Identification by high-throughput sequencing of HPV variants and quasispecies that are untypeable by linear reverse blotting assay in cervical specimens

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ABSTRACT
The linear reverse blotting assays are valid methods for accurate human papillomavirus (HPV) typing required to manage women at risk of developing cervical cancer. However, some samples showed a positive signal in HPV lines but failed to display a positive signal in subsequent typing lines (designated as HPV-X), which indicate that certain types were not available on the respective typing blots. The aim of this study is to elucidate the types or variants of HPV through the high-throughput sequencing (HTS) of 54 ASCUS cervical samples in which the viruses remained untypeable with INNO LiPA HPV® assays. Low-risk (LR)-HPV types (HPV6, 30, 42, 62, 67, 72, 74, 81, 83, 84, 87, 89, 90 and 114), high-risk (HR)-HPV35 and possibly (p)HR-HPV73 were detected among HPV-X. Individual multiple infections (two to seven types) were detected in 40.7% of samples. Twenty-two specimens contained variants characterised by 2–10 changes. HPV30 reached the maximal number of 17 variants with relative abundance inferior or equal to 2.7%. The presence of L1 quasispecies explains why linear reverse blotting assays fail when variants compete or do not match the specific probes. Further studies are needed to measure the LR-HPV quasispecies dynamics and its role during persistent infection.

1. Introduction
Human papillomaviruses (HPV) are DNA viruses that infect cutaneous and mucosal epithelia. The HPV genome is a double-stranded circular DNA ~8,000 base pairs (bp) in length. The prototypical HPV genome encodes 6 early genes (E1, E2, E4, E5, E6 and E7) and 2 late genes (L1 and L2). More than 225 HPV types have been characterised based on sequence information collected by the International HPV Reference Centre [1]. Classification of HPV is based on the nucleotide sequence of the L1 gene, which encodes the major capsid protein. HPV belongs to five major genera: alpha (65 types), beta (54 types), gamma (99 types), mu (3 types) and nu (1 type) [2]. Specific HPV types display less than 90% similarity to other HPV types [3]. HPV types can be further classified into lineage variants or sublineage variants; these variants possess genome sequences that empirically diverge by 1–10% or sublineage variants with genomes that are 0.5%–1% divergent [4].

The mucosal HPV types belonging to the alpha genus are divided into four groups based on their epidemiological association with cancer. (i) Group 1, which include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 that are carcinogenic to humans. (ii) The group 2A which include HPV68 that is probably carcinogenic to humans. (iii) The group 2B, which include HPV 26, 53, 66, 67, 70, 73 and 82 are possibly...
carcinogenic to humans. The HPV types 30, 34, 69, 85 and 97 in group 2B are possibly carcinogenic to humans based on their phylogenetic similarity to HPV types for which there is sufficient or limited evidence of carcinogenicity in humans. (iv) There is no epidemiological evidence that other HPV types in the alpha genus included in the group 3 (HPV6, 7, 11, 13, 32, 40, 42, 43, 44, 54, 61, 62, 71, 72, 74, 81, 83, 84, 87, 89, 90, 91, 102, 106 and 114) are carcinogenic to humans; they cause skin or genital warts, minor cytological atypia and often no apparent disease [5].

Testing for infection with high-risk (HR) HPV is an invaluable part of the clinical guidelines for cervical carcinoma screening, management and treatment [6]. Despite a similar overall level of accuracy in detecting high-grade cervical intraepithelial neoplasia (CIN), commercial HPV assays do not detect the same infections in women undergoing primary cervical screening [7-9]. Their concordance in positive results varies between 48% and 69% [10,11]. Most of these assays use sets of consensus primers or mixtures of primers to amplify a subgenomic region of L1 or E6/E7 [10]. Full HPV genotyping is based on the visualisation of amplicons generated by consensus primers using secondary probe hybridisation on solid supports or sequencing. INNO-LiPA HPV genotyping assays have been proven to be valid methods for HPV genotyping that offer performance comparable to that of other methods and licensed for in vitro diagnostic use [12,13]. However, the finding in several studies that ~1% of cervical samples showed a positive signal in HPV lines but failed to display a positive signal in subsequent typing assays and were designated as containing HPV-X may indicate that certain types or variants were not available on the respective typing blots [14,15].

High-throughput sequencing (HTS) has been efficiently used for HPV genotyping and has enabled the identification of multiple infections and the precise identification of sequences even in specimens with weak viral loads [16-18]. The aim of this study is to elucidate retrospectively by HTS the spectrum of types and variants of HPV present in cervical samples that were untypeable (HPV-X) using the INNO-LiPA HPV Genotyping systems.

