Comparison of 2 Different PCR-Based Technologies for the Detection of Human Papilloma Virus from Paraffin-Embedded Tissue: Genómica Clinical Arrays Versus SPF_{10}-$LiPA_{25}$

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Abstract: The great interest in molecular epidemiology of human papilloma virus (HPV) in cervical cancer led us to perform a thorough evaluation of 2 polymerase chain reaction (PCR)-based methods for the detection of HPV in archival formalin-fixed paraffin-embedded (FFPE) samples. Thus, the aim of this study was to compare HPV detection in FFPE samples that have histopathologic diagnosis of invasive cervical cancer using SPF10 broad-spectrum primers PCR followed by DNA enzyme immunoassay and LiPA25 (version 1: Labo Biomedical products, Rijswijk, The Netherlands version 1) and the Papillomavirus Clinical Arrays technique (Genómica, Tres Cantos, Madrid, Spain). In this study, 235 biopsies with histopathologic diagnosis of invasive cervical cancer were analyzed for the detection and genotyping of HPV by LiPA25 SPF10-PCR System (version 1) and Papillomavirus Clinical Arrays technique. The detection of HPV DNA with Genómica technique was 75.1%, and 91.9% with LiPA25 SPF10-PCR. The Genómica technique detected a higher percentage of multiple infections (35%) than LiPA25 (8.9%), with a very low agreement for the detection of multiple infections between them ($P > 0.05$). Our study highlights an important difference between 2 PCR-based methods for detection and genotyping of HPV. LiPA25 SPF10-PCR technology may be more adequate than Genómica for the detection of HPV DNA when using FFPE tissue.

Key Words: human papilloma virus, cervical cancer, PCR

(*Diagn Mol Pathol* 2012;21:45–52)

Cervical cancer is ranked the second most common cancer among women worldwide; however, in Europe, it is the fifth most common cancer in women.[1] During the last decade, epidemiological studies, supported by molecular techniques, were indicative that infection by human papilloma virus (HPV) is a "necessary cause" for the development of cervical cancer. Esta referencia está mal tendría que ser la 2.

The causal association had been demonstrated when the presence of HPV DNA was unequivocally identified in 90% to 100% of evaluable cervical samples with invasive cervical cancer (ICC).[2,3] Hence, the sensitivity and specificity of molecular biology techniques have become essential to determine the presence of HPV in cervical carcinogenesis.

Two prophylactic HPV vaccines showing promising results have been approved and are being implemented in many areas. Both of these vaccines are effective against at least 2 mucosal HPV types most commonly found in cervical cancer, including the most frequently found HPV type, HPV-16. Detection of HPV DNA could be useful as a guide to assess the impact of a vaccination program and to monitor the frequency and severity of HPV infections with genotypes not included in the vaccines.[4]

Moreover, the archival formalin-fixed paraffin-embedded (FFPE) samples are an important source for both retrospective epidemiological studies and for diagnostic purposes.

However, it is well known that the fixation time and the type of fixative used in the inclusion of FFPE samples can considerably affect the quality and quantity of DNA that can be extracted. Currently, there are 2 methods that are considered more appropriate for the detection of HPV DNA in archived FFPE samples: in situ hybridization and polymerase chain reaction (PCR). Both methods

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Supported by Foundation for Medical Research Mutua Madrilena and Foundation for Developing Applied Scientific Research and Technology in Asturias (FICYT).

Laia Alemany: has received occasional support for travel to meetings for the study or other purposes (institutional) from Sanofi Pasteur MSD. Silvia de Sanjosé my research unit is involved in vaccine trials organized by GlaxoSmithKline and Merck/Sanofi Pasteur MSD and investigators driven research partially sponsored by GlaxoSmithKline and Merck/Sanofi Pasteur MSD. Travel fund to conferences/symposia/meetings are occasionally granted by either GlaxoSmithKline, Sanofi Pasteur MSD or Qiagen.

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have the advantage that they require small amounts of tissue, and both methods tolerate having nucleic acids degraded to some degree. However, in general, PCR is more sensitive and more reproducible[3,5]; therefore, it is currently considered the method of choice.[6,7]

Our interest in the molecular epidemiology of HPV in cervical cancer led us to perform a careful evaluation of different PCR-based methods for HPV detection in FFPE samples. Thus, the purpose of this study was to determine the sensitivity, specificity, and agreement of the Papillomavirus Clinical Arrays technique (Genómica, Tres Cantos, Madrid, Spain) in comparison with the SPF_{10}-$LiPA_{25}$ PCR System (version 1: Labo Biomedical Products, Rijswijk, The Netherlands). Both techniques have the ability to detect the presence of the most clinically important HPV types by PCR amplification of HPV DNA.

