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Detection by PCR of human papillomavirus in Colombia: Comparison of GP5+/6+ and MY09/11 primer sets

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A B S T R A C T

The aims of this study were to determine the prevalence of HPV infection and evaluate the concordance and performance of two primer sets for detecting single and multiple viral infections. A total of 1810 Colombian women were enrolled in the study, and molecular, cytological and epidemiological analyses were performed. Both concordance and performance of two different PCR amplification primer sets (GP5+/6+ and MY09/11) were assessed. The results showed that 60.2% of females with positive HPV DNA were infected by more than one viral type. The OR for multiple infections was 18.2 when using the MY09/11 primer set and 6.52 with the GP5+/6+ primer set. The results also showed an association between GP5+/6+ positivity and the severity of the disease regarding the cytological findings. It was also found that using a single primer set led to underestimating the prevalence for HPV infection. The simultaneous use of these primer sets is an important tool for the detection of HPV DNA, being equally relevant for identifying multiple infections and low viral DNA copies. This study highlights the importance of suitable assessment of HPV epidemiological profiles; screening programs must also be strengthened to broaden the coverage of the most vulnerable populations.

1. Introduction

There is strong evidence implicating some human papillomaviruses (HPVs) with the induction of carcinogenesis, especially of cervical cancer which is the second most common type of cancer in females worldwide. More than 100 types of HPVs have been identified to date, but only 40 types are known to infect the genital tract (de Villiers et al., 2004).

The polymerase chain reaction (PCR) has been used extensively for HPV typing in many clinical and epidemiological studies because of its high sensitivity for detecting HPV DNA. Nevertheless, an exhaustive identification of HPV types, based solely on PCR, requires a large number of reaction per sample due the high degree of genetic heterogeneity amongst HPV types (van Doorn et al., 2006).

Different primer systems targeting relatively conserved nucleotide sequences have been developed with the aim of detecting a wide spectrum of HPV types. The sets used most frequently at present are GP5+/6+, MY09/11, PGMY and SPF10 (Iftner and Villa, 2003). The use of one or more primer sets has been recommended when designing PCR-based HPV detection methods (Karlsen et al., 1996; Remmerbach et al., 2004); this should ensure better understanding of the natural history of this particular infection and increase epidemiological studies' sensitivity. The use of more than one primer set has been shown to offer better robustness and sensitivity for detecting multiple HPV infections (i.e. infection with more than one type of HPV) as well as a low viral copy number, compared to the use of a single primer set which, in some cases, has led to underestimating the prevalence of HPV in cervical samples (Fuessel Haws et al., 2004; Iftner and Villa, 2003).

The present study was aimed at evaluating the presence of HPV DNA by using two primer sets annealing in a conserved region of the L1 gene. GP5+/6+ has been reported as a convenient method for detecting low viral loads (Remmerbach et al., 2004) whilst MY09/11 has greater sensitivity for detecting coinfections. Both primer sets

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Table 1
The primers used in this study.

Type	Primer	Primer sequence (5′–3′)	Size (bp)	Region
GH20	Pr1	GAAGAGCCAAGGACAGGTAC	268	
PC04	Pr2	CAACTTCATCCACGTTCACC		
GP5+	Pr1	TTTGTTACTGTGGTAGATACTAC	150	L1
GP6+	Pr2	GAAAAATAAACTGTAAATCATATTC		
MY09	Pr1	CGTCCMARRGGAWACTGATC	450	L1
MY11	Pr2	GCMCAGGGWCATAAYAATGG		
HPV-16	Pr1	TCAAAAGCCACTGTGTCCTGA	120	E6
	Pr2	CGTGTTCTTGATGATCTGCAA		
HPV-18	Pr1	CGACAGGAACGACTCCAACGA	202	E6–E7
	Pr2	GCTGGTAAATGTTGATGATTAACT		
HPV-31	Pr1	CTACAGTAAGCATTGTGCTAT	155	E5
	Pr2	ACGTAATGGAGAGGTTGCAATAACCC		
HPV-33	Pr1	AACGCCATGAGAGGACACAAG	212	E7
	Pr2	ACACATAAACGAACTGTGGTG		
HPV-45	Pr1	ACGGCAAGAAAGACTTCGCA	134	E6–E7
	Pr2	CACAACAGGTCAACAGGATC		
HPV-58	Pr1	CGAGGATGAAATAGGCTTGG	109	E7
	Pr2	ACACAAACGAACCGTGGTGC		
HPV-6/11	Pr1	TGCAAGAATGCACTGACCAC	334	E6–E7
	Pr2	TGCATGTTGTCCAGCAGTGT		

