



Research Article

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# New Quantitative PCR Technology for Screening Human Papillomavirus Test – Identification of 25 Genotypes: A Molecular Epidemiology and Cross-Sectional Study

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## Abstract

### Background

A kit for screening HPV-genotypes based on the prevalence of Mexican population there isn't designed

### Objective

To design a new molecular test for human papillomavirus (HPV) screening.

### Methods

Molecular epidemiology and cross-sectional study. Multicenter from referral centers. 326 women were included. The primary outcome was designed of a kit for screening HPV-genotypes based on the prevalence found in population. Oligonucleotides for different genotypes were designed. The conditions for quantitative PCR and the pools for low- and high-risk (LR and HR) of HPV-genotypes were established.

### Results

With new molecular test we detected 30 HPV-genotypes. A kit with the 25 most prevalent genotypes distributed was designed: four HR genotypes (16, 18, 31, and 59), three LR genotypes (6, 53, and 61), and two pools collectively detected. One pool of another 10 HR genotypes: 33, 35, 39, 45, 51, 52, 56, 58, 66, and 68, and finally a pool of 8 LR genotypes: 11, 40, 43, 44, 55, 70, 73, and 81. The sensitivity of the new molecular test was 97.9% and its specificity was 100% based on the sequencing results.

### Conclusion

Our findings provide evidence that the new molecular test for screening of HPV can serve as a better triage test.

**Keywords:** Human Papillomavirus; Pap Test; Quantitative PCR Technology

## Introduction

It is well established that cervical screening based on the identification of low- and high-risk (LR, HR) human papillomavirus (HPV) genotypes can identify more than 95% of precancerous cervical lesions [1-3]. There is evidence that testing for HPV E6/E7 viral messenger RNA (mRNA) is more specific than molecular tests using amplification of target DNA [1,4-5]. However, present technics cannot identify all HPV-genotypes or have a relatively low specificity for cervical intraepithelial neoplasia [CIN] grade 2 or 3 [CIN2+]) [1,6-7]. The use of primary HR-HPV screening in the cervical cancer programmes has an acceptable sensitivity, specificity, and positive predictive value (PPV), but its sensitivity limits its utility for triage. 1 HPV-genotypes 16 and 18 are the genotypes with the highest potential for oncogenesis, and genotypes 31, 33, 45, 52, and 58 explain about 18.5% of malignant neoplasms [1,8-10]. Today, vaccines (tetravalent and nonavalent) have approximately 79-89% of protection again HPV infection (even cross protection for 31, 33 and 45 genotypes), and 85-95% of potential prevention for vulvar, vaginal, and anal cancers related to this virus [14]. The aim of the current study was to design a new molecular test (care GENETM-HPV test) for HPV screening and compared versus "COBAS" test and Pap test.

## Materials and Methods

A multicenter, cross-sectional, and molecular epidemiology study was designed. The subtypes of low- and high-risk HPV genotypes more prevalent were identified in eligible population. A

cohort of patients was subjected to two molecular diagnostic tests and Pap test (as the reference test). The data collection was carried out according to a prospective cohort design. All specimens were collected from all levels of health care. We compared the HPV-genotypes identified by care GENETM-HPV test with COBAS system test and Pap test. A diagnostic test evaluation was estimated.

### Molecular and Sampling Procedures for Detection of Human Papillomavirus Infection

Two tests were used for a molecular analysis:

- i. COBAS system test.
- ii. care GENETM-HPV detection kit-I (care GENETM-HPV test).

The COBAS system uses an amplification of target DNA by the technics of PCR and nucleic acid hybridization, for detecting a group of 14 HR-HPV genotypes in a single analysis: genotypes 16 and 18 (HPV-16 and HPV-18), and a pool of other HR-HPV genotypes: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 [10]. The new test "care GENETM-HPV detection kit-I" employs TaqMan probe qPCR technology for the amplified HPV-DNA detection. The kit was initially designed to detect 33 genotypes, of which 22 are high risk and 11 are low risk. In preliminary tests, 29 genotypes out of 33 possible were detected. Finally, we designed a kit with the 25 most prevalent genotypes, distributed as follows: four HR genotypes (16, 18, 31, and 59), three LR genotypes (6, 53, and 61), and two pools collectively detected. One pool of another 10 HR genotypes: 33, 35,

