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Accurate human papillomavirus genotyping by 454 pyrosequencing

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Abstract

Accurate HPV typing is essential for evaluation and monitoring of HPV vaccines, for second-line testing in cervical cancer screening, and in epidemiological surveys. In this study, we set up and assessed in clinical samples a new HPV typing method based on 454 next-generation sequencing (NGS) of HPV L1 amplicons, generated by using a modified PGMY primer set with improved sensitivity for some HPV types that are not targeted by standard PGMY primers. By using a median 12 800-fold coverage, the NGS method allowed us to correctly identify all high-risk HPV types, in either single or multiple infections, with a sensitivity of 50 genome equivalents, as demonstrated by testing WHO LabNet EQA sample panels. Analysis of mixtures of HPV16- and HPV18-positive cell lines demonstrated that the NGS method could reproducibly quantify the proportion of each HPV type in multiple infections in a wide dynamic range. Testing of HPV-positive clinical samples showed that NGS could correctly identify a high number of HPV types in multiple infections. The NGS method was also effective in the analysis of a set of cervical specimens with discordant results at hybrid capture 2 and line probe assays. In conclusion, a new HPV typing method based on 454 pyrosequencing was set up. This method was sensitive, specific, quantitative and precise in both single and multiple infections. It could identify a wide range of HPV types and might potentially discover new HPV types.

Keywords: 454 pyrosequencing, diagnostic accuracy, genotyping, human papillomavirus, multiple infection, next generation sequencing, sensitivity and specificity

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Introduction

With the implementation of prophylactic HPV vaccination programmes, accurate HPV typing methods are needed for the evaluation of HPV vaccine efficacy and for monitoring the distribution of HPV types in the general population and in vaccinated cohorts [1]. In addition, HPV typing has been introduced in cervical cancer screening programmes as a second line test for the management of women with high-risk HPV infection and could be relevant in the follow-up of HPV-positive (and cytology-negative) women in screening with HPV as primary screening test [2,3]. As individual high-risk HPV types differ in their oncogenic potential [4], identification of the presence of high-risk HPV types that are more strongly associated with invasive cervical cancer (i.e. HPV16, HPV18 and HPV45) [5], may improve risk stratification and management of HPV-positive women.

The definition of the oncogenic potential of individual HPV types is based mainly on the epidemiological evidence of the association between HPV DNA detection and invasive cancer; furthermore, for some rare HPV types phylogenetic similarities have been considered for attributing the risk class [4,5]. Thus, the accuracy of HPV detection and typing is also important because it might affect some of these evaluations. In this regard, the WHO Global Proficiency Studies demonstrated the need to improve methods for reliable HPV genotyping, as many false-positive and false-negative results were reported from participating laboratories [6,7]. A recent large study on invasive cervical cancer addressed the importance of accurate broad-spectrum HPV detection and typing.

In this study, out of 10 575 globally collected invasive cervical carcinoma specimens, the presence of HPV DNA could be detected in 84.9% of specimens and HPV types could be identified in 96.3% of the positive cases [8]. In the same case series, a further analysis of the HPV-positive invasive cancers with unidentified HPV types, that used novel primer sets for genotyping, demonstrated the presence of possibly carcinogenic IARC group 2B HPV types and rare variants of carcinogenic HPV types that have infrequently or never been detected in invasive cervical cancer and that are usually not targeted by currently used hybridization-based genotyping assays [9].

We recently reported the proof of principle that next-generation sequencing (NGS) may be used for HPV typing [10]. Potential advantages of an NGS typing method compared with hybridization-based assays are (i) the high specificity of a sequencing-based method; (ii) the broad spectrum of detectable HPV types, including not yet discovered HPV types; (iii) the ability to identify sequence subtypes, variants and mutations; and (iv) the ability to identify different HPV types and to estimate their relative amount in multiple infections. The NGS HPV typing method we developed was based on 454 pyrosequencing of HPV L1 amplicons generated by PCR amplification with MY09/11 degenerate consensus primers [10]. This method could identify HPV types in multiple infections with relatively high sensitivity; however, the low sensitivity of the MY09/11 primer set for some HPV types was not increased by deep-sequencing [10]. In the present study, we improved the HPV NGS typing protocol by designing a new primer set based on modified PGMY multiplex primers and by analysing longer reads generated by the 454 titanium chemistry. The analytical performance of the new protocol was assessed in reference and clinical specimens and its analytical accuracy was compared with hybridization-based assays. The clinical diagnostic accuracy of the HPV NGS typing protocol was not evaluated in this study.

