than the cut-off value for genotyping in this experiment. The increased number of MFI values may reflect an overall higher sensitivity of the Luminex-based HPV assay.

As shown in Table 4, multiple infections were detected in 42 samples by these assays. Among these samples, 90 samples had a single infection, 35 samples had double infections, six samples had a triple infection, and one sample was infected with six different types of HPV. HPV type 16 was the most common type identified in the test, followed by 18, 58, 31, and so on. The 132 samples contained a total of 15 different HPV types, present in single or multiple infections.

Detection sensitivity. To determine the detection limits of the Luminex-based HPV genotyping assay, a HPV plasmid clone was used. The serial dilutions of the plasmids were amplified in a PCR reaction, and the PCR products were labeled with biotin-dCTP. Next, the amplicons were hybridized. We used the HPV type 18 plasmid as a template, as shown in Fig. 1, compared to HPV DNA microarray. The two assay methods showed a significant relationship between the input HPV copy number and the signal intensities. The HPV DNA microarray efficiently detected 500 copies of the HPV 18 template, whereas Luminex detected a highly significant sensitivity at between 500 and 5000 copies with the HPV 18-specific probe. This result indicates that the detection limit of the Luminex assay was less than 5000 copies with a significant intensity. The signals were maintained

Table 3. Representative examples for multiplex human papillomavirus (HPV) genotyping results with clinical specimens

Clinical sample number	HPV type-specific probe and GAPDH probe												GAPDH	Result HPV type
	6	16	18	31	35	42	52	56	58	59	66	68		
288	1413	4	19	6	8	32	1	-4	-1	25	14	-15	617	6
341	-27	2675	9	-27	-84	15	15	-18	-17	-5	-185	1	532	16
411	21	13	4186	9	-72	19	24	9	17	10	-177	-11	944	18
320	-9	-38	-11	1080	-70	4	-11	-12	-7	8	-6	-43	325	31
265	-10	-19	-6	-1	440	-2	-12	-7	12	-6	24	21	358	35
385	-1	-2	12	4	-84	1979	5	2	-12	-6	-180	-24	1262	42
223	8	-41	1	-6	-91	13	256	-4	19	10	-16	-45	246	52
309	-11	-55	1	-12	-92	-4	-13	817	25	13	-17	-42	360	56
211	-5	-40	-5	-20	-67	-3	-13	-12	1887	-6	-20	-45	555	58
231	-1	-58	-9	-18	-76	20	-19	-20	17	1602	-26	-68	778	59
234	-1	-53	11	6	-94	-12	9	-1	2	14	4748	-51	560	66
270	-4	-50	11	-20	-96	9	-6	-9	25	26	-21	2917	1091	68
Cut-off	200	200	200	200	200	200	200	200	200	200	200	200	200	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 4. Detection of human papillomavirus (HPV) types among a total of 132 clinical samples

Infection type	HPV type															Total
	6	16	18	31	35	42	51	52	55	56	58	59	66	67	68	
Single-infection	1	41	8	6	8	1	0	6	0	2	8	2	1	1	5	90
Multi-infection	3	20	12	6	2	2	3	2	1	4	5	4	3	1	2	70 [†]
Total	4	61	20	12	10	3	3	8	1	6	13	6	4	2	7	
Incidence (%)	2.5	38.1	12.5	7.5	6.3	1.9	1.9	5.0	0.6	3.8	8.1	3.8	2.5	1.3	4.4	
Multi-infection (%)	4.3	28.6	17.1	8.6	2.9	2.9	4.3	2.9	1.4	5.7	7.1	5.7	4.3	1.4	2.9	

[†]Double infection, 35; triple infection, 6; six infections, 1.