2. Material and methods

2.1. DNA specimens and controls

A selection of 252 HPV DNA-positive cervical samples with squamous cells of undetermined significance (ASCUS) cytology was obtained from the laboratory of Virology at the Dupuytren Hospital (Limoges, France) and the Bicêtre Hospital (Kremlin-Bicêtre, France). The 252 samples were positive for HPV with indeterminate genotype (HPV-X) according to the INNO-LiPA HPV Genotyping Extra assay (Fujirebio®). INNO-LiPA HPV Genotyping Extra permits the amplification of 65 pb SPF10 fragment. Blot strips that include two generic HPV positive control probe lines allow the identification of 28 HPV genotypes: HPV-6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 54, 56, 58, 59, 66, 68, 69/71, 73, 70, 74 and 82. Of note, all the samples were labelled as HPV-X alone, detected without other associated HR- or LR-HPV types. HPV16 DNA extracted from a cervical cytology specimen (sample S53) and a purified plasmid encompassing the full-length genome of HPV16 (GenBank accession number K02718; ATCC® 45113D™) were used as positive controls.

2.2. Protocol for DNA sample library preparation and HPV typing by Illumina® MiSeq HTS

HPV DNA was amplified by PCR using modified GP5+/GP6+ primer pairs extended with overhang adapter sequences required to bind the Illumina® indexes and sequencing [19]. PCR was performed in 50 μL reaction mixtures containing 1X High-Fidelity Platinum buffer, 3.5 mM MgCl2, 0.6 μM forward and reverse primers and 1U of proof-reading Taq polymerase (Platinum™ Taq DNA Polymerase, Thermo Fisher®). The cycling protocol consisted of an activation step (15 min at 95°C), 21 cycles (1 min at 94°C and 2 min at 50°C, decreased by 0.5°C per cycle to 40°C and 1.5 min at 72°C), 24 cycles (1 min at 94°C, 2 min at 40°C, 1.5 min at 72°C), and a final elongation step (4 min at 72°C) [20]. Each PCR product was dual-indexed using the KAPA HiFi HotStar Uracil + ReadyMix (Roche Diagnostics®). The Agencourt Ampure XP beads system (Beckman Coulter®) was used to purify the DNA libraries. The concentrations of the DNA libraries were normalised prior to pooling and sequencing to ensure equal representation of each sample. A PhiX Control (Illumina®) spike-in at 5% was used as an internal control to monitor sequencing quality. The combined library and the PhiX controls were loaded at 8 pM final concentration on MiSeq using the v3 reagent kit and sequenced by Illumina MiSeq 2 x 300-bp paired-end sequencing.

2.3. Analysis of HTS data

FastQC were processed using CLC Genomics Workbench software V10.0.1 (Qiagen Bioinformatics). The reads were submitted to quality control and discarded if the quality score was less than 0.05 (QC30) and/or if more than 2 ambiguous nucleotides were detected. Reads with lengths of less than 100 nucleotides were removed for further analysis. The Qiagen GS reference mapper (CLC HTS Core Tools, Qiagen) was used to identify HPV reads by aligning the trimmed reads to the L1 region of 184 HPV reference sequences downloaded from the HPV episteme database (PaVE) (https://pave.niaid.nih.gov/). To be assigned to an HPV type, each read was required to align with at least 90% identity. The reads were clustered into operational taxonomic units (OTU) at 99% similarity (CLC Genomics Workbench (Qiagen Bioinformatics). OTU containing at least 200 reads were analysed, and representative sequences for each OTU were thus extracted.

2.4. Phylogenetic analysis and detection of variants

Phylogenetic analysis was performed using the maximum likelihood method based on the Tamura-Nei model using Mega 7 software, allowing the classification of OTU sequences into HPV type clusters [21]. Multiple alignments were performed for the OTU sequences that had been grouped in the same HPV cluster using ClustalW2 and BioEdit v7.2.5 [22,23].

3. Results

3.1. HPV amplification and illumina sequencing

The modified GP5+/GP6+ fusion primers amplified 58 (23.0%) of 252 selected samples. A total of 32,881,974 reads were obtained (Fig. 1). After trimming, 10,987,925 (75.8%) of the reads mapped to references. The median length of HPV reads was 139.3 bp ranging from 138.6 to 147.4 bp. Four samples (S33, S35, S40 and S41) did not meet the quality criteria to be considered as positive samples; they were excluded from the study (Supplementary Table S1).