Methods

Specimens

The following groups of samples were analysed in order to evaluate distinct features of the 454 NGS-based HPV genotyping test. The different experiments that were performed, with their design and objectives, are summarized in Table 1.

HPV-positive cell lines. To assess the reproducibility and linearity of the NGS test, HPV16 and HPV18 DNA-positive controls were prepared with DNA purified from CaSki and HeLa cells (which contain *c.* 500 and 50 copies of HPV16 and HPV18 genome per cell, respectively) and diluted in DNA

TABLE 1. Summary of the experimental design, objectives and samples that were analysed by the NGS HPV genotyping method in this study

Objective	Experimental design	Type and No. of samples	HPV typing method	Data processing	Data analysis	End-point
Analytical validation: Sensitivity and specificity for high-risk HPV types and for HPV6 and HPV11	Testing reference samples at low concentrations	Specimens of the 2010 and 2011 WHO proficiency study of HPV genotyping (n = 46 samples of the WHO Panel 2010 and n = 46 samples of the WHO Panel 2011)	Amplification and 454 deep sequencing with PGMY primers	Preprocessing of raw reads and genotype assignment	Comparison of results vs. WHO benchmark	Identification of HPV types
Analytical validation: Sensitivity and Specificity for HPV68a and 68b	Testing reference samples with HPV68 and 68b	A group of 18 specimens of the 2010 WHO proficiency study of HPV genotyping (n = 10 samples at different concentrations without HPV68; n = 8 samples at different concentrations containing HPV68a and HPV68b)				
Analytical validation: Reproducibility and quantitative analysis of HPV16 and HPV18	Testing samples with different proportions of HPV16 and HPV18	CaSki (HPV16-positive) and HeLa (HPV18-positive) cells mixed in six different proportions and tested in duplicate (n = 12 tests)	Amplification and 454 deep sequencing with modified PGMY primers		Evaluation of dependency between HPV GE and read number	Quantification of HPV types in multiple infections
Clinical application for analysis of samples with multiple HPV infections	Testing clinical samples with a high number of different HPV types	N = 10 pooled samples generated from 30 genital swabs with multiple HPV infection.			Comparison of results vs. SPF10-LiPA results	Number of identified HPV types in clinical samples with multiple infections and in pooled samples
Clinical application as reference test	Discrimination analysis of samples with discordant results from other tests	N = 8 cervical swabs with discordant HC2 and SPF10-LiPA results			Comparison of results vs. HC2, SPF10-LiPA and HPV type-specific real-time PCR	Identification of HPV types in clinical samples

carrier obtained from the HPV-negative HEK293 cells, according to the WHO Human Papillomavirus Laboratory Manual [11]. CaSki and HeLa cell DNA was mixed in six different proportions to generate multiple infection control samples, corresponding to the following proportions of HPV16 to HPV18 genome equivalents (GE): 1000:1, 500:1, 100:1, 10:1, 1:1 and 1:5.

WHO proficiency panel specimens. To benchmark the ability of genotyping multiple infections, HPV DNA of the 2010 and 2011 WHO proficiency study of HPV genotyping were used as reference samples [6]. The panels were composed of purified plasmid DNA in which genomic DNA of different HPV types was cloned and diluted in a background of human placental DNA as previously described [7,11]. Each panel was composed of 43 reference samples that contained different amounts of plasmids (5 and 50 IU per 5 μL for HPV16 and HPV18 and 50, and 500 GE per 5 μL for the other high-risk HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a and 68b, and the low-risk HPV types 6 and 11) and three reference samples that contained HPV-positive and HPV-negative cell lines, as described in the report of the WHO proficiency study [7]. Some samples contained different HPV types to mimic multiple infections.