have been used extensively in numerous HPV identification studies (Qu et al., 1997). The study has evaluated the concordance and performance of these two primer systems for detecting HPV DNA in single and multiple infections. Similar studies have been carried out in Colombia; however, the present work has included cytology samples collected from a heterogeneous group of women from different economic, socio-demographic and cultural backgrounds whose epidemiological profiles have not been described previously and who are considered to be vulnerable populations having limited access to healthcare services.

2. Materials and methods

2.1. The population studied

This study involved 1810, 14–77 year-old females (mean age = 38.9, SD = 11.9) from five Colombian departments who attended regular cervical cancer screening sessions between April and September 2007. This population consisted of females attending the League Against Cancer, Leticia, Amazonas ($n = 140$), Hospital San Juan Bautista, Chaparral, Tolima ($n = 148$), Hospital Engativa, Bogotá ($n = 796$), Hospital San Rafael, Girardot, Cundinamarca ($n = 321$) and Hospital San Andrés, Tumaco, Nariño ($n = 405$). Stata Software (version 11) was used for estimating population size (1778 individuals, 95% confidence interval, plus or minus 0.02, estimated proportion was 0.25) (Mendez et al., 2005). The number of samples was adjusted in line with a proportional allocation in which the sample size depended on the number of cytologies being taken in each department.

2.2. Ethical approval

All the women were informed about the study by the nurses coordinating the project and then gave their written consent. They all filled out a questionnaire regarding their socio-demographic characteristics, sexual behaviour and risk factor data before undergoing a gynaecological examination. Each participating institution's Ethics Committee approved and supervised all the procedures carried out in this study.

2.3. Collecting cervical samples and PCR-based HPV detection

A cytobrush was used for collecting the cervical samples for Papanicolau testing and HPV-DNA detection; they were stored at 4 °C in 95% ethanol until processing. The Pap test cytological findings were classified according to the Bethesda system. The PCR assay samples were digested in lysis buffer containing 10 mM Tris–HCl (pH 7.9), 0.45% Nonidet P-40, 0.45% Tween 20 and 60 μg ml Proteinase K, first at 60 °C for 1 h and then at 95 °C for 10 min (Nelson et al., 2000).

The human β-globin housekeeping gene was amplified in all samples using GH20/PC04 primers to check DNA integrity (Table 1). The PCR mix contained: 1× amplification buffer, 100 μM each dNTP, 2.5 mM $MgCl_2$, 1 U Taq Polymerase (Bioline, MA, USA), 1 μM of each primer and 0.8 μg μl bovine serum albumin (BSA) as buffer additive (final volume = 10 μl) (Kreader, 1996). The thermal cycling conditions consisted of initial denaturing for 10 min at 94 °C, followed by 35 amplification cycles for 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, followed by a final extension step for 7 min at 72 °C (de Roda Husman et al., 1995).

The HPV DNA was detected using generic GP5+/6+ and MY09/11 primer sets annealing in the L1 gene (Table 1) according to published protocols (de Roda Husman et al., 1995; Manos et al., 1989) which had some modifications made to it. The GP5+/6+ PCR assays were carried out in a 20 μl volume mixture containing: 1× amplification buffer, 100 μM each dNTP, 3 mM $MgCl_2$, 1 U Taq polymerase (Bioline, MA, USA) and 2 μM of each primer. Thermal cycling conditions consisted of initial denaturing for 10 min at 94 °C, followed by

Table 2
Comparing HPV detection using two generic primers in single and multiple infections.

HPV infection status ($n = 894$)	GP5+/6+		MY09/11	
	Negative *n* (%)[a]	Positive *n* (%)[a]	Negative *n* (%)[a]	Positive *n* (%)[a]
Single infections (n = 356)	169 (47.5)	187 (52.5)	85 (23.9)	271 (76.1)
Multiple infections ($n = 538$)	269 (50.0)	269 (50.0)	110 (20.4)	428 (79.6)
Total	438	456	195	699

[a] Total percentages were calculated by rows.