3.2. Deciphering of HPV types among 54 HPV-X sequencing samples

Among the 54 sequencing samples, 16 different types were detected (Fig. 2). HPV83 (21/54) and HPV42 (20/54) were the most frequently detected, followed by HPV81, HPV67, HPV90, HPV74 and HPV87. All of the HPV types identified by HTS are unclassifiable as to carcinogenicity in humans (group 3) except for the high-risk (HR) HPV35 found in one sample (S59) and a possibly HR (pHR) HPV73 in co-infection with HPV90 with or multiple genotypes in S17 and S18, respectively (Table 1 and Supplementary Table S2). Twelve LR-HPV types (HPV30, 42, 62, 67, 72, 81, 83, 84, 87, 89, 90 and 114) were also identified (Fig. 2). Individual multiple infections were detected in 22/54 (40.7%) sequencing samples; the number of types present in...
multiple infections ranged from two to seven (Table 1).

### 3.3. HPV divergent sequences and variants

Reads clustering in OTU were compared to their respective HPV reference sequences; variants were detected in 22/54 (40%) specimens (Supplementary Table S2). Sequencing of the L1 region of the plasmid used as positive control (HPV16; K02718) showed only one kind of OTU with a relative abundance (OTU reads/total reads per sample) of 100%, suggesting that the variants observed in clinical samples are not due to random sequencing errors. Phylogenetic analysis allowed a classification of all OTU sequences into 16 clusters; the two positive controls (K02718) and S53 were localised in the HPV16 cluster (Fig. 3). Multiple alignments of OTU showing different homologies of sequences according to the HPV types considered and the specimens are presented in Supplementary Fig. S1. Among the variants, changes ranged from 2 to 10 nucleotides. In sample S19, two OTU sequences (S19–V3 and S19–V14) clustering with HPV30 presented the maximum of 10 changes out of 91 nucleotides. The numbers of reported variants in some samples were very high, reaching 15 for the divergent sequences clustering with HPV83 in sample (S01) and 17 for the divergent sequences (S19–V3 to S19–V19) clustering with HPV30 in sample (S19), as shown in Fig. 4A. In the sample S19 encompassing also a wild type HPV42 (RA 79.8%) and HPV67 (7.7%), the 17 minority variants of HPV30 have relative abundance values ranging from 0.3% to 2.7% (Supplementary Table S2). The 16/19 recorded changes in HPV30 L1 sequences corresponded to C to T transitions. On the other hand, among the OTUs found in 20 specimens clustering with HPV42, all 21 amplimers except S49–V2 were highly conserved; these amplimers displayed sequences that were 100% similar to the reference sequence (Fig. 4B).

### 4. Discussion

#### 4.1. Identification of 54 HPV-X by HTS

In our study, we were able to identify HPV genotypes in 54 of the 252 pre-selected HPV-X DNA specimens. We found individual multiple infections (from two to seven different types) in 40.7% of the specimens, consistent with data reported in the literature. Indeed, 20–40% of HPV-positive women harbour in their cervix at least two HPV types [24]. The original SPF10 system was designed at the end of the 90s as an ultra-sensitive and broad-spectrum PCR-based assay for the detection by DNA-enzyme immunoassay with consensus probes of a wide range of HPV types [15,25]. It has been shown that the SPF10 LiPA25—(Version 1, Labo Biomedical Products, based on licensed Innogenetics technology), the later version of the SPF10 system developed for the identification of 25 HPV types—was more sensitive and better suited for epidemiologic HPV research than the INNO-LiPA HPV Genotyping Extra ® [26]. The CE-IVD INNO-LiPA system is a multiplex PCR containing an internal human DNA control. It utilizes uracil-N-glycosylase (UNG) to eliminate carryover of PCR products prior amplification. These technical differences may finally result in a reduced sensitivity to

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**Table 1**

| HPV type identified | Number of detections among 54 specimens |
|---------------------|----------------------------------------|
| 35                  | 1                                      |
| 42                  | 4                                      |
| 67                  | 3                                      |
| 74                  | 1                                      |
| 81                  | 2                                      |
| 83                  | 15                                     |
| 87                  | 3                                      |
| 89                  | 1                                      |
| 90                  | 1                                      |
| 114                 | 1                                      |
| 6, 42               | 1                                      |
| 42, 74              | 1                                      |
| 42, 81              | 1                                      |
| 42, 83              | 3                                      |
| 42, 84              | 2                                      |
| 42, 90              | 1                                      |
| 62, 67              | 1                                      |
| 62, 81              | 1                                      |
| 62, 89              | 1                                      |
| 73, 90              | 1                                      |
| 87, 90              | 1                                      |
| 30, 42, 67          | 1                                      |
| 42, 67, 81          | 1                                      |
| 42, 74, 83          | 1                                      |
| 42, 74, 87          | 1                                      |
| 42, 81, 83          | 1                                      |
| 74, 81, 83          | 1                                      |
| 42, 67, 72, 89, 90  | 1                                      |
| 42, 67, 72, 74, 81, 84, 90 | 1 |