Pools of HPV-positive genital swabs. A group of 30 genital swabs collected at Padova University Hospital from patients with a positive result at the INNO-LiPA HPV Genotyping Extra assay (Innogenetics, Gent, Belgium), which is based on SPF10 primers (SPF10-LiPA), were analysed retrospectively to assess the ability of the 454 pyrosequencing method to identify HPV types in multiple infections. Samples were selected consecutively among those with multiple infections and representative of different HPV types. Nucleic acids purified from these samples were pooled in equal amounts to generate 10 samples with a large number of different HPV types.

Cervical swabs with discordant hybrid capture and SPF10-LiPA results. A group of eight consecutive cervical cytology specimens with discordant results at hybrid capture 2 (HC2; Qiagen Corporation, Gaithersburg, MD, USA) and SPF10-LiPA testing were retrospectively selected for typing with the 454 pyrosequencing method. The HC2 assay and SPF10-LiPA were carried out according to the manufacturer's instructions. Discordant samples were also tested with previously described HPV type-specific real-time PCR assays targeting HPV types 16, 18, 31, 33, 45, 52 and 58 [12,13].

Protocol for HPV typing by 454 pyrosequencing

Total DNA was purified from anogenital swab specimens by using the MagNA Pure 96 Viral NA Small Volume Kit® on a MagNA Pure 96™ instrument (Roche Diagnostics, Monza, Italy). To generate amplicons for the 454 NGS library, *c.* 100 ng of purified DNA was amplified by PCR with fusion primers and AmpliTaq Gold® DNA Polymerase (Life Technologies Italia, Monza, Italy). Multiplex fusion primers were designed in order to contain the PGMY primer sequences reported in the WHO Human Papillomavirus Laboratory Manual [11], the A e B sequencing adapters (i.e. 5′-CGTATCGCCTCCCTCGCGCCA-3′ and 5′-CTATGCGCCTTGCCAGCCCGC-3′, respectively) and the key sequence (i.e. 5′-TCAG-3′) required for 454 NGS, and one of eight different 10 bp multiplex identifier (MID) barcodes, according to the manufacturer's GS FLX Standard sequencing method. As the PGMY primers set recommended by the WHO manual cannot detect HPV68a, we designed an additional primer pair specific for this high-risk HPV type (i.e. PGMY09 5′-CGTCCTAATGGGAATTGGTC-3′ and PGMY11 5′-GCACAGGGACACAACAATGG-3′). PCR conditions were one cycle of 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 55°C for 90 s and 72°C for 2 min, and a final 7-min step at 72°C. PCR products were purified by AMPure beads (Agencourt Bioscience Corporation, Beverly, MA, USA), quantified, diluted to 4 $\times$ 10^6 molecules/μL, and pooled according to MID barcode compatibility. Each pool was amplified by emulsion PCR (emPCR) using the GS-FLX emPCR Kit II and III and sequenced at 5000$\times$ in both forward and reverse directions coverage on a Genome Sequencer FLX Instrument by using the GS FLX Titanium chemistry (Roche 454 Life Sciences, Branford, CT, USA). The average read length obtained from NGS runs was 358.3 $\pm$ 129.5 nucleotides, in agreement with the median read size yielded by the GS FLX Titanium protocol. The median number of reads per sample was 12 806, range 3685–34990. Raw data will be submitted to the SRA database.

Analysis of 454 pyrosequencing data

HPV typing was carried out by means of a custom analysis pipeline, as described in [10]. A minimum cut-off of five reads was used to define genotype detection. Reads that did not align with known HPV genotypes were further analysed by means of BLAST searches against the non-redundant database of nucleotide sequences and were identified as human genomic DNA contaminant. Analysis of 454 pyrosequencing data was performed blind to the results of the other HPV detection and genotyping tests.

