Comparison of DNA Sequencing and Roche Linear Array® in Human papillomavirus (HPV) Genotyping

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Abstract. *Background: Human papillomavirus (HPV) is the etiological agent of cervical cancer. HPV genotyping is important to determine the presence of high-risk types. Recently, a new HPV genotyping method, the Roche Linear array® genotyping test, was introduced and is compared here with a sequencing-based HPV genotyping system. Materials and Methods: A series of 102 women (age range 30-55 years) shown to be HPV DNA-positive by PCR were typed by sequencing and the Linear array genotyping assay. Results: The sequence analysis revealed the presence of 80 single high-risk types and 22 single low-risk types. With the Linear array, single infections were found in 46 cases, double infections in 37 cases, triple infections in 12 cases, and more than three in 6 cases. One case positive by sequencing gave a negative result by Linear array. Altogether, a concordant single genotype was found in 93 (91.2%) out of the 102 cases and the single-type concordance between the two assays was significant (Spearman rho=0.849, p=0.0001; intraclass correlation coefficient (ICC) (ICC=0.924, 95%CI 0.888-0.949) (p=0.0001). The majority of the disparate results were due to the detection of multiple types by the Linear array. Conclusion: The Roche Linear array® is a highly accurate assay for HPV genotyping. This is particularly true in the presence of multiple infections which DNA sequencing is unable to resolve.*

Currently, more than 100 human papillomaviruses (HPVs) have been identified (1) and about 40 of them can infect the genital mucosa. However, only a restricted group of HPVs are associated with high-grade cervical precancer lesions (CIN) or cervical cancer (2, 3). These viruses are termed high-risk (HR) HPVs, while the low-risk types (LR) are generally associated with condylomas or other benign epithelial lesions. Since it is extremely difficult to culture HPV *in vitro*, PCR testing is a sensitive and non-invasive method for determining the presence of a cervical infection. Applications of an HPV-type testing include monitoring the acquisition and clearance of specific HPV types (4), as well as type-specific persistence of HPV types associated with higher risk of developing cervical cancer (5, 6).

The determination of HPV genotypes can be accomplished using different techniques: sequencing, Line Probe assay (LiPA) (7) or genotype-specific PCR. The aim of the present study was to evaluate and compare the specificity of a new genotyping method, the Linear array HPV genotyping assay, to a sequencing-based genotyping test.

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Materials and Methods

Patient selection. Among the 700 women who attended our Colposcopy Clinic in the past two years due to a Pap-smear suggestive of ASCUS (atypical squamous cells of undetermined significance), LSIL (CIN1) or HSIL (CIN2/3), 102 women who tested positive for HPV-DNA by PCR using the degenerate primers MY09/11 (8) were selected.

DNA extraction. Cervical cells collected with cytobrush and Ayre's spatula were placed in sterile phosphate-buffered saline (PBS) and transferred to our laboratory. The cells were centrifuged and the supernatant was discarded. The DNA was then extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions and was resuspended in 50 μl.

HPV genotyping by sequencing. Five microliters of the extracted DNA were amplified with the degenerate primers MY09/11 (8). The obtained 450 bp fragment underwent a sequencing reaction using the CEQ DTCS-Quick Start kit (Beckman Coulter, Fullerton, CA, USA).

HPV genotyping by the Roche Linear array®. The Linear array genotyping assay (Roche Diagnostics, Indianapolis, IN, USA) is based on PCR amplification of target DNA using HPV primers, hybridization of the amplified product to oligonucleotide probes and their detection by colorimetric reaction. Particularly, the master mix contains primers for the amplification of a 450-bp fragment of the L1 region of more than 37 HPV genotypes (9) and a 268-bp fragment of the human β-globin gene. The detection and genotype determination was performed using the denaturated amplified DNA and an array of oligonucleotide probes, located in the polymorphic region of L1 that permitted independent identification of individual HPV genotypes. Negative samples were included in each assay to demonstrate the specificity of the test.

Results

Detection of HPV types by sequencing and Linear array assay. Sequence analysis of the 102 selected HPV-positive samples showed the presence of 80 single HR HPVs and 22 single LR HPVs (Table I). The genotype was assigned based on the match between the obtained sequence and those deposited in Genebank (nucleotide-nucleotide BLAST; http://www.ncbi.nlm.nih.gov/BLAST), and was confirmed by two alignment sequences (BLAST 2 sequence) against the consensus sequence of the indicated genotype.

When the same specimens were analyzed by Linear array genotyping assay, single infections were found in 46 cases, double infections in 37 cases, triple infections in 12 cases and more than three HPV types in 6 cases. One additional sample positive by sequencing was negative when tested by the Linear array. The concordant and discordant cases shown by the two methods are reported in Table II. A genuinely different genotype was detected in only 6 out of 102 (5.8%) cases, Table III.