40 amplification cycles for 1 min at 94 °C, 2 min at 40 °C and 1.5 min at 72 °C, followed by a final extension step for 7 min at 72 °C (de Roda Husman et al., 1995). The PCR assays with MY09/11 primers were carried out in a 20 μl volume mixture, exactly as described above. Thermal cycling conditions consisted of initial denaturing for 5 min at 94 °C, followed by 40 amplification cycles for 30 s at 94 °C, 1 min at 45 °C and 1 min at 72 °C; a final extension step lasted 7 min at 72 °C. Positive and negative controls were used in each assay to assess whether the DNA was contaminated. The PCR amplification using generic primers was carried out twice, at different times, to rule out sample contamination and reduce the number of false positives (Remmerbach et al., 2004). The amplification products obtained from all the PCRs (5 μl) were run on 2% agarose gels stained with SYBR safe (Invitrogen, CA, USA) and then visualised on an ultraviolet transilluminator.

Each set of generic primers' (GP5+/6+ or MY09/11) analytical sensitivity was only calculated for HPV 16 using plasmid DNA, meaning that these values could not be extrapolated to other HPV types. Serial dilutions of 100 ng of plasmid DNA containing the HPV-16 *L1* gene were thus used; these were extracted from transfected Sf21 cells. This procedure was carried out in duplicate and the same DNA was used as positive control.

The samples that proved positive by generic primers (when using one or both sets) were then tested with type-specific primers which had been designed to anneal in early protein genes (E5–E7) (Table 1) (Karlsen et al., 1996; Lee et al., 2001; Sotlar et al., 2004) for genotyping the two most prevalent low-risk types (HPV-6/11) and six high-risk types associated with ~90% of cervical cancers reported in Latin-America and worldwide (i.e. HPV-16, -18, -31, -33, -45, -58) (Parkin et al., 2008; WHO/ICO, 2010). The high-risk types mentioned above had the highest incidence and prevalence according to previous studies carried out in Colombia (Molano et al., 2002).

2.4. Statistical analysis

The concordance between results obtained with the two generic primer sets (GP5+/6+ and MY09/11) was assessed by using kappa statistics (κ) with 95% confidence intervals (95% CI). The difference between the percentages of cases detected by each method was analysed by applying the McNemar chi-square test. The strength of the association between each method and the variables of interest, such as coinfection and cytology findings, were measured using ORs (95% CI) and adjusting OR by logistical regression. The relationship between cytological findings and detection by each primer set was explored by using the test of trends in odds ratio (normal cytology was used as reference category). The diagnostic value for each primer set for detecting infection and coinfection was assessed by calculating their sensitivity, specificity and area under their corresponding receiver operating characteristic (ROC) curves. Areas under the ROC curves were compared by using chi square methods. Each estimator was reported with its 95% confidence interval and the hypotheses were tested at 5% significance level. STATA 9 Software was used for all statistical analysis.

3. Results

All the 1810 samples were β-globin gene positive (268-bp DNA fragment) and thus were suitable for further PCR analysis. HPV DNA was detected in 894 (49.4%) of the samples having good DNA quality (positive for β-globin gene); 699 (38.6%) of these were detected using MY09/11 and 456 (25.2%) using GP5+/GP6+ (Table 2) whilst 261 (14.4%) samples were detected with both generic primer sets. Coinfection was detected in 538 (60.2%) out of 894 HPV

Table 3
Comparison of HPV detection using the GP5+/6+ and MY09/11 primers regarding cytological findings.

Pap smear result[a] (n = 1718)	GP5+/6+		MY09/11		Crude ORs (95% CI)		Adjusted ORs (95% CI)[b]	
	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)	GP5+/6+	MY09/11	GP5+/6+	MY09/11
Normal cytology (1564)[c]	1186 (75.8)	378 (24.2)	952 (60.9)	612 (39.1)	–	–	–	–
ASCUS (92)	61 (66.3)	31 (33.7)	52 (56.5)	40 (43.5)	**1.59 (1.01–2.49)**	1.19 (0.78–1.82)	**1.82 (1.10–2.99)**	1.46 (0.91–2.35)
LSIL (49)	26 (53.1)	23 (46.9)	26 (53.1)	23 (46.9)	**2.77 (1.56–4.93)**	1.37 (0.77–2.43)	**2.85 (1.53–5.27)**	1.24 (0.67–2.30)
HSIL (13)	6 (46.2)	7 (53.8)	8 (61.5)	5 (38.5)	**3.66 (1.21–10.9)**	0.97 (0.31–2.98)	**4.80 (1.47–15.6)**	1.34 (0.41–4.36)
Total	1279 (74.4)	439 (25.6)	1038 (60.4)	680 (39.6)				