Results

Sensitivity and specificity in WHO Panel 2010 and 2011

In a first pyrosequencing run, a PGMY multiplex primer set, which was designed based on the sequences recommended in the WHO Laboratory manual [11], was used to analyse the

WHO 2010 and 2011 proficiency panel samples. In all samples, HPV types were correctly detected and typed at both 10 and 100 GE/μL concentrations, with the exception of HPV68a, which was never detected. In samples with multiple HPV types, the number of reads obtained for HPV31, HPV33, HPV51 and HPV56 was *c.* ten-fold lower than other HPV types, suggesting a different sensitivity of the method for these HPV types.

As the PGMY primers were shown to be unable to detect HPV68a due to several mismatches also in the WHO proficiency study [7], we designed a new primer pair targeting HPV68a and added it to the PGMY primer mix. The sensitivity and specificity of the modified PGMY primer set for HPV68a was investigated with a new NGS run in eight samples of the WHO 2010 proficiency panel containing HPV68a or HPV68b as single or multiple infections and in ten samples negative for HPV68a and HPV68b (Table 1). The results of the 454 NGS run demonstrated that the modified PGMY primer mix allowed 100% correct identification of all genotypes in all samples, including HPV68a at both 10 and 100 GE/μL, both in single and in multiple infections (Table 2). The number of HPV68a reads was higher than the reads of other genotypes in multiple infections. This was probably due to the different sensitivity of primers, because those targeting HPV68a had no mismatches with their target, at variance with other target HPV types (Table 2).

TABLE 2. Results of HPV genotyping of WHO 2010 proficiency panel samples by using a modified PGMY primer mix and 454 pyrosequencing

HPV type	HPV GE/μl	Primer MID	454 pyrosequencing results (No. reads)
HPV11	10	MID4	HPV11 (6498)
HPV11	100	MID6	HPV11 (5177)
HPV18	1	MID8	HPV18 (6208)
HPV18	10	MID7	HPV18 (9756)
HPV59	10	MID1	HPV59 (6754)
HPV59	100	MID8	HPV59 (12535)
HPV68a	10	MID2	HPV68a (7934)
HPV68a	100	MID3	HPV68a (6780)
HPV68b	10	MID6	HPV68b (8668)
HPV68b	100	MID5	HPV68b (8675)
HPV6, 16, 18, 51	10	MID8	HPV6b (950), HPV16 (825), HPV18 (454), HPV51 (52)
HPV6, 16, 18, 51	100	MID6	HPV6b (1237), HPV16 (972), HPV18 (740), HPV51 (67)
HPV11, 16, 31, 33, 58	10	MID7	HPV11 (3447), HPV16 (3326), HPV31 (21), HPV33 (86), HPV58 (1655)
HPV11, 16, 31, 33, 58	100	MID4	HPV11 (2876), HPV16 (3165), HPV31 (144), HPV33 (236), HPV58 (2054)
HPV39, 45, 52, 56, 68a	10	MID5	HPV39 (22), HPV45 (129), HPV52 (391), HPV56 (58), HPV68a (8195)
HPV39, 45, 52, 56, 68a	100	MID1	HPV39 (49), HPV45 (566), HPV52 (178), HPV56 (41), HPV68a (6327)
HPV35, 59, 66, 68b	10	MID2	HPV35 (36), HPV59 (5782), HPV66 (949), HPV68b (4590)
HPV35, 59, 66, 68b	100	MID3	HPV35 (72), HPV59 (3145), HPV66 (125), HPV68b (1292)

Reproducibility and quantitative analysis of HPV16- and HPV18-positive samples

To analyse the reproducibility of the 454 pyrosequencing method, nucleic acids purified from CaSki and HeLa cell lines were mixed in different proportions, in order to obtain six different percentages of HPV16 and HPV18 genome copies, ranging from 0.1% to 80% of HPV16 genome equivalents of the total number of HPV16 and HPV18 genome equivalents. The samples were both amplified and sequenced in duplicate with fusion primers tagged with MID1 and MID2. The results of this test are shown in Fig. 1. Not only the percentage (Fig. 1b) but also the absolute number of reads (Fig. 1a) of the two HPV types obtained by 454 pyrosequencing was highly reproducible and the percentage of reads correlated with the percentage of each HPV type that was present in the sample mix (regression analysis, $R^2 = 0.97$).