*a Numbers in bold indicate HPV types that are carcinogenic to humans (group 1).
b Numbers in bold and italic indicate HPV types that are possibly carcinogenic to humans (group 2B).
InnoLiPA [26]. However, among the HPV-X studied here, the twelve LR-HPVs detected by HTS (HPV30, 42, 62, 67, 81, 83, 87, 89, 90 and 114) cannot be clearly identified by using INNO-LiPA HPV Genotyping Extra® strips, as respective probes are not included in the hybridization strip. Of note, six of them (HPV42, 62, 67, 81, 83 and 89) have been added to the more recent version of the test (INNO-LiPA HPV Genotyping Extra II®, Fujirebio®), which is designed for the genotyping of 13 HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), 6 possibly (p)-HR-HPV (26, 53, 66, 70, 73, and 82), 13 low-risk (LR) HPV or genotypes with unknown significance (6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 81, 83 and 89) [27]. Nevertheless, HTS technologies have become indispensable to considering HPV types that are not apparent by using commercial LiPA assays in epidemiological studies to determine their significance in the context of their association to cervical disease, at any stage of the disease, a fortiori in the vaccine area [28].

4.2. Why some HPV-X still remained untypeable in our study

In our study, we used the very common GP5+/GP6+ primer pair, which allow the amplification of a broad range of mucosal HPV types in a single reaction [9,19]. However, these primers amplified only 58/252 (23.0%) of our pre-selected HPV SPF10 PCR positive samples, question the efficiency of GP5+/GP6+ in detecting all types from clinical samples. It has been proposed recently that the performance of these PCR systems should be improved by the addition of eight forward broad-spectrum primers and two backward broad-spectrum primers (BSGP5+ and BSGP6+); this has led to more sensitivity in the detection of genital HPV types [24]. Furthermore, wide variations in sensitivities across mixed HPV types or variants are a known concern in consensus-primer-based PCR assays [29–31]. Lastly, in our study, GP5+/GP6+ primers cannot amplify any hypothetical beta and gamma HPV species. The sum of these technical limitations may partially explain why some HPV still remained unexplored in clinical samples.

4.3. HTS facilitates the characterisation of HPV quasispecies in cervical samples

Our HTS results clearly illustrate that the limitations in HPV LiPA typing could also be linked to the presence of numerous variants sources of quasispecies in clinical samples. HTS sequencing became a common method for the identification of HPV types, the metagenomic detection of novel putative HPV types and the analysis of minor HPV variants in genotype-phenotype studies [32,33]. It facilitates the characterisation of viral quasispecies as a whole; rather than focusing on dominant viral haplotypes (e.g., sequences shared by a significant proportion of the population) or a consensus sequence. The concept of quasispecies consisting of numerous variants has already been proposed...
for HPV16 [34,35]. The existence of quasispecies is difficult to understand based on the high fidelity of the host DNA replication machinery and the general low evolutionary rate of nucleotide substitutions in HPV, which is estimated to be $2 \times 10^{-8}$ per site per year [34]. Neither mutagenic mechanisms leading to quasispecies nor the impact of intratype variations in HPVs genome on anocervical cancer development have been extensively explored [36]. For the authors, L1 hypermutation may alter the specific loop structure of the capsid protein that contains type-specific immunodominant epitopes [37,38]. Selective hypermutations have been proposed to be involved in the evolution of the HPV16 genomes and in the rapid escape of the virus from the innate and adaptive immune response [38,39]. Our results show that the concept of quasispecies can be extended to the L1 sequences of alpha LR-HPV, but this observation remains inconstant among LR-HPV according to the types as already shown for HR-HPV [36,40].

5. Conclusion

In conclusion, this study identified frequent individual multiple infections with LR-HPV in HPV X/ASCUS cervical samples and revealed that the presence of numerous minority variants is not uncommon in the LR-HPV detected. The presence of L1 quasispecies can explain why genotyping by linear reverse blotting assays fails when variants compete with or do not match the specific hybridisation probes. Pending a better understanding of the mutagenic processes in HPV genomes and the unknown role played by the L1 quasispecies, HPV types that are unapparent via LiPA assays should be considered in epidemiologic and clinical studies.

Declaration of competing interest

The authors have no conflicts of interest to declare.
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