Novel Microsphere-Based Method for Detection and Typing of 46 Mucosal Human Papillomavirus Types

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We have developed a novel microsphere-based genotyping method for 46 mucosal human papillomavirus (HPV) types. HPV DNA was amplified by PCR using general primers and typed by hybridization to HPV type-specific probes coupled to sortable microspheres based on the Luminex xMAP technology. Hybridization to each probe was specific for each HPV type without cross-hybridization and sensitive enough to allow typing of HPV contained in clinical specimens. The method was validated with direct sequencing and the Roche Linear Array genotyping method.

About 50 human papillomavirus (HPV) types are associated with infections of the genital, anal, and oropharyngeal mucosae (2, 8, 10). A few of these are known to be high-risk oncogenic types and the cause of cervical cancer (16), other anogenital cancers, and head and neck malignancies (3, 8). Detection of high-risk HPV types collectively is being considered as a screening method for cervical cancer, with the promise of improving the sensitivity and cost-effectiveness of cervical cancer screening programs (6). In recent years, there is also mounting evidence for the utility of type-specific identification of HPV type 16 (HVP16) and HPV18, as these two types are significantly associated with persistent infection and lesion progression, thus conferring a higher risk for cancer than other oncogenic types (15). As HPV16 and -18 account for 70% of the cervical cancers worldwide (2), two HPV vaccines targeting these types have been developed and shown to be highly efficacious in preventing both persistent infection with the types and the associated dysplastic changes in the cervical epithelium that lead to malignant transformation (12, 22). Since these vaccines are type specific, it is important to know the distribution of the various HPV types in a population, as well as to have a surveillance system in place to monitor vaccine efficacy over time and any unexpected shifts in the frequency of HPV types not covered by the vaccines. The above highlights the need for and the importance of using type-specific tests for HPV.

Numerous HPV typing methods based on a variety of detection platforms have been described in the literature, and some are commercially available (reviewed in reference 19). At the Canadian National Microbiology Laboratory (NML), we have developed a Luminex-based method (NML Luminex assay) to meet our need for sensitive and extensive typing of HPV in the context of epidemiological and molecular surveillance studies at the national level. The assay is based on the xMAP platform (Luminex Corporation, Austin, TX), and it detects 46 mucosal types. In this report, we describe its design and performance.

HPV DNA either cloned in plasmids (for validation of the probes) or from clinical cervical cancer specimens (for assay validation) was amplified by a nested PCR method using the PGMY primers for the first step (5, 11) and the GP5+/GP6+ primers (7)

for the second step. The GP6+ primer (Invitrogen, Burlington, ON, Canada) carried a 5′ biotin label and phosphorothioate bonds in the first 4 nucleotides (nt) on the 5′ end. Exact PCR conditions, which are important for maximum sensitivity of HPV DNA detection, are shown in Table S1 in the supplemental material.

For the detection of the PCR products by the xMAP technology, microspheres labeled with different ratios of red and infrared fluorophores (Luminex Corporation, Austin, TX) were coupled, according to the manufacturer's instructions, to HPV type-specific probes carrying a $5'$-C_{12} amino modification that reacts with the carboxyl groups on the microspheres. For hybridization, PCR products in a total volume of 17 μl in a 96-well PCR microplate (Fisher, Ottawa, ON, Canada) were denatured at 95°C for 10 min; 33 μl of the microsphere mix (15 microspheres/μl for each set) was then added. The samples were incubated at the hybridization temperature of 60°C for 10 min, and after addition of 25 μl of a 0.04-μg/μl solution of streptavidin-phycoerythrin (PE) (Invitrogen) in 1× tetramethyl ammonium chloride (TMAC) (Sigma), they were incubated for 5 more minutes at 60°C. The optimum concentration of streptavidin-PE was determined as the one giving the highest signal-to-background ratio. The hybridization temperature of 60°C was chosen because it eliminated cross-hybridization among probes. Hybridization was analyzed on a Luminex Liquid Chip 200 flow cytometer (Qiagen) using the Luminex IS software (Luminex). The analysis was carried out at 60°C with a maximum volume of 50 μl of sample and a minimum count of 100 microspheres per type, with settings of 8,300 and 16,500 for the lower and upper gates, respectively, as recommended by the manufacturer. In order to increase the hybridization of the