39, 45, 51, 52, 56, 58, 66, and 68, and finally a pool of 8 LR genotypes: 11, 40, 43, 44, 55, 70, 73, and 81 from crude cervical scraping. Probes for HR-HPV genotypes specific sequences were labelled with the fluorophore Fam (10 HR-HPV genotypes), VIC (HPV-16), Cy5 (HPV-18), ROX (HPV-31), and Quasar705 (HPV-59). Probes for LR-HPV genotypes specific sequence were labelled with the fluorophore FAM (eight LR-HPV genotypes), HEX (HPV-6), Cy5 (HPV-53), and Quasar 705 (HPV-61). The probe for internal control (IC) was labelled with fluorophore ROX (Table S1). The probe specifically binds to target sequences and fluorescence increases due to

the separation of fluorescent dye and quencher by Taq polymerase exonuclease activity during amplification. This test consists of two reaction mixture (with Taq polymerase, dNTP mixture, UDG, and reaction buffer), Mix H (4x Primer/Probe Mixture with HR-HPV specific primer and TaqMan probe), Mix L (4x Prime/Probe Mixture with LR-HPV specific primer and TaqMan probe), PC1 (Positive control for Mix H), PC2 (Positive control for Mix L), and Nuclease-free Water. HPV type-specific signals are detection from fluorescent dye on TaqMan probe, Ct (Cycle threshold) value from qPCR machine represents the relative degree of infection.

**Table S1:** Fluorescent Reporter.

	High Set	Low Set
Dye	Genotypes	
Fam	33, 35, 39, 45, 51, 52, 56, 58, 66, 68	11, 40, 43, 44, 55, 70, 73, 81
Vic/Hex	HPV-16	HPV-6
Cy5	HPV-18	HPV-53
Quasar 705	HPV-59	HPV-61
Rox	HPV-31	IC

HVP: human papillomavirus.

### Sample Preparation, Storage Condition, and Setting Analysis and Cut Off Value by Qpcr Technology

The sample is human genomic DNA prepared by a genomic DNA isolation kit (QIAamp DSP DNA mini kit or equivalent). Isolated genomic DNA has to be stored below -20°C (sealed). It is stable and can be used for 12 months from date of manufacture. The Real-time

PCR reaction Mix and PCR amplification steps were done according to manufacturer's recommendations (Table S2). The analysis setting and acceptance criteria employed also were the recommended by fabricant (Positive: Ct value of signal is 45 or less. Negative: Ct value of signal is not detected according to the fluorescence results, (Table S3)).

**Table S2:** PCR amplification steps.

Stage	UDG activation	Pre-denaturation	Amplification	
Temp	50°C	95°C	95°C	57 °C*
Time	4 min	10 min	20 seg	1 min
Cycles	1	1	45	

\*Fluorescence measure at 57°C step of amplification stage.

**Table S3:** Acceptance criteria.

	High set	Low set
Fluorescence	Interpretation	
Fam, <45	Positive for other high-risk HPV-genotypes	Positive for other low-risk HPV-genotypes
Fam, N/A	Negative for other high-risk HPV-genotypes	Negative for other low-risk HPV-genotypes
VIC/HEX, <45	Positive for HPV-16	Positive for HPV-6
VIC/HEX, N/A	Negative for HPV-16	Negative for HPV-6
Cy5, <45	Positive for HPV-18	Positive for HPV-53
Cy5, N/A	Negative for HPV-18	Negative for HPV-53
Quasar705, <45	Positive for HPV-59	Positive for HPV-61
Quasar705, N/A	Negative for HPV-59	Negative for HPV-61

ROX, <45	Positive for HPV-31	HPV genotype result valid
ROX, N/A	Negative for HPV-31	HPV genotype result invalid

HR: High-risk pool; (Others 10 genotypes): genotype 31, 35, 39, 45, 51, 52, 56, 58, 66, and 68.; LR: Low-risk pool; (Others 8 genotypes): genotypes 11, 40, 43, 44, 55, 70, 73, 81.