Analysis of multiple HPV infections

Ten pools of samples with multiple HPV infection identified by SPF10-LiPA were generated to assess the ability of the 454 pyrosequencing method to detect HPV types in multiple infections. The results of this analysis are reported in Table 3. In most cases, the 454 pyrosequencing method detected all expected HPV types identified by SPF10-LiPA. In addition, in some samples, the 454 pyrosequencing method detected HPV types that are not targeted by the SPF-10 LiPA method (e.g. HPV55 and HPV83). In some cases, 454 pyrosequencing did not detect HPV types identified by SPF10-LiPA. These discrepancies were mainly due to the different abilities of the two methods to amplify particular HPV types [6,7]. In addition, some discrepancies were associated with questionable genotyping results generated by the SPF10-LiPA due to the line-probe interpretation algorithm of the assay, at variance with the 454 pyrosequencing that generated unambiguous genotyping results.

Analysis of samples with discordant HC2 and SPF10-LiPA results

Eight samples with discordant HC2 and SPF10-LiPA results were analysed by 454 pyrosequencing (Table 4). Type-specific real-time PCR was carried out to further investigate discrepant findings. HPV detection and typing by 454 pyrosequencing appeared an accurate and sensitive method, which could detect the presence of high-risk HPV infection not detected by HC2 or the presence of some high-risk HPV types, such as HPV59, for which the SPF10-LiPA system has low sensitivity. The 454 pyrosequencing system also indicated false-positive results by either HC2 (e.g. HC2 positivity in the presence of low-risk or possibly high-risk HPV types such as HPV53 and HPV66) or SPF10-LiPA