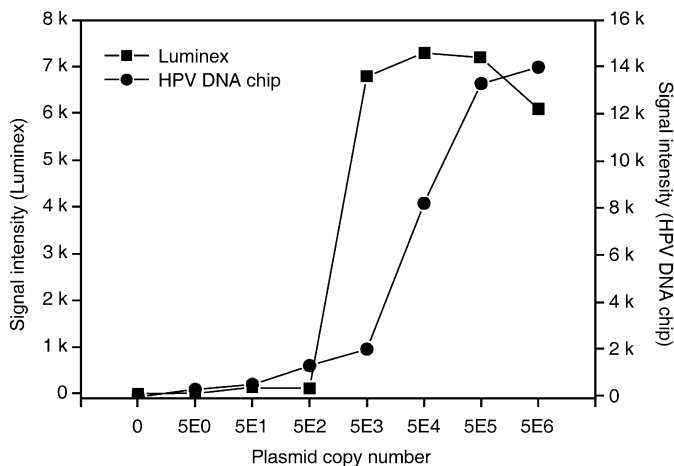


Fig. 1. Detection limit analysis of the Luminex assay with serial dilutions of 5 to 5×10^6 human papillomavirus (HPV) 18 plasmid copies. The detection limit was determined using cut-off 200 mean fluorescence intensity (MFI) in the Luminex assay. In HPV DNA microarray, the signal intensity of each copy number was calculated at 635 nm.

steadily with HPV 18 copy number above 10^5 , whereas the signals for low copy number were not detected.

HPV detection accuracy. The Luminex-based HPV genotyping assays showed a good accordance with the HPV DNA microarray for the detection of HPV types (Table 5). Only 88 single-infection samples were tested by the HPV DNA microarray because there was no HPV 6 and 67 types on the microarray. Both methods

showed good accordance signals with 65 HPV-positive samples. Therefore, these data demonstrated good agreement of our Luminex-based HPV assay with HPV DNA microarray. These findings suggest that the Luminex-based HPV assay is a useful method for diagnosing HPV infection in the clinical setting.

Discussion

Although the majority of HPV infections resolve spontaneously, persistent infection with known high-risk HPV types is a significant risk factor for cervical cancer, and is increasingly recognized as playing a role in other cancers. The accurate identification of high-risk HPV genotypes is critical for defining a woman's risk for progression to cervical cancer. Major improvements in the development of innovative tests for clinical use have allowed for determination and implementation of the best treatment options for patients. Several nucleic acid-based methods have been utilized to identify and quantify specific HPV types in clinical samples, such as detection of viral nucleic acid by *in situ* hybridization,⁽¹⁸⁾ Southern blot analysis,⁽¹⁹⁾ hybrid capture and PCR.^(20,21) The PCR-based methods have shown differences in amplification efficiencies among HPV types, with reduced amplification of the minor HPV types observed.⁽²²⁾ In addition, another disadvantage of single-locus assays is the high degree of homology among specific HPV genes, from one HPV type to another, which leads to an excessive rate of false-positive results.⁽²³⁾ As a result of HPV sequence integration into host DNA in advanced tumor stages, the open reading frame of specific HPV genes, such as the L1 gene, may become disrupted. Such disruption of HPV gene sequences may lead to false-negative results in assays that target the disrupted gene sequence.^(24,25)

Here, we demonstrated the efficacy of the PCR-based fluorescent Luminex assay to detect the presence of HPV types in clinical

Table 5. A comparative table of accordance rates between the methods of DNA chip and Luminex, and clinical samples[†]

Rate	HPV type											Total
	16	18	31	35	42	52	56	58	59	66	68	
Accord	28	6	6	2	1	6	2	8	1	1	4	65
Accordance rate (%)	68.3	75	100	25	100	100	100	100	50	100	80	73.9

[†]Only single infection data were analyzed.

samples; multiple fluorophores were used to simultaneously detect a number of HPV loci in a single PCR reaction tube. This technique was highly sensitive, reproducible and simultaneously genotyped all clinically relevant genital HPV types in a single reaction. Comparing this assay to an established HPV DNA microarray,⁽²⁶⁾ we found excellent agreement between both methods. We obtained evidence for the higher sensitivity of this Luminex-based assay with the detection of 11 additional HPV types in a single infection. Several factors likely contributed to the higher sensitivity: (1) a biotin-14-dCTP labeled with a fluorophore resulting in maximum emissions; and (2) improved compatibility with YBT L1 F/GP6-1 PCR products that have a size of approximately 190 nucleotides. Thus, the present assay provided a method for simultaneously detecting and amplifying a number of distinct HPV genes from different HPV types, substantially reducing the occurrence of false-positive results.

