Genotyping of human papillomavirus in cervical lesions by L1 consensus PCR and the Luminex xMAP system

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Infection with human papillomavirus (HPV) is the main cause of cervical cancer, the principal cancer in women in most developing countries. Molecular epidemiologic evidence clearly indicates that certain types of HPV are the principal cause of invasive cervical cancer and cervical intraepithelial neoplasia. Comprehensive, high-throughput typing assays for HPV, however, are not currently available. By combining L1 consensus PCR and multiplex hybridization using a Luminex xMAP system-based suspension array, the authors developed a rapid high-throughput assay, the HPV DNA suspension array (HPV-SA), capable of simultaneously typing 26 HPVs, including 18 high-risk HPV genotypes and eight low-risk HPV genotypes. The performance of the HPV-SA applied to 26 synthetic oligonucleotide targets was evaluated. The HPV-SA system perfectly discriminated 18 high-risk HPV targets from eight low-risk HPV targets. To assess the clinical applicability of the assay, the HPV-SA was performed with 133 MY09/MY11 primer set-mediated PCR (MY-PCR)-positive clinical specimens; of the 133 samples, 121 were positive by HPV-SA. Both single and multiple types were easily identified. The authors believe that improvement of the assay may be useful for epidemiological studies, cancer-screening programmes, the monitoring of therapeutic interventions, and the evaluation of the efficacy of HPV vaccine trials.

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INTRODUCTION

Human papillomavirus (HPV) infection is a prominent concern in both the research and medical fields. Although HPV has been associated with many diseases, cervical cancer has particular significance, being the principal cancer in women in most developing countries. The data from combined case-control studies (Munoz *et al.*, 2003) suggest that some HPV genotypes should be regarded as high-risk or carcinogenic (e.g. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) and that some should be considered probably carcinogenic (HPV26, 53 and 66), whereas the relative risk associated with certain other HPV genotypes may be low.

There is a strong need to identify individual HPV types to investigate the epidemiology and clinical behaviour of particular types. At present, several strategies are used to detect and type HPVs. Several consensus PCR systems have been conveniently used in a number of large-scale epidemiological studies (Gravitt *et al.*, 2000; Hart *et al.*, 2001). However,

consensus PCR products do not provide genotype information (Vernon *et al.*, 2000). In addition, a number of HPV typing assays have recently been reported, such as solid-phase microarrays (Klaassen *et al.*, 2004; Oh *et al.*, 2004), GP5 +/6 + -linked enzyme immunoassay (EIA) (van den Brule *et al.*, 2002), the Roche AMPLICOR HPV test, and the INNO-LiPA HPV assay (Labo Biomedical Products; van Ham *et al.*, 2005). Although these assays are capable of typing a relatively large spectrum of HPV genotypes, they cannot be automated or deployed in a high-throughput platform.

Here, we report an improved genotyping method based on the Luminex xMAP system, also called the HPV DNA suspension array (HPV-SA). The HPV-SA provides a rapid and cost-effective method to simultaneously detect different HPV genotypes (Fig. 1). The Luminex xMAP system (Dunbar *et al.*, 2003) incorporates a proprietary process to internally dye polystyrene microspheres with two spectrally distinct fluorochromes. The HPV-SA is created using precise ratios of these fluorochromes, and consists of 26 different microsphere sets with specific spectral addresses. Each microsphere set possesses an HPV type-specific probe on its surface, and they can be combined, allowing up to 26

Abbreviations: HPV, human papillomavirus; HPV-SA, human papillomavirus DNA suspension array; MY-PCR, MY09/MY11 primer set-mediated PCR.