PCR products to the probes, we removed the nonlabeled strand of the PCR product using bacteriophage T7 gene 6 exonuclease (New England BioLabs, Pickering, ON, Canada) according to the method described previously (17). T7 exonuclease is a $5' \rightarrow 3'$ processive enzyme that rapidly degrades one of the strands on a duplex DNA molecule (14). In order to protect the GP6+ strand carrying the biotin label and selectively digest only the GP5+ strand, the first 4 nucleotides at the 5' end of the molecule were modified to include phosphorothioate bonds between the deoxyribose moieties instead of the usual phosphodiester bonds (17). Optimal digestion conditions were determined by incubating 40 units of T7 exonuclease with 100 μl of PCR product for various times and then measuring the fluorescence on the Luminex system. T7 exonuclease digestion increased the hybridization signal by about 2-fold, reaching a plateau from about 20 min to about 60 min of incubation. We chose for subsequent experiments 40 min of incubation. Under these conditions, the limit of the detection by Luminex, after PCR amplification, was between 5 and 10 ng of HPV16 DNA.

Unique probes, 30 nucleotides (nt) in length with a 5'-C$_{12}$ amino linker modification (kindly provided by the NML DNA Core Laboratory), were targeted at the region of the L1 gene comprised between the GP5+ and GP6+ primers (7). Unsuitable probes (not sensitive or not specific) were redesigned typically by shifting their position 10 nucleotides to the right or to the left along the variable region of the GP5+/GP6+ fragment. The final set of probes is shown in Table 1. Sensitivity and specificity for each type were determined by using DNA from genomic or L1 clones from defined HPV types, procured as described in Table S2 in the supplemental material. For all assays, four negative controls containing only host cell DNA were run alongside the samples. The average background fluorescence of each bead in the controls was subtracted from the fluorescence of each bead of the samples. This correction normalizes the background variations that occur between runs and between microsphere types. For example, while in one experiment the average background (all microspheres of 8 negative controls) was 12 ± 47, the backgrounds for the microspheres for HPV33 and -72 were 66 ± 18 and 318 ± 45, respectively. After correction, the average background became 0.4 ± 8.5 overall and 0.4 ± 9.0 and 0.6 ± 12 for the HPV33 and -72 microspheres, respectively. This correction avoided the need for a bead washing step used in other Luminex procedures (13, 18, 20, 21, 23). A fluorescence signal greater than 100 fluorescence units (FU) after correction was chosen as threshold for positivity.

The results of a typical experiment are shown in Fig. 1. All 46 probes strongly hybridized only with the corresponding HPV DNA and not with HPV DNA of any other types. In the particular experiment shown in Fig. 1, the microspheres for HPV89, HPV72, and HPV81 showed fluorescence above 100 FU with probes for other types. This should be interpreted as random fluctuations, rather than systematic cross-reactivity, because the abnormal fluorescence reading was not reproducible in other experiments. This corresponds to a false-positive rate of 0.15% (3/1,980 measurements).

Validation against clinical specimens was performed by comparing the results of the NML Luminex assay with direct sequencing of the amplified products utilizing 795 archived