**Table 1:** Comparison and evaluation of different diagnostic molecular tests for detection of human papillomavirus.

Reference	Comparison Test	DP	RP / CN	RN / CP	DN	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	AUC $\pm$ SE (95% CI)
Pap test	Cobas	3	169	2	152	1.74 (0.45-5.42)	98.70 (94.90-99.77)	60.00 (17.04-92.74)	47.35 (41.80-52.97)	0.502 $\pm$ 0.032 (0.439-0.565)
Pap test	careGE-NETM-HPV test	57	115	42	112	33.14 (26.27-40.77)	72.73 (64.86-79.44)	57.58 (47.24-67.32)	49.34 (42.68-56.02)	0.529 $\pm$ 0.032 (0.467-0.592)
Cobas	careGE-NETM-HPV test *	5	0	94	227	100.00 (46.29-98.13)	70.72 (65.36-75.57)	5.05 (1.87-11.94)	100.00 (97.93-99.96)	0.854 $\pm$ 0.041 (0.773-0.934)

DP: Double positive; RP: Reference-positive; CN: Comparison-negative; RN: Reference-negative; CP: Comparison-positive; DN: Doble negative; PPV: Positive predictive value; NPV: Negative predictive value; %: percentage, CI: Confidence interval; AUC: area under curve.

Parameters for calculated the standard error of the area under curve, assumption non-parametric distribution. \* Statistical Significance <0.01