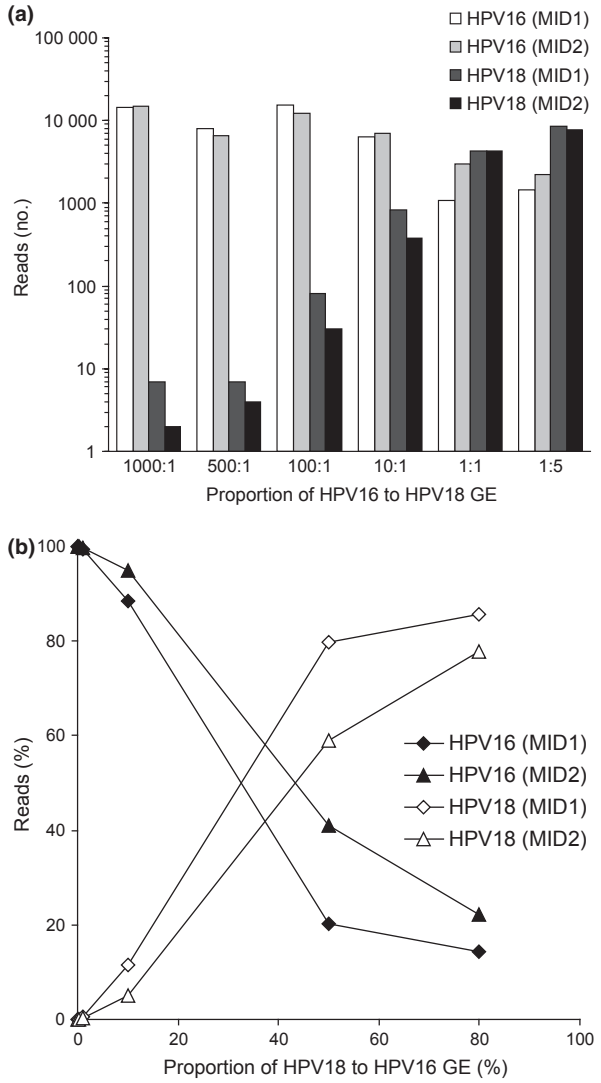

FIG. 1. Results of 454 pyrosequencing analysis of pools of DNA purified from CaSki (HPV16-positive) and HeLa (HPV18-positive) cells. The analysis was performed in duplicate pool samples with PCR primers containing either the multiplex identified MID1 or MID2. Pools contained different proportions of DNA purified from cell lines (and corresponding HPV genome equivalents, GE) ranging from 0.1% (i.e. 1000:1 HPV16 to HPV18) to 80% (i.e. 5:1) HPV16 GE of the total amount of HPV16 and HPV18 GE in samples. Results are represented as absolute number (a) and percentage (b) of reads of HPV18 and HPV16 obtained in samples amplified in duplicate with MID1 and MID2 barcoded primers.

(e.g. HPV51), although we cannot completely exclude the presence of low level infection by some HPV types not detected by 454 pyrosequencing. Finally, the 454 pyrosequencing method allowed the detection of HPV types that cannot be identified by SPF10-LiPA (e.g. HPV61, HPV62 and HPV67).

TABLE 3. Results of HPV genotyping of pools of HPV-positive genital swab specimens by using a modified PGMY primer mix and 454 pyrosequencing

HPV types (SPF-10 LiPA)	Primer MID	454 PGMY results (No. reads)
HPV6, 16, 31[a], 51, 52, 56	MID3	HPV6 (6557), HPV16 (203), HPV51 (18), HPV56 (22), **HPV58 (21)**, **HPV61 (92)**, **HPV73 (38)**
HPV6, 31, 56, (52)[a,b]	MID3	HPV6 (11451), HPV31 (52), **HPV42 (38)**, HPV56 (33), **HPV83 (32)**
HPV16, 18, 52, 66[a], (51)[a,b]	MID3	HPV16 (11894), HPV18 (1316), HPV66 (41), **HPV55 (25)**, **HPV89 (37)**, **HPV11 (25)**
HPV16, 44, 53, 66	MID3	HPV16 (4357), HPV53 (251), HPV66 (14107), HPV44 (64)
HPV11, 16, 52[a], 58	MID3	HPV11 (44), HPV16 (8003), HPV58 (29)
HPV11, 52, 59, 66	MID3	HPV11 (23), HPV52 (30), HPV59 (9276), HPV66 (47)
HPV6, 11, 16, 44, 52	MID4	HPV6 (12805), HPV11 (19160), HPV16 (218), **HPV35 (10)**, **HPV39 (11)**, HPV44 (298), HPV52 (345), **HPV54 (11)**, **HPV58 (29)**, **HPV61 (176)**, **HPV62 (21)**, **HPV83 (33)**
HPV16, 44, 51, 52	MID5	HPV16 (9076), HPV44 (69), HPV51 (54), HPV52 (255), **HPV61 (105)**
HPV16, 52, 58, 70	MID7	HPV16 (1453), **HPV42 (21)**, HPV52 (2506), **HPV53 (27)**, HPV58 (1074), **HPV61 (44)**, **HPV66 (48)**, HPV70 (16063), **HPV89 (32)**
HPV6, 11, 16, 31[a], 44, 51, 53, 66	MID8	HPV6 (7950), HPV11 (870), HPV16 (1402), HPV44 (124), **HPV45 (335)**, HPV51 (212), HPV53 (117), HPV66 (5188)

HPV types marked with [a] were detected by SPF10-LiPA only, while HPV types in bold were detected by 454 only.
[b]The presence of HPV types within brackets is defined as possible by SPF10-LiPA.

Discussion

In this study, we developed a new method for HPV genotyping based on 454 pyrosequencing of HPV L1 amplicons generated with a new modified set of PGMY primers. This method was sensitive, specific, quantitative, and precise in both single and multiple infections. It could identify a wide range of HPV types and might potentially discover new HPV types.