A multiple fluorophore format has been reported that utilized a mixture of specific and degenerate primers to amplify a portion of the E1 gene in a number of HPV types.⁽²⁷⁾ When multiple fluorescent probes, each specific to a different HPV type, were used simultaneously compared to a single-probe assay, there was a substantially reduced sensitivity in detection of HPV-18 DNA. Similarly, detection of HPV-35 was somewhat reduced when a mixture of probes for HPV-16, HPV-33 and HPV-35 were used, compared to a single probe for HPV-35. A BARCODE HPV assay has been developed for HPV genotyping, and was evaluated using only six (6, 11, 16, 18, 45 and 51) of 45 different HPV types.⁽²⁸⁾ A serological multiplex method was reported for the detection of antibodies against HPV proteins based on *in situ*-purified glutathione S-transferase fusion proteins.⁽²⁹⁾ Recently, it has been reported that the design of the Luminex array, with 100 sets of beads, was easily integrated into the Luminex HPV Genotyping.⁽¹⁶⁾ The design of type-specific HPV probes allowed genotyping of an even broader spectrum of HPV types that were amplified by the PCR primer. However, these Luminex assays have shown low ability for type-specific genotyping and have missed variants with the type-specific probes.

The present study utilized a Luminex-based plurality of fluorescent probes and provided high specificity for HPV genomic DNA. Each PCR assay was confirmed using the HPV 18 loci-specific plasmid at concentrations ranging from 5 to 5 × 10⁶ copies. The high sensitivity is critical for screening clinical samples where the copy number of HPV may be low. Because the physical manifestations of HPV infection are often covert, and

the latency period prolonged,⁽³⁰⁾ infection with HPV may not be detected until the patient has been diagnosed with CIN, which can progress to carcinoma.⁽³¹⁾ Typically, higher-grade lesions (CIN2, CIN3 and carcinoma) are associated with high HPV copy number, which may be detectable by traditional methods. However, many assays currently in use are not sensitive or specific enough to detect low copy number HPV. Thus, the sensitivity of this Luminex-based HPV genotyping is critical for early detection of HPV when HPV copy numbers are low and therapeutic intervention is more likely to be effective.

Because 40% of HPV infections are mixed infections, the Luminex-based HPV genotyping can be used to differentiate between newly acquired HPV types and pre-existing infections when applied over time.^(32,33) The present results are more specifically related to a method for detecting the presence of a HPV type with a plurality of oligonucleotide sets. Each oligonucleotide set of the many available sets was specific to a single gene of the HPV type to be detected. In other words, each oligonucleotide was hybridized to nucleotide sequences derived from a single HPV gene of the same type. It was reproducible and provided potential simultaneous genotyping of all clinically relevant genital HPV types in a single reaction. Therefore, the biotin-14-dCTP and YBT L1 F/GP6-1 PCR products may provide both high hybridization signals and specificity important for use in the clinical setting.

Conclusions

There are more than 100 types of HPV that cause a wide variety of biological phenotypes, from benign proliferative warts to malignant carcinomas. Thus, high-risk HPV genotypes play a significant role in the development of cervical cancer. The Luminex-based HPV genotyping methods presented in this study provide highly specific and adaptable techniques for high-throughput screening of clinical samples for identification of nucleic acids of specific HPV types, as well as identification of patients that have been infected with low copy number of a high-risk HPV genotype.

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