MATERIALS AND METHODS

Study Materials

This is a retrospective study using consecutive FFPE biopsy cases with histopathologic diagnosis of ICC from January 1998 to December 2007. The cases were retrieved from the Pathology Departments of all 9 referral hospitals from the Autonomous Community of the Principality of Asturias (North of Spain). These include University Hospital of Asturias, San Agustín of Avilés, Cabueñes of Gijón, Jove of Gijón, Mieres, Riaño, Jarrio, Cangas of Narcea, and Arriondas. All analyzed tumor biopsy samples were obtained from women who had given informed consent.

A total of 235 biopsies with histopathologic diagnosis of ICC were included in the study. The histologic breakdowns of these ICC cases were 184 squamous cell carcinomas, 38 adenocarcinomas, 8 adenosquamous carcinomas, and 5 described as "others."

Pathology and Laboratory Procedures

All samples were analyzed by both the SPF_{10} broad-spectrum primers PCR followed by DNA enzyme immunoassay (DEIA) and genotyping by $LiPA_{25}$ (version 1: Labo Biomedical Products, Rijswijk, The Netherlands, version 1) and the Papillomavirus Clinical Arrays (Genómica, Tres Cantos, Madrid, Spain).

The following process was performed in the paraffin blocks for both techniques. The FFPE blocks were reembedded whenever they were observed to be in poor condition for cutting or when microtome cassettes for embedding were not used. At least 4 paraffin sections were obtained for each block ("sandwich" method) where the first and last sections were used for histopathologic evaluation after Hematoxylin and Eosin staining (HE). The sections in between the HE were cut at 5 μm and collected in 1.5 mL screw-type tubes for HPV DNA testing. A criterion of 1 cm^2 area was used to determine the number of sections needed to be cut for each block. Therefore, it may have been necessary to use 2 or 3 cut sections for DNA isolation if the embedded tissues were very small.

FFPE blocks were cut and processed under strict conditions to avoid potential contamination. Tissue-free paraffin blocks were cut between each study block to detect HPV carryover from block to block, which were randomly included for testing at a ratio of 10% of the total ICC cases. For each study block, a new blade was used and the paraffin was cleared with a vacuum cleaner and the microtome was cleaned with Histoclear II (a xylene substitute) and 70% alcohol.

To further determine possible sources of contamination, paraffin blocks containing non-HPV-related lesions, processed for inclusion at the same time as the cervical cancer specimens at the local pathology laboratory, were blindly included in the testing at a ratio of 5% of the total ICC cases.

HPV DNA Detection and Typing

Once HE slides were reviewed pathologically and the sample was determined to be adequate for testing (histopathology confirmation of ICC in both first and last HE slides), the sample proceeded to proteinase K digestion.

Papillomavirus Clinical Arrays by Genómica (Genómica, Spain)

This in vitro diagnosis test is based on a microarray technology that detects 35 clinically relevant HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, and 89).

DNA isolation was performed using proteinase K digestion, according to the manufacturer's instructions with some modifications. Therefore, the DNA was extracted by treating the FFPE tissue with 25 μL of freshly prepared Proteinase K solution (at a final concentration of 20 mg/mL) at 56°C for 18 hours to overnight on a heating block (according to the manufacturer's instructions, DNA incubation was for 3 hours, but we obtained better results with an overnight incubation in previous studies performed in our laboratory—data not shown). After the digestion time, Proteinase K was heat deactivated for 10 minutes at 70°C according to the manufacturer's instructions. After DNA extraction, 5 μL of the DNA isolate from each case was added to 0.2 mL amplification tubes provided (Clinical Arrays, Genómica), which had been validated in large studies of European Conformity. PCR was subsequently performed using a Perkin Elmer 9600 thermocycler. The Clinical Arrays use consensus primers MY09/11 that amplify a 450 bp fragment of the viral L1 region.

The amplification and subsequent detection of the amplified product were performed according to the manufacturer's instructions. In brief, the PCR product was denaturalized and hybridized in a 1.5 mL tube with a low-density array that had spotted triplicate oligonucleotide probes for 35 HPV types, internal genomic DNA probes (CFTR human gene of 892 bp), and control plasmids (1202 bp) to assure both the PCR procedure and the integrity of the DNA. A colorimetric detection was performed and the signal was measured in an array scanner. The Genómica technique was performed at the Centre University Hospital of Asturias.