The NGS method could correctly identify all high-risk HPV types, including HPV68a, which is not amplified by PGMY primers. Targeting of HPV68a was achieved by the addition of a new specific primer pair to the multiplex PGMY primer set.

By testing different mixtures of HPV16- and HPV18-positive cell lines, the 454 pyrosequencing method was demonstrated to reproducibly quantify the proportion of each HPV type in multiple infections, with a relatively high dynamic range. However, some HPV types, such as HPV31, HPV33, HPV51 and HPV56, appeared to be less efficiently amplified, leading to an underestimation of their amount. Nonetheless, the 454 pyrosequencing method could correctly detect all HPV types of the WHO proficiency panel at the low 50 GE/5 μL level. In addition, a wide number of different HPV types could be detected in samples with multiple infections.

TABLE 4. Results of HPV genotyping of cervical swab samples with discordant HC2 and SPF10-LiPA results by using a modified PGMY primer mix and 454 pyrosequencing

HC2	SPF10-LiPA	Real-time PCR[a]	454 NGS typing (genotype/No. reads)
Negative	HPV31, 51, 66	HPV16 and HPV31 positive	HPV6 (6410), **HPV16** (137), HPV31 (2118), HPV51 (1044), HPV66 (67)
Negative	HPV16, 31, **33**, X	HPV16 and HPV31 positive	HPV11 (5253), HPV16 (38), HPV31 (37), **HPV58** (28), **HPV59** (770), HPV66 (17)
Negative	HPV16, 51	HPV16 positive	HPV16 (638), HPV51 (947), HPV61 (3448), HPV81 (5710)
Negative	HPV11, **51**, 66	Negative	HPV11 (10117), HPV66 (538)
Positive	**Negative**	Negative	HPV59 (392), HPV62 (9015)
Positive	HPV66	Negative	HPV66 (12445)
Positive	HPV53	Negative	HPV53 (4423), HPV61 (4709)
Positive	HPVX	Negative	HPV62 (9142), HPV67 (2481)

[a]HPV types detected by type-specific real-time PCR: HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, HPV58 and HPV68b.
Discrepant results are highlighted in bold.

The method was useful for the analysis of samples with discordant results obtained by two hybridization based-methods (i.e. the HC2 and the SPF10-LiPA assay), for which problems of cross-hybridization and low-sensitivity for some HPV types, respectively, have been reported [6,7,14–16]. Indeed, methods based on sequencing are expected to be more specific than hybridization-based methods and are considered the reference standard for genotyping. Our method, based on sequencing of a 450-bp long fragment of the L1 gene, allows us to achieve a very good specificity of genotyping and, potentially, could lead to the discovery of a new HPV types, for which a long sequence might provide sufficient information for phylogenetic analysis [17,18]. This feature represents an advantage over methods based on the analysis of very short sequences, such as the commercially available HPV sign® Genotyping test (Qiagen), which is based on broad-spectrum amplification of a variable region of HPV L1 and pyrosequencing of short reads from the amplicons on the Pyromark Q24 system (Qiagen). In addition, the HPV sign® Genotyping test was demonstrated to have a low sensitivity for some HPV types and for the discrimination of HPV types in multiple infections, but, on the other hand, being based on sequencing of short amplicons, the assay was shown to be suitable for testing archival formalin-fixed paraffin-embedded biopsies [19].

Costs and turnaround time are major problems with diagnostic methods based on NGS technologies, as already discussed in our previous proof of principle study [10]. Full automation and standardization of protocols for library preparation and sequencing are required for the implementation of this technology in routine diagnostics. Possible applications of this genotyping method might include: (i) second line testing in patients with HPV-related lesions for risk stratification, (ii) accurate analysis of HPV types in HPV-related cancers, (iii) epidemiological monitoring of mucosal HPV type distribution, (iv) detection of uncommon or novel HPV types; and (v) any other application that requires accurate HPV typing and estimation of the relative load in multiple infections.

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Ethical Approval

The study was approved by the local ethics committee. All study participants provided written, informed consent.

Transparency Declaration

The authors have no conflicts of interest in this article.

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