## Outcome, Study Variables, Statistical Analysis and Ethical Considerations

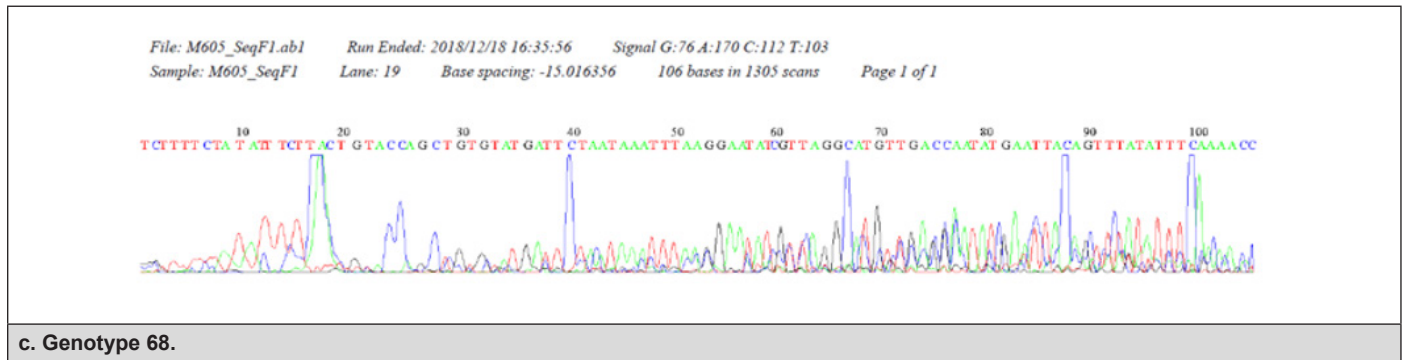
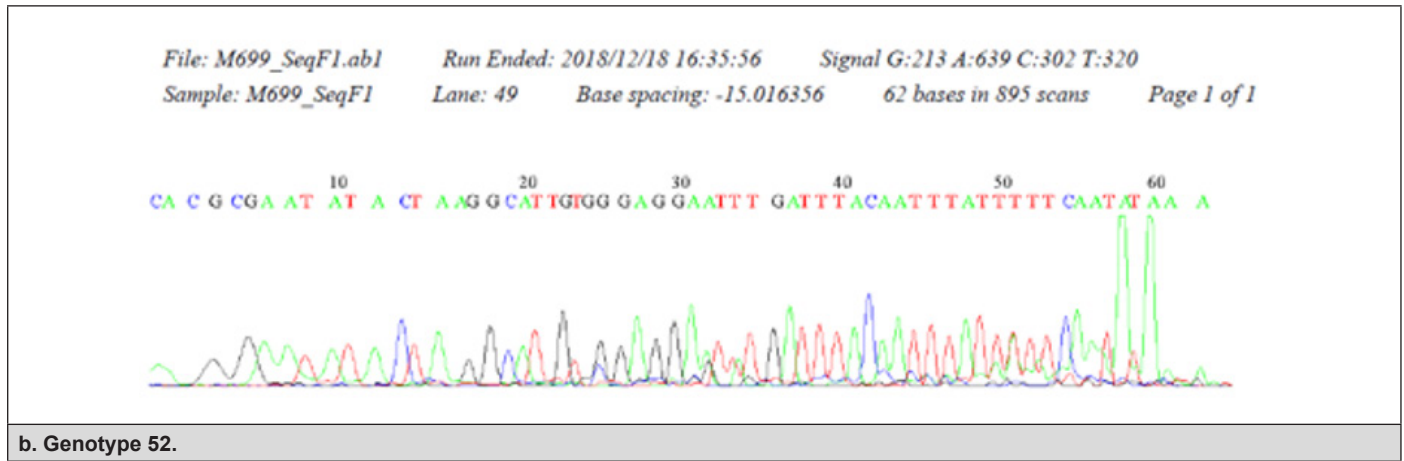
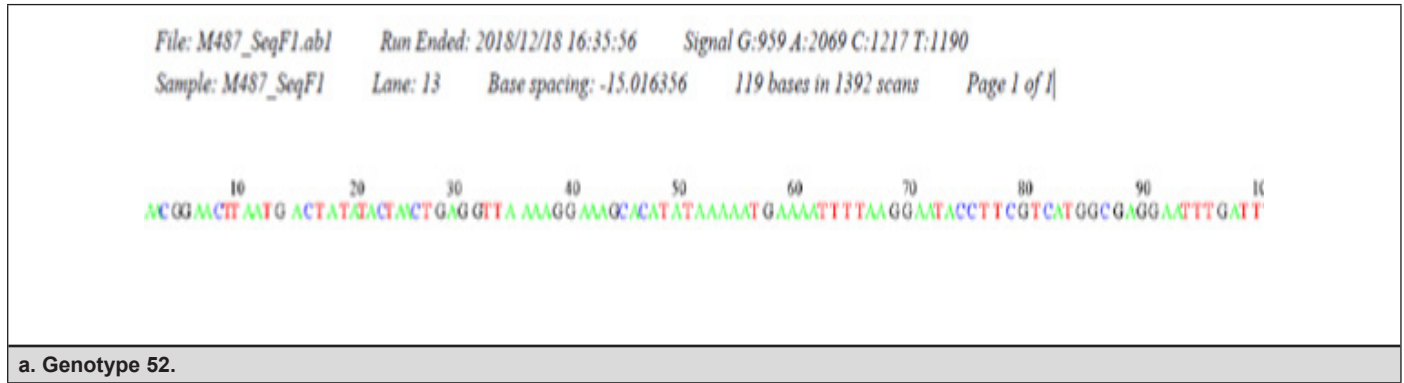
The primary outcome of the present study was detection of HPV-genotypes. We included age, HPV genotypes and place of residence as study variables. All information was included in a database. Categorical variables were described by both the absolute frequency and percentage with the corresponding 95% confidence interval (95% CI). All categorical variables were compared using chi square test or Fisher exact test as appropriate. The continuous variables were described by mean and standard deviation (SD). To calculate sensitivity, specificity, and predictive positive and negative value, 2  $\times$  2 tables were generated using results from the screening visits and HPV molecular tests. The area under the curve (AUC) was also calculated by a receiver operating characteristic curve (ROC) analysis. Analyses were performed using the total population. All statistical tests were two-sided. A p value < 0.05 (2- sided testing) was considered significant. The Research Ethics Committee, the Research Committee, and the Biosecurity Committee from ISSSTE approved the protocol.