SPF_{10}-$LiPA_{25}$ PCR System, Version 1

The DNA was extracted by treating the FFPE tissue with 250 μL of freshly prepared Proteinase K solution (1 mg/mL) at 56°C for 18 hours to overnight on a heating block. After the digestion time, the Proteinase K was heat deactivated during 10 minutes at 95°C.[8] SPF_{10} PCR, which amplifies a 65 bp fragment, was performed using 10 μL of the DNA extract in a final reaction volume of 50 μL. All samples were run at a 1:10 dilution to reduce possible inhibition during PCR. The amplified PCR products were analyzed by DEIA for the detection of HPV DNA. The DEIA assay uses a probe hybridization technique that contains a mixture HPV-specific probes that can recognize around 54 mucosal HPV genotypes. In brief, 10 μL of the PCR product was denatured using NaOH and after subsequent incubation using digoxigenin-labeled HPV-specific probes, the plates were washed using an automatic plate washer. Finally, an antidigoxigenin alkaline phosphatase substrate was added to the wells and after a short incubation time, the optical densities were determined. Optical densities (OD450) were read on a microtiter plate reader (Biotek).[9] For HPV DNA genotyping, 10 μL of the PCR product, identified previously by DEIA as positive, were subsequently analyzed by a reverse hybridization assay on a strip format ($LiPA_{25}$, version 1)[8] (Labo Biomedical Products, Rijswijk, The Netherlands). $LiPA_{25}$ can detect 25 high-risk and low-risk HPV types (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74). The sequence variation of the SPF_{10} primers can recognize these different HPV genotypes, except for types 68 and 73, as the interprimer regions of these 2 types are identical and cannot be determined separately by this test. Purple band on a probe site on the strip indicates positive hybridization. More than 1 band may be visualized for certain HPV types as it may have a combination of probe sites or confirmation probes. Specimens that were HPV DNA positive on the DEIA but did not hybridize with any of the 28 probes on the LiPA were coded as HPV type X (uncharacterized type). SPF_{10} PCR, DEIA, and $LiPA_{25}$ assay (version 1) were performed at ICO.

An inhouse β-globin PCR that amplifies a 115-bp fragment of the human β-globin gene was used to determine DNA quality on these samples as no internal DNA control is included with the SPF_{10} technology. The amplified product was run on a 3% agarose gel stained with SYBR safe DNA Gel Stain (Invitrogen) to visualize the bands.

Statistical Analysis

The data analysis was performed with the statistical package SPSS 13.0. In this study, agreement between the 2 assays was performed by comparing concordance values in positivity using the κ statistics. The McNemar test for matched pair data was used for assessing unequal distribution of discordant results.

The value of the κ index was interpreted as described by Landis and Koch, 1977[10] ($\kappa < 0.20$: very low concordance; κ 0.21 to 0.40: low concordance; κ 0.41 to 0.60: moderate concordance; κ 0.61 to 0.80: good concordance; $\kappa > 0.80$: excellent concordance).

Furthermore, analysis for the overall agreement, taking into account not only general positivity but also HPV types, was performed to assess the exact agreement between the 2 techniques. The agreement was classified as complete, partial, or disagreement according to the definitions below:

"Complete agreement" is considered when both techniques detected the same viral type/types in a given sample.

"Partial agreement" is when the agreement was not total but matched at least one of the HPV types detected in both tests.

"Complete Disagreement" is when there was no match on the types that were detected with either technique.

For all statistical analysis, the statistical significance was set at $P < 0.05$.

The sensitivity and specificity of Genómica technique for the detection of HPV in paraffin-embedded samples were analyzed taking the $SPF_{10}/LiPA_{25}$ technique as the reference for HPV detection in FFPE samples into consideration. The sensitivity was calculated as true-positive/true-positive + false-negative, whereas the specificity was calculated as true-negative/true-negative + false-positive.

Ethical Issues

The specimens were undisclosed (without name and/or original medical record number). All determinations were subject to the rules and regulations of the Data Protection Act. The procedures met all legal requirements and were approved by the Clinical Trials and Research Committee from the Central University Hospital of Asturias.

RESULTS

A total of 235 paraffin blocks from women with cervical cancer were included in this study. Five of the samples were found β-globin negative and HPV negative with the SPF_{10} PCR/$LiPA_{25}$ technique. In addition to these 5 samples, 9 other samples were found negative for both the human CFTR gene and HPV with Genómica technique. Therefore, a total of 14 samples were considered unsuitable and were excluded from the concordance analysis.

Concordance Analysis

As shown in Table 1, of the 221 samples included in the study, 160 were found positive for HPV DNA by both techniques (72.4%).

HPV prevalence determined by Genómica technique was 75.1% (166 of the 221 samples were positive), whereas the HPV prevalence by $SPF_{10}/LiPA_{25}$ was 91.9% (203 of the 221 samples were positive), giving an overall agreement of 77.8%, for HPV detection between the Genómica technique and $SPF_{10}/LiPA_{25}$. It was determined to be statistically significant but showed "low concordance" (κ index = 0.24, $P < 0.001$).