Fig. 1. Schematic representation of the HPV-SA. The HPV DNA was first extracted from the clinical specimen (A). The template was then amplified on a PCR processor with the MY09/11 primer set (B). PCR products were transferred to a new PCR tube containing the 26-plex HPV-SA and hybridized for 30 min (C). After washing, the hybridized microspheres were incubated with streptavidin-R-phycoerythrin at room temperature for 30 min (D). Finally, the mixture was analysed on the Luminex¹⁰⁰ analyser (E).

different HPV targets to be measured simultaneously in a single reaction vessel. A third fluorochrome coupled to a reporter molecule detects the hybridization occurring at the microsphere surface. Microspheres pass by two separate lasers in the Luminex¹⁰⁰ analyser. High-speed digital signal processing classifies the microsphere based on its spectral address and detects the hybridization on the surface. Thousands of microspheres are interrogated per second, resulting in an analysis system capable of analysing and reporting up to 26 different reactions in a single reaction vessel.

METHODS

Specimens. The samples used in this study were 246 clinical specimens, 113 of which were negative by MY09/MY11 primer set-mediated PCR (MY-PCR), and 133 positive. Of the 133 positive specimens, 30 were liquid-based cytology specimens which were collected as part of routine cervical-screening procedures. The remaining 103 specimens were cervical specimens which had been frozen after completion of Digene Hybrid Capture II (HCII) testing and maintained at -70°C . These cervical specimens were stored in Digene Specimen Transport Medium (STM) that also contained a denaturing reagent (diluted sodium hydroxide solution containing dark-purple indicator dye) supplied as part of the HCII kit and used in the first step of HCII testing.

Specimen processing. The extraction of DNA from the specimens without denaturing reagent was by the method of Beby-Defaux *et al.* (2004). In brief, cervical cells were removed from the cytobrush by agitation and were washed twice in PBS, pH 7.2. The pelleted cells were resuspended in 150 μl lysis buffer, pH 8.0, containing 10 mM Tris/HCl, 1 mM EDTA, 10 mM NaCl, 0.5% SDS and 200 μg proteinase K ml^{-1} . After incubation for 1 h at 56°C , the

proteinase K was inactivated at 95°C for 15 min. DNA was extracted using a standard phenol/chloroform method (Sambrook *et al.*, 1989). For the extraction of DNA from the specimens containing denaturing reagent, the standard phenol/chloroform method was also employed. Five microlitres of the DNA preparation was used for each amplification reaction.

PCR amplification. The MY09/11 primer set (Gravitt *et al.*, 2000) was synthesized with several degenerate nucleotides in each primer, and was thus a mixture of 25 primers, capable of amplifying a wide spectrum of HPV types. MY09 reverse primers were labelled at the 5' terminus with biotin (Invitrogen). The MY-PCR (Gravitt *et al.*, 2000; Oh *et al.*, 2004), with some modifications, was used for amplification of HPV DNA. In brief, a standard PCR was carried out with a 50 μl mixture containing 5 μl template DNA, 0.2 μM MY11 forward primer, 0.8 μM biotin-MY09 reverse primer, 0.2 mM deoxy-nucleoside triphosphates, 1.25 U *Pyrobest* DNA polymerase (TaKaRa Biotechnology), 1 $\times$ *Pyrobest* buffer and 4 mM MgCl_2 . Cycling conditions on a PCR processor (PE9600, Perkin-Elmer) were as follows: 5 min of denaturation at 95°C , followed by 40 cycles of 1 min of denaturation at 95°C , 1 min of annealing at 55°C , and 1 min of extension at 72°C . Before the reactions were cooled to room temperature, an additional incubation for 5 min at 72°C was performed. In order to check for the presence of PCR inhibitors in the DNA samples, an additional PCR was performed under the same experimental conditions with primers targeting the human β -globin gene. In order to check for positive PCR results, 5 μl of the PCR product was analysed by agarose gel electrophoresis according to standard procedures (Sambrook *et al.*, 1989).