## Results

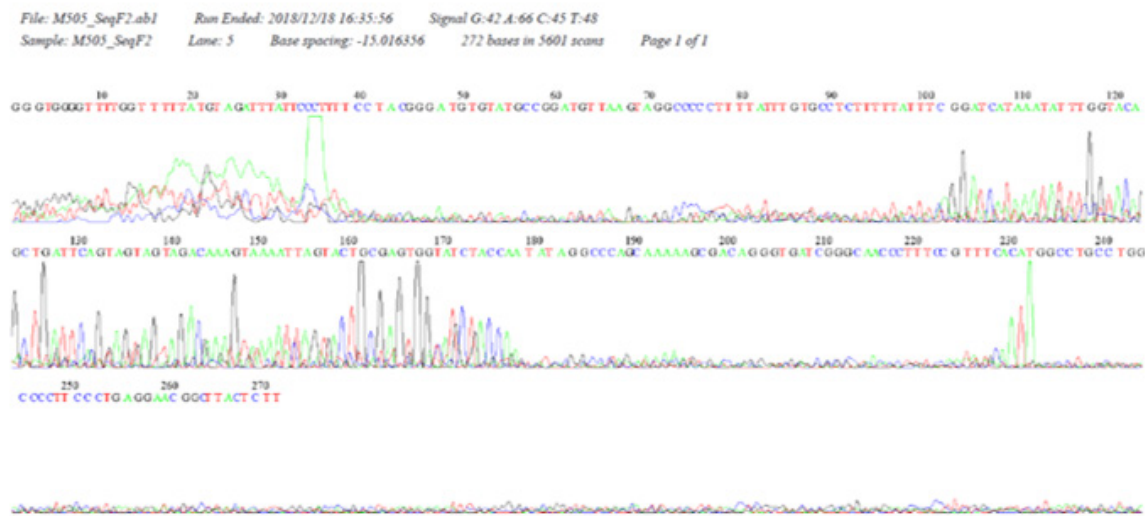
We included a total of 326 women patients with their corresponding samples. The average age of the patients was 42 [1] years old (SD= 11.2, min= 17, max= 84, range= 67, median= 45, interquartile range= 35-51). We include patient's samples from 6 states (Chiapas, Ciudad de México, Estado de México, Hidalgo, Querétaro, and Tlaxcala). We observed more that 50% samples have low

(18.4%) and high (34.4%) grade squamous intraepithelial lesions (LSIL, HSIL). Three samples (from the group of 60 samples with HSIL) had infection with HPV-16 (n= 2) and HPV-18 (n= 1). However, with care GENETM-HPV test we detect a total of 99 positive samples to 30 HPV-genotypes (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 55, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 73 and 81). The most prevalence genotypes were genotypes 6 (13.2%; 95% CI 9.5-16.9) and 16 (4.6%; 95% CI 2.5-7.1), followed by genotypes 53, 58, 59 (2.8%; 95% CI 1.2-4.6), 31, 61 (2.5%; 95% CI 0.9-4.3), 18, 51, 56 (2.1%; 95% CI 0.6-4.0), and 33 (0.3%; 95% CI 0.0-0.9). Moreover, 35.7% of samples with LSIL (NIC-1) by Pap test are positive to unique (65%) or multiples (35%) infection with HPV-genotypes. Similarly, 28.3% of samples with HSIL (NIC-2) by Pap test are positive to unique (88.2%) or multiples (11.8%) infection with HPV-genotypes (p = 0.1089). Even, 26.2% of negative samples by Pap test are positive to unique (82.4%) or multiples (17.6%) infection with HPV-genotypes. Of 99 positive samples to HPV-genotypes, 74.7% (n= 74) have infection with the presence of 1 genotype of HPV, and 25.3% (n= 25) have co-infection with two or three HPV-genotypes. Three patients had co-infection with three different HPV-genotypes (<18, 51, 56>, <6, 31, 69>, and <18, 31, 39>). The most frequent co-infection observed was the combination between genotype 16, and 61 (12%, three of the 25 patients). From care GENETM-HPV tests, we observed 40 negative samples. Twenty out of the forty-qPCR negative samples showed 150 bp amplicon. Twenty samples with band were sequenced and get the following results:

- a. Four Samples were turned out as HR-HPV (Figure 1).
- b. Eight samples were turned out as LR-HPV.
- c. No sequencing results were obtained from the rest eight samples and were excluded for the accuracy calculation.