TABLE 1. Comparison of Results Obtained by both SPF_{10}/$LiPA_{25}$ and Genómica Techniques

	$SPF_{10}/LiPA_{25}$		Total No.
GENOMICA	Positive	Negative	Samples
Positive	160*	6	166
Negative	43	12	55
Total No. samples	203	18	221

*Three of the 160 samples positive with both Genómica and $SPF_{10}/LiPA_{25}$ techniques were:
Genómica: HPV 61—SPF_{10}/LiPA25: HPV 31
Genómica: HPV 61—$SPF_{10}/LiPA_{25}$: HPV 18, 52, and 68 or 73
Genómica: HPV 61—$SPF_{10}/LiPA_{25}$: HPV 16
Should be noted that HPV 61 is not detectable with $SPF_{10}/LiPA_{25}$ technique.

Forty-three samples (19.5%) were found positive by $SPF_{10}/LiPA_{25}$ but negative by Genómica technique. However, 6 samples (2.7%) that were found positive by Genómica were found negative by $SPF_{10}/LiPA_{25}$. The difference was statistically significant (McNemar test; $P < 0.001$).

The validity parameters of the Genómica technique for the detection of HPV in paraffin-embedded samples were analyzed taking into account that the $SPF_{10}/LiPA_{25}$ technique is thought to be a "gold standard" method, as mentioned previously in Methods section. Thus, the Genómica technique had a sensitivity of 78.8% (160/(160 + 43)) and a specificity of 66.7% (12/(12 + 6)).

Regarding HPV positivity, we found some differences by histologic diagnosis, with the proportion of HPV-positive cases by $SPF_{10}/LiPA_{25}$ (being considered "the most sensitive PCR assay" for the detection of HPV in these type of samples) in decreasing order of 92.8% in squamous cell carcinomas, 85.7% in adenosquamous, 73.0% in adenocarcinomas, and 40% in other histologies. In Table 2, more details are shown on the HPV-positive rate by histologic subtypes in adenocarcinomas.

TABLE 2. Prevalence of HPV in Adenocarcinomas According to Histologic Subtype

	Analyzed cases (N)	Positive HPV cases (N)	HPV Detection (95% CI)
Mucinous Adenocarcinoma	26	21	80.8 (60.6-93.4)
Endocervical	18	17	94.4 (72.7-99.9)
Intestinal	3	2	66.7 (9.4-99.2)
Villoglandular	2	1	50.0 (1.3-98.7)
Signet Ring Cell	1	1	100.0 (2.5-100.0)*
Minimal Deviation	1	0	0.0 (0.0-97.5)*
NOS	1	0	0.0 (0.0-97.5)*
Clear cell adenocarinoma	4	1	25.0 (0.6-80.6)
Adenocarcinoma NOS	3	3	100.0 (29.2-100.0)*
Serous	3	1	33.3 (0.8-90.6)
Endometrioid	1	1	100.0 (2.5-100.0)*
Total	37	27	73.0 (55.9-86.2)

CI indicates confidence interval; HPV, human papilloma virus; NOS, not otherwise specified.
*One-sided, 97.5% confidence interval.

TABLE 3. Concordance Between Single or Multiple Infections Using Both Techniques: $SPF_{10}/LiPA_{25}$ and Genómica

	$SPF_{10}/LiPA_{25}$		
GENOMICA	Single Infection	Multiple Infection	Total No. Samples
Single Infection	95	9	104
Multiple Infection	51*	5	56
Total No. samples	146	14	160

*Of the 51 samples that are discordant for single and multiple infections:
10 samples by Genómica were: HPV 16 and 61—but by $SPF_{10}/LiPA_{25}$:HPV 16
4 samples by Genómica were: HPV 16 and 84—but by $SPF_{10}/LiPA_{25}$: HPV 16
It is important to note that neither HPV 61 nor HPV 84 are detectable by $SPF_{10}/LiPA_{25}$ technique.

HPV Detection of a Single or Multiple Infections

In this study, the Genómica technique detected a higher percentage of multiple HPV infections, 35% (58 multiple infections from the 166 positive samples tested by Genómica) in contrast to 8.9% by $SPF_{10}/LiPA_{25}$ (18 multiple infections of the 203 positive samples by $SPF_{10}/LiPA_{25}$). To calculate the concordance index for the detection of single or multiple infections between both techniques, only the samples with positive results for both $SPF_{10}/LiPA_{25}$ and Genómica techniques (N = 160) were considered. The overall agreement between the 2 techniques was 62.5%, as shown in Table 3. The result was not statistically significant, and showed very low concordance (κ index = 0.003, $P > 0.05$). Fifty-one samples detected as multiple HPV infections by Genómica were detected as single HPV infection by $SPF_{10}/LiPA_{25}$. In contrast, 9 samples detected as multiple HPV infections by $SPF_{10}/LiPA_{25}$ were detected as single HPV infection by Genómica (McNemar; $P < 0.001$).