Percentages were calculated by rows.
Values in bold had $p < 0.05$.
Abbreviations: ASCUS, atypical squamous cells of undetermined significance; LSIL, low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion.
[a] Female samples were classified according to the Bethesda system.
[b] OR adjusted for age, number of pregnancies, age at first intercourse, number of lifetime sexual partners, ethnicity, geographical region, contraceptive method used. Confidence intervals for crude and adjusted ORs are shown in parentheses.
[c] Normal cytology was used as reference category.

Table 4
Crude and adjusted odds ratios (ORs) for HPV detection, using two generic PCR primers, in samples with known co-infections.

	Crude ORs (95% CI)		Adjusted ORs (95% CI)[a]	
	GP5+/6+	MY09/11	GP5+/6+	MY09/11
Negative for multiple infections[b] ($n = 1272$)	–	–	–	–
Positive for multiple infections ($n = 538$)	5.8 (4.53–7.42)	14.3 (10.67–19.34)	6.52 (5.03–8.47)	18.2 (13.58–24.50)

All p values were <0.05.
[a] OR adjusted for age, number of pregnancies, age at first intercourse, number of lifetime sexual partners, ethnicity, geographical region and contraceptive method used.
[b] Negative for multiple infections was used as the reference category.

DNA positive samples. The results of this study had a 5×10^{-6} ng detection limit for GP5+/GP6+ and 2×10^{-3} ng for MY09/11.

As can be observed in the Papanicolau test results (Table 3), only 92 cervical samples were unsatisfactory and they were thus excluded from the analysis. The OR values for the association between GP5+/GP6+ and the severity of the disease according to the cytological findings revealed a significant trend for detecting HPV DNA with this primer set (test of trends in odds: chi^2 (1) = 21.87, $p = 0.00$) which remained significant when ORs were adjusted. The same association with MY09/11 also showed an increasing trend; however, such trend was not statistically significant (test of trends for odds: chi^2 (1) = 1.20, $p = 0.27$).

The case detection percentages with each primer set revealed statistically significant differences (McNemar chi^2 (1) = 196.53, $p = 0.000$). The kappa statistics revealed 65% concordance between both primer systems ($\kappa = 0.22$; 0.16–0.25, 95% CI) Regarding HPV infected females, total concordance became reduced to 27% in the single infection group ($\kappa = -0.46$; −0.54 to 0.38, 95% CI) and to 30.4% in the coinfection group ($\kappa = -0.42$; −0.48 to 0.36, 95% CI). Concordance between both primer systems was 69.7% in the group of females suffering cytological abnormalities ($\kappa = 0.38$; 0.23–0.52, 95% CI) whereas it was 63.9% ($\kappa = 0.19$; 0.14–0.24, 95% CI) in the normal cytology group.

The GP5+/6+ set had 51% sensitivity for detecting HPV infections (ROC area = 0.75; 0.73–0.77 95% CI) whereas MY09/11 had 72.2% sensitivity (ROC area 0.89; 0.87–0.90 95% CI); both systems had 100% specificity. A comparison between the areas under the ROC curve for each primer system revealed significantly higher values for MY09/11 in detecting HPV infections (chi^2 (1) = 104.04, $p = 0.00$).

However, when both methods were analysed regarding their performance for detecting HPV positivity in co-infected samples, GP5+/6+ had 50% sensitivity and 85.3% specificity (ROC area = 0.67; 0.65–0.7 95% CI) whereas MY09/11 had 79.6% sensitivity and 78.7% specificity (ROC area = 0.79; 0.77–0.81 95% CI). The crude and adjusted ORs were calculated for evaluating the ability of each primer set to detect either single or multiple infections. The OR results regarding coinfection detection were 18.2 when using the MY09/11 primer set and 6.52 when using the GP5+/6+ primer set (Table 4). Comparison of the areas under the ROC curves showed that the MY09/11 set had significantly higher values for detecting coinfections (chi^2 (1) = 40.43, $p = 0.00$).