#### d. Genotype 68.

**Figure 1:** Sequencing to detect samples positive to HPV-genotype 52, and 68.

Four Samples were turned out as HR-HPV from COBAS system tests. Those four samples are different from the four previous samples of sequencing results. The difference observed might come from the different sensitivity of the two methods. The eight samples excluded for the accuracy calculation in sequencing turned out negative in COBAS system tests. The percentage of infected samples with HPV genotype 33 was 2.2% (5/255), for HPV genotype 31 was 11.5% (12/104), for HPV genotype 58 was 8.6% (22/255) and for HPV genotype 11 was 0.4% (1/255). On the other hand, the percentage of infected samples with HPV-genotype 53 was 2.9% (3/104), and for HPV-genotype 59 was 4.8% (5/104). The ratio of infection of HPV-16 and -18 were 2:1 in simple infections, and 3:1 in co-infections. Generally, it was confirmed that the replaced HPV types are well detected. The sensitivity of the care GENETM-HPV test was 97.9% and its specificity was 100% based on the sequencing results. Data showed that the sensitivity and specificity of the care GENETM-HPV test were 100 and 70.72% compared to COBAS system test, respectively (Table 1). Even more, the area under curve was higher compared to COBAS test (AUC  $\pm$  SE= 0.854  $\pm$  0.041; 95% CI 0.773-0.934). The kit was designed with the 25 most prevalent genotypes. In relation to low risk-, and high-risk genotypes, we design by each group two pools. We included genotypes 16 and 18 for their oncogenic capacity, with genotypes 31 and 59 (by the distribution observed). Another pool included 10 HR-genotypes (33, 35, 39, 45, 51, 52, 56, 58, 66, and 68). The most prevalence LR-genotypes were included in the same pool (6, 53, and 61). Finally, a pool of 8 LR-genotypes were included: 11, 40, 43, 44, 55, 70, 73, and 81. We included genotype 81 due to the possible epidemiological and public health transcendence.

## Discussion

According to *Bruni, et al.* [9], the estimated global HPV infection prevailed in women with normal Pap test was 11.7% (95% CI

11.6%-11.7%), different from our findings (26.2%; 95% CI 19.2-33.8). Our prevalence is similar to the prevalence observed by SubSaharan Africa (24.0%), and Eastern Europe (21.4%), but higher compared with the prevalence in Latin America (16.1%) [15,16]. In contrast to *Gallegos-Bolaños, et al.* our findings shown that the prevalence of HPV co-infection was lower than mono-infection and the most prevalent genotypes were HPV-6, and -16 [17]. In addition, the co-infection with HPV-16, and -61 genotypes were the most frequent combination contrary to reported by these authors, who indicated that the co-infection with HPV-51 and -52 genotypes was the most frequent combination in all their cases [17]. Similarly, *Aziz H, et al.* reported in a population of Punjab, Pakistan that HPV-6 was the most frequent HPV-genotype found in 25% of infected women, however, the prevalence observed in our population was lower (13.2%) [18]. Even more, HPV-16, -58, -59, and -31 genotypes were the most common HR-genotypes in patients with cervical lesions, while, types HPV-6, 61, and 53 were the most common LR-genotypes in the same group of patients (similar to a previously published study) [19]. Contrary to the results reported by *Jácome Galarza, et al.* [20], cytological examination revealed normal Pap test in 36% of samples (35.7% with LSIL, and 28.3% with HSIL), and a different double infections combination (to except of genotypes combination between HPV-53 and -62) [20]. In relation to triple infections, our findings were totally different from reported in the population of Michoacan [20]. Even, it was strange that the Cobas HPV test only identified 5 positives specimens among all samples. The detection rate of Cobas in our specimens is extremely low, however, we repeat all test (we check the reagents and procedure of our experiment). On the one hand, there are countries where the HPV co-infection is less frequent than the mono-infection, but there are regions where co-infection is higher [17, 21-22]. In Mexico the prevalence and patterns of HPV infection and cervical cancer distribution differ according to the geographic