HPV-type Specific Concordance

The concordance of specific HPV-type detection between the 2 techniques is shown in Table 4. The samples that were found positive using either of the 2 techniques were included in this analysis, taking into consideration only those cases where the viral types were detectable by both techniques (eg, HPV 61 and 84 that are included in Genómica, cannot be detected by $SPF_{10}/LiPA_{25}$).

Table 5 describes the discordant and partially concordant cases for both techniques, again taking into

TABLE 4. Type-specific Concordance between $SPF_{10}/LiPA_{25}$ and Genómica Technique, with One or the Either Technique Being Positive for HPV

	N	%
Complete agreement	102	49.5
Partial agreement	45	21.9
Complete disagreement*	59	28.6
Total	206	100.0

Samples were considered for analysis when HPV was positive for at least one of both techniques. HPV indicates human papilloma virus.
*Of the 59 discordant samples, 43 are negative by Genómica technique but positive with $SPF_{10}/LiPA_{25}$, and 6 samples are positive by Genómica but negative by $SPF_{10}/LiPA_{25}$ technique.

TABLE 5. Description of Discordant and Partially Concordant Cases

Discordant			Partially Concordant		
N	GENOMICA	$SPF_{10}/LiPA_{25}$	N	GENOMICA	$SPF_{10}/LiPA_{25}$
16	Negative	16	5	16, 18	16
5	Negative	45	5	16, 58	16
4	Negative	18	3	16, 53	16
4	Negative	33	2	11, 16	16
3	Negative	31	2	16, 33	33
2	Negative	39	1	16	16, 51
1	Negative	52	1	16	16, 45
1	Negative	51	1	16	16, 52
1	Negative	35	1	16	16, 53
1	Negative	X	1	16	16, 56
1	Negative	16, 18	1	16	16, 33
1	Negative	68 or 73	1	18	18, 52
1	Negative	16, 52	1	45	35, 45
1	Negative	11, 39	1	11, 16	11, 16, 35
1	Negative	35, 68, or 73	1	16, 33	16
2	16	Negative	1	16, 35	16
1	18	Negative	1	16, 35	35
1	52	Negative	1	16, 39	39
1	16	Negative	1	16, 45	16
1	18	Negative	1	16, 45	45
1	16	52	1	16, 51	16
1	16	33	1	16, 59	59
1	16	X	1	16, 66	16
1	16	68 or 73	1	18, 58	18
1	16	45	1	33, 58	33
1	58	56	1	35, 58	35
1	16, 58	45	1	33, 58	58
1	16	18	1	45	16, 45
1	6, 31	16	1	58, 66	16, 58
1	33, 59	35	1	11, 16	16
			1	16, 58	16
			1	11, 18, 58	18
			1	18, 33	16, 33

account only those cases that were positive for at least one of the techniques but detectable by both $SPF_{10}/LiPA_{25}$ and Genómica techniques.

Figure 1 shows the distribution of HPV types detected by $SPF_{10}/LiPA_{25}$ and by Genómica as single infections of the 221 samples included in the study. In both techniques, HPV 16 and 18 were found to be the most frequent types. It can also be inferred that the $SPF_{10}/LiPA_{25}$ technique detected more HPV infections as single infections than Genómica.

Genómica technique detected a larger number of multiple infections than $SPF_{10}/LiPA_{25}$, as reported in Table 3. Figure 2 shows the distribution of the multiple HPV types individually, but were found in combination of 2 or more types. It can be seen that HPV 16 was still the most frequently detected viral type in either of the techniques used.

DISCUSSION

In this study, we have analyzed 2 different techniques that are used for HPV DNA detection (Papillomavirus Clinical Arrays and SPF_{10} PCR-$LiPA_{25}$). Our results have translational implications for clinical practice when we aim to reach maximum sensitivity.

This is the first large study comparing Genómica and SPF_{10} PCR-$LiPA_{25}$ techniques for detection of HPV in FFPE samples. A large number of samples were collected over a long period of time and were analyzed in this study. However, it is important to note that there was no heterogeneity in the HPV detection by years since diagnosis (data not shown).

The difficulty in performing the procedures was similar for both methods. However, the interpretation of results was, perhaps, less subjective with Genómica than for $SPF_{10}/LiPA_{25}$. This is because Genómica relies on a computer to provide the readout of the results, whereas for the $SPF_{10}/LiPA_{25}$, the results depend on visual interpretation of the test strips by the user. The risk of contamination was also similar in both techniques, as it is for all PCR-based technologies. A strict compliance with specific protocols for preventing possible contamination and good laboratory practice were followed to minimize possible erroneous results.