TABLE 1 Probes used to detect 46 mucosal HPV types by the NML Luminex method

HPV type	Oligonucleotide sequence, 5' to 3'
6	CATCTTCCACATACACCAATTCTGATTATA
11	ACTATGTGCATCTGTGTCTAAATCTGCTAC
13	GTGTGTGCAGCCACTACATCATCTCTTTCA
16	AAAATATGTCATTATGTGCTGCCATATCTAC
18	ATATGTGCTTCTACACAGTCTCCTGTACCT
26	CCTTACCATTAGTACATTATCTGCAGCATC
30	ATCTGCAACCACACAAACGTTATCCACATA
31	CAATATGTCTGTTTGTGCTGCAATTGCAAA
32	ACTGTAACAACTGAAGACACATACAAGTCT
33	TAATATGACTTTATGCACACAAGTAACTAG
34	TAGGTACACAATCCACAAGTACAACTGCAC
35	AAAATATGTCTGTGTGTTCTGCTGTGTCTTC
39	ATCTACCTCTATAGAGTCTTCCATACCTTC
40	CTTATGTGCTGCCACACAGTCCCCCACACC
42	TCTGGTGATACATATACAGCTGCTAATTTT
43	AAACTTAACGTTATGTGCCTCTACTGACCC
44	AAACATGACAATATGTGCTGCCACTACACA
45	TAATTTAACATTATGTGCCTCTACACAAAA
51	GCCACTGCTGCGGTTTCCCCACATTTACTC
52	GACTTTATGTGCTGAGGTTAAAAAGGAAAG
53	CGCAACCACACAGTCTATGTCTACATATAA
54	ACAGCATCCACGCAGGATAGCTTTAATAAT
56	CATGACTATTAGTACTGCTACCAGAACAGT
58	ATGACATTATGCACTGAAGTAACTAAGGAA
59	CTTTCTGTGTGTGCTTCTACTACTTCTTCT
61	CATTTGTACTGCTACATCCCCCCCCTGTATC
62	ACCGCCTCCACTGCTGCAGCAGAATACACG
66	GACTATTAATGCAGCTAAAAGCACATTAAC
67	TCTGAGGAAAAATCAGAGGCTACATACAAA
68	ATTGTCCACTACTACAGACTCTACTGTACC
69	ACTGTATCTGCACAATCTGCATCTGCCACT
70	GTCTGCCTGCACCGAAACGGCCATACCTGC
71	ACCAAAACTGTTGAGTCTACATATAAAGCC
72	CAGCTTCTAATTTTCGTGAGTATCTTCGCC
73	TAGGTACACAGGCTAGTAGCTCTACTACAA
74	TAACATGACTGTGTGTGCTCCTACCTCACA
81	TACTATTTGCACAGCTACATCTGCTGCTGC
82	GCTGTTACTCCATCTGTTGCACAAACATTT
83	CAGCTGCTGCTACACAGGCTAATGAATACA
84	GCTACCAACACCGAATCAGAATATAAACCT
85	TGCAACTACTAATCCAGTTCCATCTATATA
86	TAATTTTACTATTAGTGCCGCTACCCAGAA
87	CAATTTTACTATTAGTGCTGCCACTCAAAC
89	GCTTCCCAGTCTGGCACAGAATAC
90	CACCAATATGACTATTTGTGCCACACAAAC
91	TAACTTAACCTTGTGTGCATCCACTGAGTC
97	TCTACACAAAATGGCGTAGCTACCACATAT