Table 1. Sequences (30 bp) of HPV type-specific probes

HPV type	Sequence (5'→3')
6	ATCCGTAACATACATCTTCCACATACACCAA
11	ATCTGTGTCTAAATCTGCTACATACACTAA
16	GTCATTATGTGCTGCCATATCTACTTCAGA
18	TGCTTCTACACAGTCTCCTGTACCTGGGCA
26	AGTACATTATCTGCAGCATCTGCATCCACT
31	TGTTTGTGCTGCAATTGCAAACAGTGATAC
33	TTTATGCACACAAGTAAGTAGTGACAGTAC
34	TACACAATCCACAAGTACAAATGCACCATA
35	GTCTGTGTGTTCTGCTGTGTCTTCTAGTGA
39	TCTACCTCTATAGAGTCTTCCATACCTTCT
40	GCTGCCACACAGTCCCCACACCAACCCCA
42	CTGCAACATCTGGTGATACATATACAGCTG
43	TCTACTGACCCTACTGTGCCAGTACATAT
44	GCCACTACACAGTCCCCTCCGTCTACATAT
45	ACACAAAATCCTGTGCCAAGTACATATGAC
51	AGCACTGCCACTGCTGCGGTTTCCCCAACA
52	TGCTGAGGTTAAAAAGGAAAGCACATATAA
53	GTCTATGTCTACATATAATTCAAAGCAAAT
54	TACAGCATCCACGAGGATAGCTTTAATAA
56	GTACTGCTACACGACGTTAAGTAAATATG
58	ATTATGCATGAAGTAAGTAAGGAAGGTAC
59	TCTACTACTGCTTCTATTCTTAATGTATAC
66	TATTAATGCAGCTAAAAGCACATTAACATAA
68	TCTACTACTACTGAATCAGCTGTACCAAAT
73	GTGTAGGTACACAGGCTAGTAGCTCTACTA
82	CGACTCGTGTACTCAATCTGTTGCACAAA

Fig. 2. Establishing an HPV-SA. Twenty-six HPV type-specific probes were coupled to 26 microsphere sets. The HPV-SA was hybridized to each synthetic oligonucleotide target (SOT). Only microsphere sets that generated a signal of >2000 median intensity of fluorophore (MIF) when hybridized to the target at 2 nmol l^{-1} were included in the array. The excellent signal-to-noise ratio of the array and the absence of cross-hybridization signals may be observed.

Preparation of the HPV-SA. Type-specific 30 bp sequences of probes specific for HPV types 6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 73 and 82 were selected, as reported previously (Jacobs *et al.*, 1995; van den Brule *et al.*, 2002). The 30 bp type-specific probe sequences are listed in Table 1. All probes used for covalent coupling to carboxylated microspheres were synthesized with a 5' amine group and a T18 spacer, and contained 30 nucleotides (Invitrogen). Probes were covalently coupled to carboxylated microspheres using a previously described carbodiimide coupling method (Dunbar *et al.*, 2003; Fulton *et al.*, 1997). Synthetic oligonucleotide targets that primed the strand complementary to the sequence of the HPV type-specific probe attached to carboxylated microspheres carried a biotin group in their 5' ends serving as a reporter for hybridization.

HPV-SA hybridization and analysis. Hybridization was performed using a method described elsewhere (Iannone *et al.*, 2000), with some modifications. Thirty-eight microlitres of $5 \times$ SSC hybridization solution containing 5000 of each probe-coupled microsphere set were transferred to a 0.2 ml thin-walled PCR tube. Then, 12 μl of control (negative or positive) or PCR product was added (50 μl total volume). To denature the PCR products, the tube was initially heated to 99°C for 5 min in a PE Biosystems 9600 thermocycler and then cooled to 55°C for 30 min. Microspheres were washed twice with 100 μl $2 \times$ SSC/0.02% Tween-20 in 96-well microtitre plates (Millipore). Hybridized microspheres were resuspended in 75 μl $2 \times$ SSC/0.02% Tween-20. Twenty-five microlitres of streptavidin-R-phycoerythrin ($10 \mu\text{g ml}^{-1}$ in $2 \times$ SSC/0.02%

Fig. 3. HPV-SA analysis with clinical specimens. Amplicons from MY-PCR were hybridized to the HPV-SA and analysed in the Luminex¹⁰⁰ analyser. Specimen 1 had multiple infections with HPV 6 and 11. Specimen 2 had multiple infections with HPV 11, 33 and 58. Specimens 3, 4 and 5 had single infections with HPV 39, 58 and 66, respectively.