We evaluated the concordance of the results of Genómica in the detection of HPV in FFPE with histopathologic diagnosis of ICC compared with SPF_{10} PCR-$LiPA_{25}$, the latter being considered "the most sensitive PCR assay" for the detection of HPV in these type of samples. We have observed and confirmed that the SPF_{10} PCR-$LiPA_{25}$ technique was more sensitive for detecting HPV DNA in FFPE tissues than the Genómica technique. In fact, the prevalence of HPV detected with the SPF_{10} PCR-$LiPA_{25}$ technique was of 91.9% versus 75.1% with Genómica.

It has been well established that archived paraffin-embedded tissues are an important source of material for performing retrospective and clinical studies. However, the process of fixation of the samples can significantly affect the quality of nucleic acids[11]; therefore, PCR primers that amplify relatively long DNA segments, such as the MY09/11 (used with Genómica technique) and GP5+/6+, may not be the most appropriate assay to use for analyzing FFPE samples as they are subject to DNA damage. In contrast, the use of primers such as that of SPF_{10} primers, which amplify short DNA fragment size, could considerably improve the sensitivity of the HPV DNA detection both in fresh samples and FFPE tissues.[8]

In agreement with our observations, several authors have reported that the fixation time and type of fixative used can greatly affect the quality of extracted nucleic acids, DNA degradation being the most common damage.[9,12,13,14] It has been reported that longer than 24 hours of fixation time in formalin, or using a fixative other than the 10% buffered formalin, could affect the conditions of DNA as well. In such cases, the amplification of the sample DNA may not be optimal as the primers used in the test may not be able to recognize the target DNA.[6,14] It has also been reported that DNA samples extracted from paraffin-embedded tissue may contain traces of the paraffin, resulting in inhibition during PCR amplification.[14]

Furthermore, several authors have reported relatively low rates of HPV detection in FFPE material using MY09/11