cervical specimens. These were amplified by nested PCR, and the products were typed with the NML Luminex assay and sequenced. The results showed a concordance of 97.7% between the two methods for the detection of HPV regardless of type. The sensitivity and specificity of the NML Luminex assay, using direct sequencing as a gold standard, were 98.8% (95% confidence interval [CI], 97.1 to 99.6) and 96.4% (95% CI, 96.4 to 93.8), respectively. Comparison of the distribution of HPV types detected by NML Luminex and direct sequencing methods is shown in Table 2. The direct sequencing method could not determine the sequences of 34 HPV positive samples, 32 of
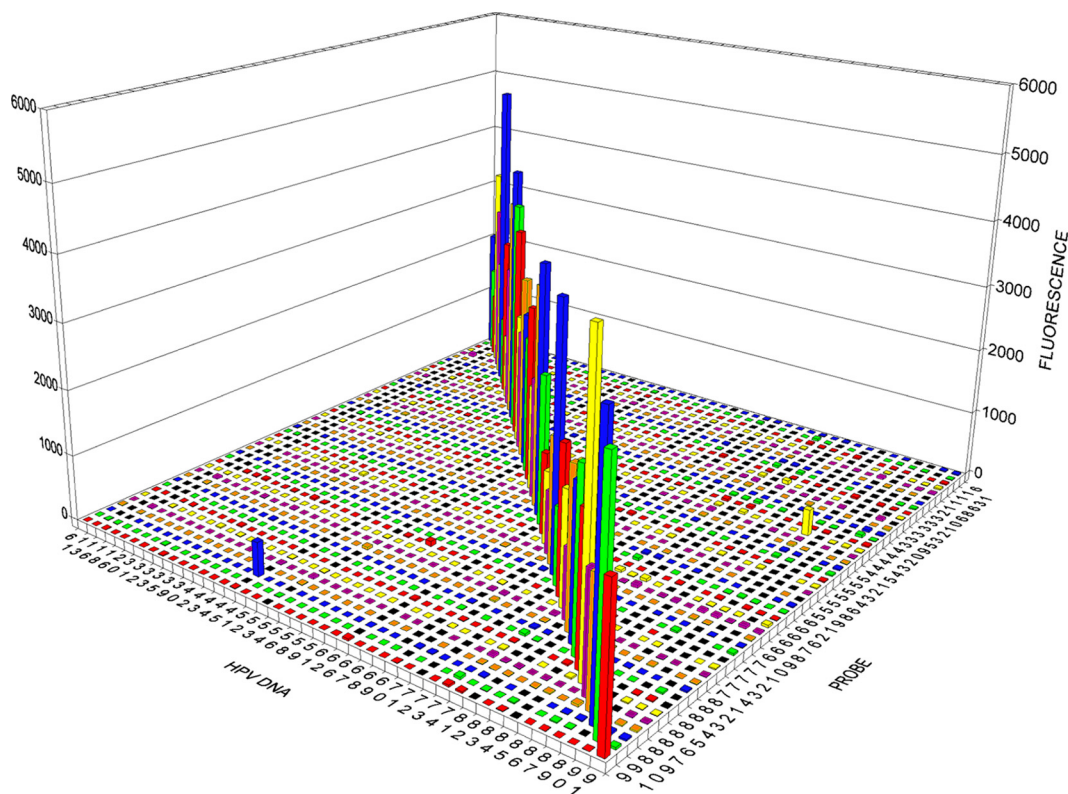
FIG 1 Sensitivity and specificity of the 46 probes for their cognate HPV types. The probes are on the right axis, and the HPV DNAs are on the left axis. The vertical axis represents the fluorescence read for each microsphere carrying a specific HPV probe. The bars on the diagonal represent the hybridization of HPV DNA type with the intended cognate probe. The average background of four negative controls was subtracted for each microsphere, as described in the text.

which were typeable by the Luminex method. There was no agreement on the HPV type detected for 13 of 429 (3.0%) samples positive by both methods. The NML Luminex assay detected a total of 793 HPV types versus 577 for direct sequencing. This discrepancy is due to the fact that direct sequencing cannot detect multiple HPV types present in the same sample.

To test the ability of the NML Luminex assay to detect multiple types in the same sample, we amplified DNAs from different HPV types separately and then mixed them together in a single Luminex detection reaction. The total amount of DNA was kept constant to simulate the situation of clinical samples, in which a mixture of different DNAs is amplified to the maximum capacity of the PCR regardless of the number of types present. The results showed that at least 30 different types can be detected simultaneously by the Luminex method with no cross-hybridization except for some false positives for HPV type 72 in samples which contained more than 20 HPV types (data not shown).

The NML Luminex method was compared with the Linear Array method (Roche Molecular System Inc., Branchburg, NJ), a leading commercial genotyping method, utilizing 150 archival cervical specimens. The linear array HPV52 result, which may be due to cross-hybridization with types 33, 35, and 58, was confirmed by an HPV52-specific PCR method (4). The results showed that the NML Luminex assay was slightly more

sensitive than Linear Array for detection of specimens positive for HPV but less sensitive for detecting multiple types (Table 3), but none of these differences were statistically significant. The lower sensitivity in detecting multiple types is likely due to limitations in the PCR method used, since we have shown that the NML Luminex assay can detect at least 30 different types of HPV simultaneously, if the DNAs are amplified separately and then mixed together. However, it is possible that a higher sensitivity for minute amounts of DNA of the reverse line blot platform may be the reason for the better performance of the Linear Array in detecting multiple types. A larger comparison study between NML Luminex and Linear Array may help in resolving this point.