location analysed and determinants of health associated [17,19,23-30]. It is well established that co-infection among HPV-genotypes is common in women, and men [17]. However, the knowledge of their epidemiological distribution is scarce. Some studies show that co-infection increases the risk of cervical cancer and is associated with both a low response and survival rate in those patients with cervical cancer that are receiving radiotherapy [17]. Our data in conjunction with other reports support evidence [17,27-28] to establish new appropriate prevention strategies for the design of new vaccines according to each population, as well as introduced new national cervical cancer screening programs and therefore restructure or design new national immunization programs. On the other hand, the present study provides epidemiological evidence indicating the need to include screening tests containing the most prevalent HPV-genotypes into national cervical cancer screening programs. As well as this study allowed established two pools of HR genotypes and two pools of LR genotypes, resulting in the screening of 25 different subtypes, which makes it more specific than any of the other tests available on the market. The pools were established according to the distribution of genotypes, clinical relevance, and technical procedure. In relation to genotype distribution, the main 10 genotypes were HPV-6, HPV-16, HPV-53, HPV-58, HPV-59, HPV-31, HPV-61, HPV-18, HPV-51, and HPV-56. Thus, one pool was established with the four most prevalent and relevant HR genotypes (16, 18, 31, and 59). Due to probes of HPV-58 and HPV-59 are similar, both were placed in different pool, and HPV-18 was included by its oncogenic relevance. The second pool of HR-genotypes was established for other 10 genotypes (33, 35, 39, 45, 51, 52, 56, 58, 66, and 68) based on their distribution. Likewise, the first pool of LR-genotypes was established with the main three genotypes (6, 53, and 61). Finally, the last pool of LR-genotypes includes eight genotypes (11, 40, 43, 44, 55, 70, 73, and 81) based on their distribution and clinical relevance (previous studies) [22]. As a result, it is clear that using an HPV test that includes more genotypes will detect more cases of pre-cancerous cervical lesions, even than with traditional cytology. Similarly, it is feasible to use this test at the population level making use of the infrastructure and resources already available within a cervical cancer screening program, and consequently the number of women referred for colposcopy owing to a positive result of HPV infection may be increased. According to the results reported by *Koliopoulos, et al.* [31], in a systematic literature search of 40 studies with more than 140,000 women aged between 20 and 70 years old, for cervical intraepithelial neoplasias (CIN) of grade 2 or worse (CIN 2+) the pooled sensitivity for hybrid capture 2 (HC2), conventional cytology (CC) and liquid-based cytology (LBC) (atypical squamous cells of undetermined significance ASCUS+) were 89.9%, 62.5% and 72.9%, respectively, different to our findings (care GENETM-HPV test sensitivity 100% compared with COBAS system test) [31]. While pooled specificity estimates were 89.9%, 96.6%, and 90.3%, respectively [31]. Others authors report that the sensitivity of thin-layer Pap (with a result of  $\geq$ ASCUS) for identifying women with CIN 3 or higher was only 61.3% compared with 88.2% for HPV testing by PCR and 90.8% by signal amplification [32-33]. Furthermore, *Mayrand, et al.* [34,35] report that in women with HSIL, the sensitivity of HPV testing was 94.6%, where-

as that of Pap testing was 55.4%.<sup>34</sup> The specificity was 94.1% for HPV testing and 96.8% for Pap smears [34]. Compared with COBAS system test, the careGENETM-HPV test has a higher sensibility and a significantly more specific AUC. Immunization is the most cost-effective public health intervention. Nowadays, HPV vaccines in use around the world protect against infection from different genotypes: 16/18 (bivalent vaccine, Cervarix, GSK, Belgium), 6/11/16/18 (tetravalent vaccine, Gardasil, Merck, USA), and 6/11/16/18/31/33/45/52/58 (nonavalent vaccine, Gardasil 9, Merck, USA) [13-14, 35-36]. Present vaccination programmes against HPV do not include all prevalent genotypes of HPV, for instance, vaccines do not contain HPV-59 considered a HR-genotype. As a result, many patients infected with other HPV-genotypes do not benefit from current HPV vaccine programs. Even, the World Health Organization recommends HPV vaccines to be introduced into national immunization programs, where the cervical cancer prevention is a public health priority. Nonetheless, in order to meet this objective, it is necessary to have a broad coverage considering both, the age of the patients and the distribution of HPV genotypes. Our findings indicate that current vaccination programmes do not include several of the most prevalent types of HPV-genotypes, therefore, and unfortunately many adolescents and adults' women fall outside the protection induced by vaccines. Consequently, our data show that it is more feasible to produce a vaccine with the prevalent genotypes in the population. Even thinking about producing the vaccine in the country based on the observed prevalence. In addition, for our population, follow-up studies will have to be developed to determine which genotype is the most oncogenic. In conclusion, we observed a different prevalence of HPV genotypes. Moreover, our study provides evidence that the "care GENETM-HPV detection-Kit-I test" for screening of human papillomavirus has more sensibility than other commercial tests such as COBAS system tests and was similar to Pap test. Consequently, this test can serve as a better triage test, because with this technique we can detect almost 90% of the HPV genotypes present in the population, and it is cheaper. Nevertheless, due to its low sensitivity compared to Pap test, it demands a strict follow-up of negative cases.

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### Conflict of Interest

The authors declare that they have no conflicts of interest.

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