All samples that had a positive result for viral DNA presence (894 that amplified in GP, MY or both PCRs) were amplified successfully by type-specific primers, thereby revealing that HPV-16 was the most prevalent type as it was detected in 654 females (36.1%), followed, in decreasing order, by HPV-31, detected in 336 (18.6%), HPV-18 detected in 274 (15.1%), HPV-33 detected in 223 (12.3%), HPV-45 detected in 193 (10.6%), HPV-58 detected in 127 (7.0%) and HPV-6/11 detected in 100 females (5.5%).

4. Discussion

Two generic primer sets were used in the present study for detecting HPV DNA in Colombian females which led to a higher prevalence of HPV infection was found compared to previous studies carried out in Colombia. A previous study where a single primer set was used for viral identification in an urban population only showed 14% HPV prevalence (Molano et al., 2002). Another study that included both rural and urban areas in a culturally diverse, low educational level population revealed a higher prevalence of viral infection (36%) (Sierra-Torres et al., 2006) even though such prevalence was lower than that reported here.

A higher prevalence of HPV infection has been reported in several studies carried out in around the world. African countries such as Comoros, the Congo, Djibouti, Eritrea and Ethiopia have reported a prevalence of infection ranging from 23.0% to 35.4% (WHO, 2007) and 45.3% and 68.9% prevalence has been reported for France and Italy (Bello et al., 2009) whilst some Latin-American countries (Costa Rica, Honduras, Nicaragua and Panama) sharing socio-demographic characteristics similar to those described in this study, have reported 20.5–51.0% viral prevalence (Ferrera et al., 2000; Herrero et al., 2008; Safaeian et al., 2007; Tabora et al., 2009; WHO, 2007). The conclusions drawn from the present study agree with studies indicating that the use of a single primer set leads to underestimating the prevalence of HPV infection, mainly in cases of multiple infection (Fuessel Haws et al., 2004).

Women infected by HPV were detected more efficiently by MY09/11 than by GP5+/6+ according to the analytical sensitivity results calculated for each primer set. MY09/11 was the more efficient system for detecting females infected with more than one HPV type, since 428 out of 538 (79.6%) infected females were detected, whereas only 269 (50%) of the same females were detected by GP5+/6+. This finding agreed with previous reports (Qu et al., 1997) and was consistent with MY09/11 consisting of a system of degenerated primers thereby promoting the identification of multiple infections (Chaiwongkot et al., 2007).

Although it has been reported that the concordance between two generic primer sets decreases in patients having coinfection (Qu et al., 1997), this study also had low concordance in the single infection group. Concordance between both primer sets increased in females having abnormal cytological findings (this being higher for GP5+/6+), thereby agreeing with previous reports. Such relationship between lesion severity and HPV detection is consistent with the HPV life-cycle since viral DNA remains integrated within a host cell's DNA when lesion severity is high; this can result in the loss, truncation or poor transcription of the L1 gene (Depuydt et al., 2007). The GP5+/6+ set is thus more sensitive for detecting a low viral copy number of HPV DNA, even if the L1 gene is truncated, because the amplification product obtained with this primer set is just one third of that obtained when using MY09/11 (Sotlar et al., 2004). False negative results when using the MY09/11 set have been associated with the loss of the L1 ORF during HPV DNA integration into host-cell DNA (Depuydt et al., 2007).

Many commercial systems for the generic identification of HPV-infected samples are available, such as PGMY-LB (Roche Molecular Systems Inc., Branchburg, NJ), SPF10-LiPA25 (Labo Bio-Medical 221 Products, Rijswijk, the Netherlands), the Amplicor HPV test and the LINEAR ARRAY HPV genotyping test (both from Roche) and PapilloCheck HPV-Screening Test (Greiner Bio-One GmbH, Frickenhausen, Germany); these identification systems' performance

Table 5
Advantages and disadvantages of different HPV detection techniques in clinical samples.