In conclusion, the NML Luminex assay is a promising novel method for genotyping 46 mucosal HPV types associated with human infections with a high specificity and sensitivity. In our laboratory, we have used the NML Luminex assay for typing over 15,000 specimens from numerous HPV prevalence studies in Canada and elsewhere, with a number of manuscripts in preparation and two already published (1, 24). The use of T7 gene 6 exonuclease and the subtraction of the background for each bead eliminate the need for a washing step, which is required for other HPV typing methods based on Luminex. In addition, our method has the most comprehensive coverage of HPV types compared with other published methods to date: the method of Oh et al. (18) detects 15 HPV types, that of

TABLE 2 Distribution of HPV types detected by NML Luminex method and direct sequencing

	NML Luminex			Direct sequencing		
HPV type	n	% of types	% of positive samples	n	% of types	% of positive samples
Negative	353	NA[a]	NA	361	NA	NA
Any type	442	NA	NA	434	NA	NA
6	43	7.5	5.4	39	4.9	4.9
11	12	2.1	1.5	11	1.4	1.4
13	0	0.0	0.0	0	0.0	0.0
16	87	15.1	10.9	68	8.6	8.6
18	26	4.5	3.3	15	1.9	1.9
26	0	0.0	0.0	0	0.0	0.0
30	2	0.3	0.3	0	0.0	0.0
31	29	5.0	3.6	29	3.7	3.6
32	3	0.5	0.4	2	0.3	0.3
33	11	1.9	1.4	14	1.8	1.8
35	8	1.4	1.0	2	0.3	0.3
39	22	3.8	2.8	16	2.0	2.0
40	9	1.6	1.1	5	0.6	0.6
42	13	2.3	1.6	5	0.6	0.6
43	0	0.0	0.0	1	0.1	0.1
44	3	0.5	0.4	1	0.1	0.1
45	12	2.1	1.5	10	1.3	1.3
51	16	2.8	2.0	9	1.1	1.1
52	33	5.7	4.2	17	2.1	2.1
53	25	4.3	3.1	12	1.5	1.5
54	11	1.9	1.4	8	1.0	1.0
56	10	1.7	1.3	4	0.5	0.5
58	28	4.9	3.5	25	3.2	3.1
59	11	1.9	1.4	5	0.6	0.6
61	7	1.2	0.9	1	0.1	0.1
62	26	4.5	3.3	17	2.1	2.1
66	39	6.8	4.9	30	3.8	3.8
67	7	1.2	0.9	8	1.0	1.0
68	2	0.3	0.3	4	0.5	0.5
69	3	0.5	0.4	2	0.3	0.3
70	11	1.9	1.4	10	1.3	1.3
71	3	0.5	0.4	0	0.0	0.0
72	5	0.9	0.6	4	0.5	0.5
73	5	0.9	0.6	3	0.4	0.4
74	1	0.2	0.1	0	0.0	0.0
81	8	1.4	1.0	6	0.8	0.8
82	7	1.2	0.9	5	0.6	0.6
83	11	1.9	1.4	5	0.6	0.6
84	12	2.1	1.5	1	0.1	0.1
85	2	0.3	0.3	2	0.3	0.3
86	1	0.2	0.1	1	0.1	0.1
87	2	0.3	0.3	3	0.4	0.4
89	8	1.4	1.0	4	0.5	0.5
90	3	0.5	0.4	1	0.1	0.1
91	1	0.2	0.1	0	0.0	0.0
97	0	0.0	0.0	0	0.0	0.0
102	NA	NA	NA	2	0.3	0.3

[a] NA, not applicable.

TABLE 3 Comparison of NML Luminex and Linear Array methods to detect multiple HPV infections

	No. of samples	
Parameter	Luminex	Linear Array
Positive for any type	171	164
Total HPV types detected	63	75
Single infections	85	73
Multiple infections	47	47
2 types	28	21
3 types	9	14
4+ types	10	12

certain variants of HPV68 (9). Future optimization of the PCR system may overcome these limitations.

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Schmitt et al. (20) covers 22 HPV types, the method of Jiang et al. (13) detects 26 HPV types, and a commercial method from Qiagen covers 18 HPV types (21). The limitations of our method are a lower sensitivity for multiple types compared to Linear Array and the inability of the PCR system to amplify

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