Tween-20) were added. The mixture was incubated at room temperature for 30 min. Finally, the mixture was analysed on the Luminex¹⁰⁰ analyser according to the system manual. A cutoff value of greater than twice the background fluorescence was used to indicate a positive reaction.

Genotyping by HPV E7 type-specific PCR. An HPV E7 type-specific PCR method was used to identify individual HPV genotypes. HPV type-specific primers were chosen to amplify approximately 100 bp in the E7 ORF of HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68, as reported by Walboomers *et al.* (1999).

Genotyping by DNA sequencing. Specimens that revealed a band of approximately 450 bp by PCR, and could not be genotyped by HPV E7 type-specific PCR, were subjected to DNA sequencing. The products were purified using the QIAquick PCR Purification kit (Qiagen). Purified amplicons were sequenced with the respective amplification primers on a Megabase 500 automated DNA analysis platform, as recommended by the manufacturer (Amersham Biosciences). Otherwise, when mixed HPV infections were suspected, PCR products were first cloned (TOPO TA cloning kit, Invitrogen) according to the instructions of the manufacturer, followed by DNA sequencing. The identification of the obtained sequence was verified by using alignment search tool (BLAST) analysis (www.ncbi.nlm.nih.gov).

RESULTS

To evaluate the specificity of the HPV type-specific probes, we performed HPV-SA hybridization with synthetic oligonucleotide targets (Fig. 2). Analysis of these data indicated that our 26-plex HPV-SA system perfectly discriminated all 18 high-risk HPV targets and eight low-risk HPV targets. As

Table 2. Results of HPV-SA and E7 type-specific PCR (or DNA sequencing) for 133 samples

+, Type identified in a sample. Only 36 of 133 specimens are shown. The two columns on the right show the results of E7 type-specific PCR and DNA sequencing, and allow a quick visual check on the agreement between HPV-SA and E7 type-specific PCR (or DNA sequencing). Samples 22, 104, 107, 115, 120, 136, 146, 150, 151, 161, 162 and 167 contained HPV genotypes not included in the 26 detected by our HPV-SA method.

Sample	HPV-SA result																												E7 type-specific PCR result	DNA sequencing result
	6	11	16	18	26	31	33	34	35	39	40	42	43	44	45	51	52	53	54	56	58	59	66	68	73	82				
1		+																										11		
2			+																								16			
3				+																							18			
8						+																					31			
14						+																	+		+		31,66			
16							+																				33			
17																								+			68			
19										+																	39			
22																												67		
30																			+								54			
101		+																		+							56	11		
104																												70		
107																												84		
109		+								+									+					+			39,68	11,54		
110	+																										6	6		
113	+	+	+	+		+																					16,18,31	6,11		
115																												61		
120																												70		
126																					+						58			
132																							+				66			
136																												61		
141																									+			82		
142																+											51			
146																												84		
150																												67		
151																												70		
161																												67		
162																												67		
167																												67		
168			+																	+	+						16,56,58			
169								+																				34		
173																		+										53		
194	+	+	+		+	+															+						16,31,58	6,11,26		
195			+												+												16	45		
197																						+					59			
203																		+					+				66	53		

shown in Fig. 2, all HPV-specific probes hybridized specifically to the corresponding targets of each of the HPV genotypes, and no cross-hybridization with other HPV types was observed.