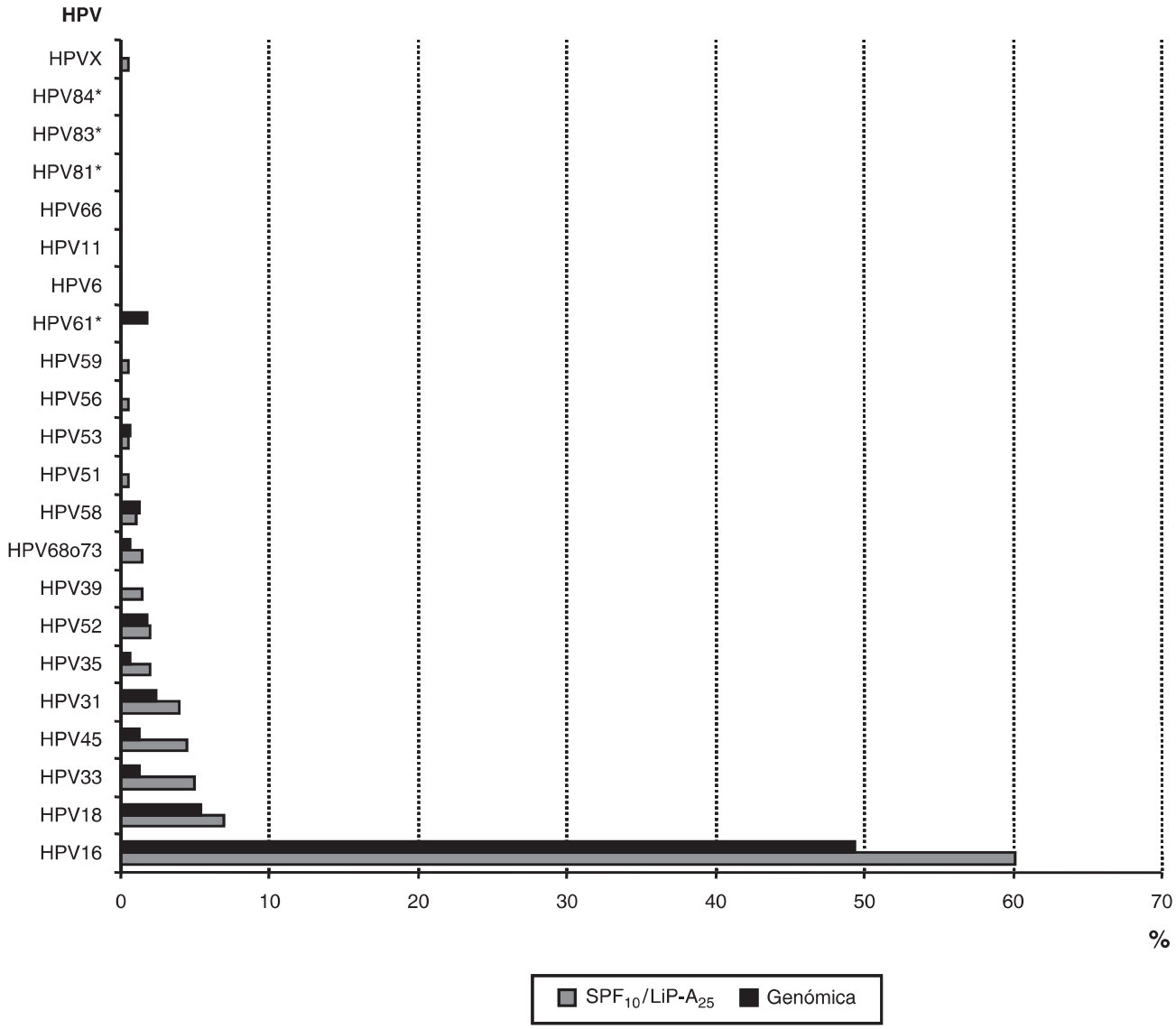

FIGURE 1. Distribution of human papilloma virus (HPV) types by Genómica and $SPF_{10}/LiPA_{25}$ techniques within single infections. *HPV 61, 81, 83, and 84 are detectable only by the Genómica technique but not by $SPF_{10}/LiPA_{25}$.

consensus primers.[6,13,15] This may be because the fixation causes strong cross-linkage and the DNA can break down during the isolation process; the amplification of sequences larger than 200 bp fragments becomes more difficult.[4,6,12] In contrast, the fragments of 65 bp amplified using SPF_{10} primers are probably less difficult to detect than the fragments of 450 bp amplified with MY09/11.

DNA extraction method itself may also play a role. Although some researchers use phenol extraction,[12] our experiments, in agreement with other authors,[16,17] show that optimal results are obtained without treatment with phenol.

In our study, squamous cell carcinomas had the highest percentage of HPV positivity, this percentage being relatively low among the adenocarcinomas. We carried out an analysis of the positivity of each subtype of adenocarcinoma, which showed a low prevalence of HPV DNA in cases of mucinous adenocarcinoma subtype "villoglandular," "minimal deviation" and "NOS," and the "clear cell adenocarcinoma" and "serous." In this connection, it has been previously described in the literature that 17.0% of cases with a diagnosis of "minimal deviation" are related to genetic changes such as Peutz-Jeghers syndrome, or in the case of "clear cell adenocarcinomas" it is well known to its relationship in the past with exposure to synthetic estrogen (diethylstilbestrol) in young women.[18]

We observed that Genómica technique detects a higher percentage of multiple HPV infections (35.0% vs. 8.9%) with a concordance of 62.5%. In a previously published study[19] describing HPV distribution in precancerous lesions in fresh samples (nonparaffin samples), a high percentage of multiple HPV infection (22.0%) was reported, which is in concordance with our study.