Method of HPV detection	Country	Population	HPV prevalence %	Sensitivity %	Specificity %	Advantages	Disadvantages	Reference
GP5+/6+	Greece	1270 liquid-based cytology	31.3	100	70.2 (CIN2) 69.6 (CIN3)	Offers higher sensitivity, especially with samples containing low copy numbers. Due to their low cost and easy implementation, they have been widely used in clinical and epidemiological studies	Can be ineffective in the amplification of some HPV types such as HPV-53 and -61. Not suitable for type-specific identification	Tsiodras et al. (2010)
	Russia	1511 gynaecological outpatients	36.6	74.0	64.1			Kulmala et al. (2004)
	Colombia	1810 gynaecology outpatients	25.2	51	100			This study
MY09/11	Australia	834 cervical specimens	37.4	63.1 (HGS test) 64.7 (hcII)	90.6 (HGS test) 84.6 (hcII)	Detects more than 25 genotypes simultaneously. High sensitivity in detecting different genotypes. Due to their low cost and easy implementation, they have been widely used in clinical and epidemiological studies	Relatively large size of the PCR fragment, especially in samples that yield poorly amplifiable DNA, such as formalin-fixed, paraffin-embedded materials, or having a high rate of viral integration, loss of target amplification region. Not suitable for type-specific identification	Baleriola et al. (2008)
	Colombia	1810 gynaecology outpatients	38.6	72.2	100			This study
PGMY-LB	Canada	157 participants from other research studies	61.5	95.6	–	Combines PCR assays with type-specific radiolabelled oligonucleotide probes to increase HPV detection sensitivity and reproducibility	Since the β-globin gene is co-amplified with HPV in PGMY assays, these assays could also be more sensitive to the effects of inhibitors	Coutlee et al. (2002)
SPF10-LiPA25	Costa Rica	5659 participants from other research studies	35.3	–	–	System of detection/genotyping capable of amplifying up to 43 different genotypes and providing type-specific genotype information for 25 different HPV genotypes simultaneously	Might have sensibility issues in samples having more than one HPV type because of competition between the different HPV genotypes present in the same and with relative concentrations	Castle et al. (2008)
Amplicor HPV test	France	470 gynaecology outpatients	51.3	96.4	100	Simultaneously detects 13 HR-HPV genotypes with assessment of the presence of the human β-globin gene as positive control	Cross-hybridisation between primers, mainly with HPV56, and requires instrumentation and skilled technologists to extract and perform amplification. Longer time taken to obtain results	Mo et al. (2008)
LINEAR ARRAY HPV genotyping test	United States	5060 females referred to colposcopy	55	89.1	46.5	Detects 37 HPV genotypes individually, including the main 14 carcinogenic HPV genotypes. Uses an internal β-globin control to verify specimen suitability	Lower clinical sensitivity (identification in females with disease). Samples have to be maintained in special media, such as STM, otherwise sensitivity might be affected	Gravitt et al. (2008)
PapilloCheck HPV-Screening Test	France	144 gynaecology outpatients	66.4	–	–	Simultaneously detects and identifies 25 different HPV genotypes, 15 high-risk HPV genotypes (HPV-HR), 2 probably high-risk as well as 8 low-risk (HPV-LR)	Might report detection discrepancies with other methods in multiple infections. Might yield false positives. Detection is directed towards E1 which is more susceptible to DNA sequence modifications (partial deletions) compared to the L1 region	Dalstein et al. (2009)

Abbreviations: SPF: short PCR fragment; LiPA: reverse hybridisation line probe assay.

seems to be affected by competition between the different HPV types present in the same sample, which could thus lead to underestimating HPV genotype prevalence (Table 5) (van Doorn et al., 2006).

The data reported in this study support using GP5+/6+ and MY09/11 as a highly sensitive tool for the PCR detection of HPV infections and coinfections. Such generic viral identification systems can be easily implemented and have a moderate cost. When used together (to avoid underestimating viral infections), they have a wide detection range enabling the identification of more HPV-infected females and have better robustness for determining the epidemiological profiles of HPV types, especially in regions having cervical cancer-associated high morbidity and mortality rates. These profiles would allow a more rational design of screening programs aimed at broader coverage of the most vulnerable populations, especially those having limited access to health services and cutting-edge technology, in turn promoting increased cervical cancer-associated death rates.

Conflict of interest

All the authors involved in this study declare having no conflicting or dual interests.

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