Our assay could type 26 genital HPVs amplified by the MY-PCR, and could detect infection with single as well as multiple HPV genotypes (Fig. 3). The hybridization results showed a high degree of reproducibility. The β -globin PCR verified that all 133 specimens contained PCR-quality DNA. Positive PCR products derived from the 133 specimens were hybridized three times by HPV-SA. HPV-SA gave a positive result for 121 of 133 specimens positive by gel electrophoresis (Table 2), but a negative result for 12 of 133 specimens. DNA sequence analysis of these 12 specimens indicated that they contained HPV genotypes not included in the 26 detected by our HPV-SA (Table 2). The 121 specimens positive by HPV-SA were also detected either by E7 type-specific PCR or by DNA sequencing, and for 120 specimens the detection results were identical with the results of HPV-SA. One specimen could not be completely identified by DNA sequencing or E7 type-specific PCR (Table 2, sample 14). HPV-SA analysis indicated that this specimen had multiple infections with HPV 31, 66 and 73 genotypes. HPV 31 and 66 infection in this specimen was detected by E7 type-specific PCR. HPV 73 was subjected to DNA sequencing, because HPV 73 cannot be genotyped by HPV E7 type-specific PCR, since it has not been possible to obtain a TA clone that contains the DNA sequence of HPV 73. Further work will be required to solve this problem. In general, the results of HPV-SA and the E7 type-specific PCR (or DNA sequencing) were highly correlated (McNemar's test for correlated proportions >0.9).

Of the 26 HPV genotypes included in the HPV-SA, the following 20 HPV types were present in our clinical specimen collection: HPV 6, 11, 16, 18, 26, 31, 33, 34, 39, 45, 51, 53, 54, 56, 58, 59, 66, 68, 73 and 82. HPV 16, 58, 11 and 18 were the most frequently occurring HPV types in this study. As in many other studies, the most prevalent HPV across all patient groups studied was HPV 16. However, HPV 58 was the second most prevalent high-risk virus among patients in our study. Interestingly, HPV 35, a high-risk virus frequently reported in the literature, was not detected in our study. Although the synthetic target of HPV 35 could be easily detected by HPV-SA (Fig. 2), false-negative results for this viral type with our assay cannot be ruled out. HPV 31 and 33, two common high-risk viruses in the literature, were found at a lower frequency than HPV 58. These apparent discrepancies may be due to differences in the local prevalence of HPV genotypes.

DISCUSSION

It is important that HPV genotypes be determined by as precise a method as possible, because the HPV genotype provides information useful for the prognosis of malignant progression. In this report, we demonstrate the feasibility of typing genital HPV by using the Luminex xMAP system

as a readout platform. HPV-SA itself is a high-throughput assay that offers significant advantages. Our assay compares favourably with well-established generic assays for high-risk genotypes, such as E7 type-specific PCR and DNA sequencing. There are good reasons to believe that HPV-SA or optimized versions of it will be of great utility in several areas of HPV research. Because of its favourable cost/benefit ratio and high sample throughput, it could be used to further define the validity of epidemiological and phylogenetic risk-classification schemes in specific geographical areas and/or ethnic populations (Munoz *et al.*, 2003). HPV-SA could also be a valuable tool in the evaluation of women enrolled in clinical trials of HPV vaccines, as it could help in defining the specificity of the immune-protective responses, cross-immunity and the durability of responses. Undoubtedly type-specific assays will be critical in the future, and HPV-SA represents a significant step in this direction.

Although reports of HPV-typing assays based on solid-phase microarrays have been published (Cho *et al.*, 2003; Kim *et al.*, 2003; Vernon *et al.*, 2003), and others are being developed, suspension arrays have some advantages over solid-phase microarrays. Hybridization kinetics with suspension arrays closely approximate the kinetics of solution-phase hybridization (Wallace *et al.*, 2005). This results in short hybridization times and faster turn-around times than those possible with solid-phase microarrays. HPV-SA uses low-volume hybridization, fast instrument readout (30 s for each specimen) and rapid and automated analysis (30 s for a set of 90 samples). The cost per well (per patient) in reagents and consumables (DNA isolation, PCR, array microspheres, plasticware, etc.) is approximately \$4 (US). This compares favourably with other available commercial HPV assays.

In conclusion, we have developed an improved method for HPV genotyping. Our data demonstrate that HPV-SA analysis coupled with MY-PCR can be applied to HPV detection and genotyping. This diagnostic tool will undoubtedly be useful for the clinical diagnosis of HPV infection and large-scale epidemiological studies.

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