Table I. *HPV typing by sequencing.*

HPV type	HPV	
	Low-risk	High-risk
HPV6	2	
HPV16		51
HPV18		7
HPV66		3
HPV31		11
HPV62	3	
HPV52		6
HPV58		2
HPV53	5	
HPV84	3	
HPV61	3	
HPV54	2	
HPV91	1	
HPV83	1	
HPV81	1	
HPV32	1	
Total	22	80

Discussion

HPV infection is frequent among young women. However, most of the infections are cleared over a period of 6-12 months and only a small percentage of the women develop a persistent infection. It has been shown that persistent infection with the same genotypes strongly increases the risk of developing high-grade squamous intra-epithelial lesions in young women (10). HPV genotyping has, thus, become a critical test in the management of women with genital HPV infection. Furthermore, women with persistent infection often present a mixed infection.

Here, we compared the specificity of the Linear array genotyping assay to a sequencing-based genotyping test.

Among the 102 samples that tested HPV-DNA positive by PCR, HPV typing performed by sequencing revealed the presence of 80 single HR types and 22 single LR types. When the same samples were analyzed by the Linear array genotyping assay, mixed infections were revealed in 55 cases and single infections were found in 46 cases, while one case was negative (Table II). Altogether, a concordant genotype was found in 93 (91.2%) out of the 102 cases (40 single infections + 53 multiple infections) and the single-type concordance between the two assays was significant (Spearman rho=0.849, p=0.0001; and ICC=0.924, 95%CI 0.888-0.949) (p=0.0001). This concordance droped dramatically, however, when cases with multiple types detected by the Linear array in single-type positive (sequencing) cases were considered discordant observations [(ICC=0.492, 95%CI 0.248-0.657) and Spearman rho=0.112 (p=0.102)].

The majority of the discrepant results were due to the detection of multiple types by the Linear array, whereas a genuinely different genotype was detected in only 6 out of 102 (5.8%) cases (Table III). The different results obtained with the two methods could be explained by the differences in type-specific sensitivity observed with the two sets of primers used in this study, *i.e.*, MY09/11 and PGMY09/11. The L1 consensus primers MY09/11 have been widely used to study the natural history of HPVs and their role in cervical cancer (8, 11). However, Gravitt *et al.* showed that the PGMY09/11 primers presented an increased type-specific sensitivity compared to MY09/11, particularly with samples infected with multiple types (9). This explains the increased number of multiple infections observed by the Linear array and the discordant results reported in Table III, with the exception of sample 66 which was negative when assayed with the Linear array even though the same sample tested positive for the β-globin gene. This discrepancy could be explained by the loss of the viral genome due to freeze-thawing of the sample.

In conclusion, Roche Linear array® genotyping is an applicable and highly accurate assay for HPV genotype detection in clinical samples and, unlike DNA sequencing, is able to identify mixed infections. Since women infected with multiple types are at increased risk of developing cervical cancer (12), the Linear array could be a valuable tool in the monitoring of such patients.

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Table II. *HPV typing by sequencing and Linear array.*

Sequencing		Linear array		Discordance due to:	
HPV type	No of cases	No of concordant cases	No of discordant cases	Different single genotype*	Multiple genotypes detected
HPV6	2	1	1	0	1
HPV16	51	21	29	1	28
HPV18	7	4	3	1	2
HPV66	3	1	2	0	2
HPV31	11	3	8	1	7
HPV62	3	1	2	0	2
HPV52	6	2	4	1	3
HPV58	2	0	2	0	2
HPV53	5	2	3	0	3
HPV84	3	2	1	0	1
HPV61	3	0	3	0	3
HPV54	2	2	0	0	0
HPV91	1	0	1	0	1
HPV83	1	1	0	0	0
HPV81	1	0	1	NEG	0
HPV32	1	0	1	1	0
Total	102	40/102	62/102	5/102	55/102

*Concordance for single-genotype detection: Spearman rho=0.849 (p=0.0001), and intraclass correlation coefficient (ICC)(ICC=0.924, 95%CI 0.888-0.949) (p=0.0001). Regular Cohen's kappa not computable, due to empty cells in the 2-way table.

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Table III. *Discordant results in sequencing and Linear array.*

Sample	HPV typing	
	Sequencing	Linear array assay
19	HPV16	HPV33
54	HPV91	CP6108/84/62/16
66	HPV81	Neg
67	HPV32	HPV40
71	HPV31	HPV11/51/54/61/72/39/CP6108
76	HPV18	HPV73

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