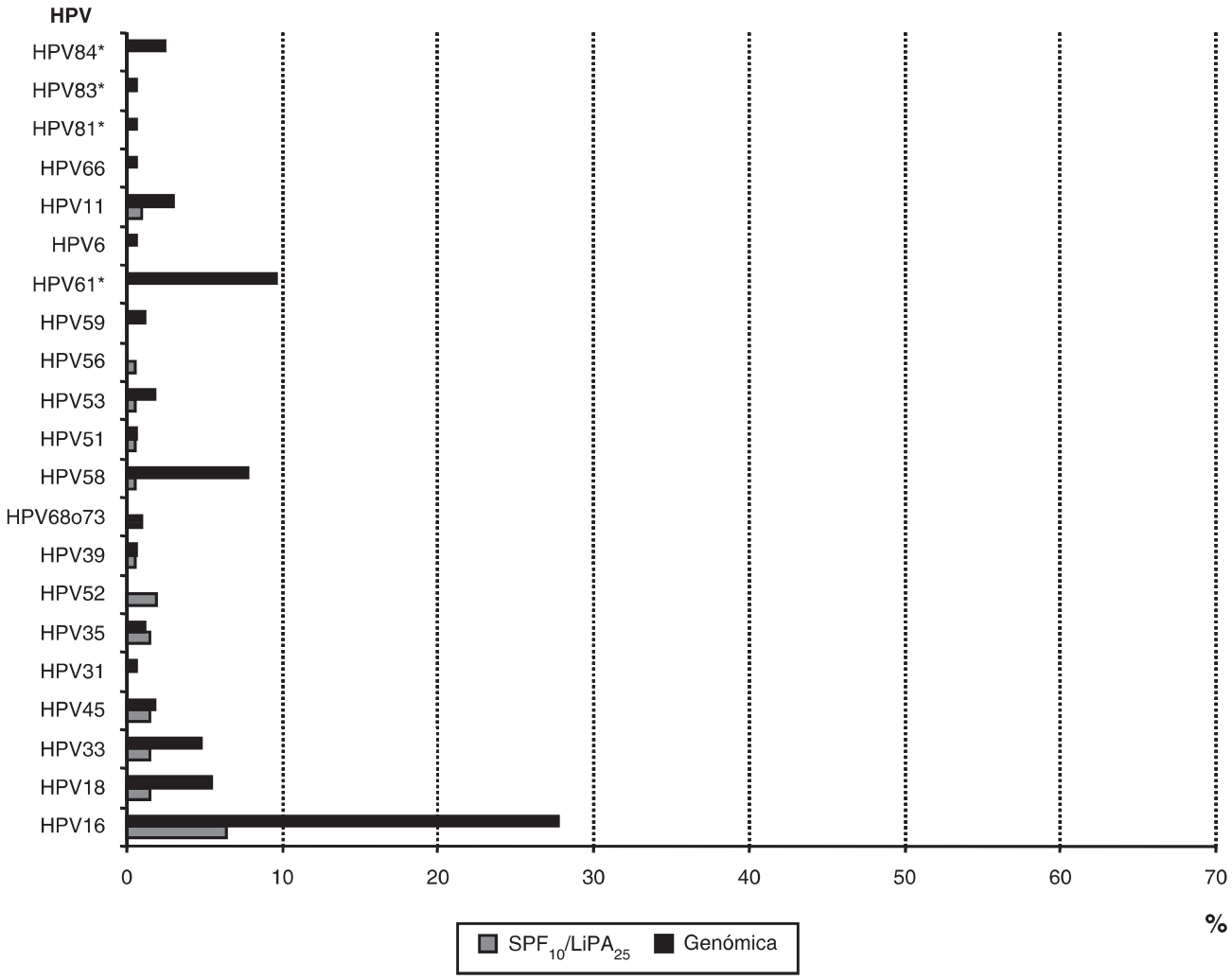

FIGURE 2. Distribution of human papilloma virus (HPV) types by Genómica and $SPF_{10}/LiPA_{25}$ techniques within multiples infections. *HPV 61, 81, 83, and 84 are detectable only by the Genómica technique but not by $SPF_{10}/LiPA_{25}$.

The difference found between the 2 techniques regarding the detection of multiple HPV infections could be due to the design of the primers. Genómica uses degenerate primers to detect a broad spectrum of HPV genotypes but lack the specificity in determining the genotype.[20] These primers were designed to amplify HPV types present in a PCR reaction indiscriminately. Therefore, cross-reaction of primers and DNA may occur, reducing the specificity of the detection of viral genotypes.[21] Otherwise, several authors have described high specificity of the SPF_{10} PCR-$LiPA_{25}$ technique in determination of the different HPV genotypes.[8] The same authors have found 4.4% of prevalence of multiple HPV infections in ICC analyzed with the SPF_{10} PCR-$LiPA_{25}$ technique in FFPE tissues.[8]

A possible explanation for the discrepancy in the HPV genotypes found between the 2 methods could be due to low viral load in the sample. That is to say, SPF_{10} PCR-$LiPA_{25}$, which can amplify very small fragments of the viral L1 region allowing remarkably sensitive detection of a broad spectrum of HPV genotypes even at low viral load[8,9] was indeed able to amplify; however, the small fragments went undetected by Genómica. Another possible explanation could, again, be due to the design of the primers. MY09/11 primers used in Genómica technology are degenerate consensus primers, which allow amplification of broad spectrum of HPV genotypes but with various levels of sensitivity among different HPV types.[21]

Despite progress in understanding the association between HPV and cervical lesions using molecular techniques, obvious limitations still exist, particularly with respect to variability in the sensitivity and specificity for the detection of specific types of HPV.[19] False-negative results can be common for certain HPV types as a result of lower sensitivity of the primers used.[2] Both methods use consensus primer sets from the viral L1 region; however, SPF_{10} primers are not degenerate primers, and therefore they can be more specific in detecting certain HPV types.[9]

We conclude that the SPF_{10} PCR-$LiPA_{25}$ System may be more appropriate technology than Genómica to determine the presence of HPV DNA and genotype in FFPE samples.

ACKNOWLEDGMENTS

The authors thank Genómica SAU (Tres Cantos, Madrid, Spain) for assistance and financial aid, Wim Quint from DDL Diagnostic Laboratory (Voorburg, The Netherlands) and ICO laboratories for handling and analysis of specimens.

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