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Original article

Comparison of five protocols to extract DNA from paraffin-embedded tissues for the detection of human papillomavirus

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ABSTRACT

Formalin-fixed paraffin-embedded (FFPE) tissues are a valuable source of DNA with which to perform large retrospective studies on the epidemiology of HPV infection. Five different DNA extraction protocols were carried out to evaluate the DNA obtained from FFPE samples with polymerase chain reaction (PCR) using two primer sets to amplify a constitutive human gene, β-globin, and two primer sets to detect the L1 and E6 HPV genes. From the five DNA extraction protocols evaluated, the best results were obtained with protocol A, corresponding to a crude extract from the sample. With the procedures described herein, we were able to amplify DNA extracted from archival paraffin blocks stored for six years. However, the amplification products were more efficiently obtained with primers that amplified shorter fragments. This result indicates that a major factor limiting the extraction process in these samples is DNA fragmentation, a factor that will naturally vary between the different specimens evaluated. Also, depending upon the extraction method, PCR amplification of a human gene does not necessarily guarantee the successful extraction of viral DNA. In conclusion, different DNA and HPV detection methods can significantly influence the results. Therefore, the DNA extraction methods and primers used for DNA amplification in fixed tissues need to be chosen carefully, depending on the specific requirements of the study being carried out.

Introduction

Human papillomaviruses (HPVs) are known most commonly for their benign and neoplastic diseases of the anogenital tract [12]. Over 150 types of HPV have been fully characterized; approximately sixty of these are predominantly detected in the cervical epithelia and belong to the *Alphapapillomavirus* genus [10].

In the majority of individuals, HPV infections are transient and asymptomatic but the persistent infection by a subgroup of so-called high-risk HPVs that have a tropism for mucosal epithelia has been identified as the cause of more than 98% of cervical carcinomas as well as a high proportion of other cancers of the anogenital region [42]. Cervical cancer is the third most common cancer among women worldwide and the majority of cases occur in the developing world [19,20].

The primary screening for cervical cancer is the Papanicolau test (Pap test). This test uses cytological interpretation of cervical cells to screen for cervical dysplasia and cervical cancer, with a sensitivity of 81% and a specificity of 77% for the detection of severe cervical dysplasia [5]. Using HPV DNA testing as the primary screening method or combining it with the Pap test every 2, 3 or 5 years is more cost-effective than the Pap test alone [11].

Cytological or histopathological examination of cervical samples does not allow viral characterization, while molecular methods allow both viral DNA detection and typing [37]. Polymerase chain reaction (PCR) is very sensitive in detecting HPV in fresh clinical specimens such as blood, cervical swabs, cervical smears and oral mucosa brushing or rinsing [8,30,36,37,57]; however, such tissues are not always available. Formalin-fixed paraffin-embedded (FFPE) tissues are a valuable source of DNA for retrospective molecular studies and identification of specific molecular markers [32].

There is a huge reservoir of FFPE tissue specimens stored in histopathology laboratories around the world. These samples provide the opportunity to perform large retrospective studies on the epidemiology of HPV infection, to determine the geographical distribution of HPV genotypes [16], to search for the association of

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HPV with cancers other than cervical cancer [39,56] and to correlate HPV typing information with pathology in order to evaluate the impact of vaccination [35]. However, FFPE tissue specimens have potential problems, including the following: (i) paraffin as a physical barrier; (ii) DNA cross-linking; (iii) DNA-protein cross-linking; and (iv) the presence of PCR inhibitors. Also, DNA fragmentation induced by fixation limits viral DNA amplification by PCR [33,45]. The aim of this study was to compare the success of DNA extraction from FFPE cervical tissues using five different DNA extraction protocols in order to detect HPV using PCR.

The results presented here are of clinical relevance because HPV detection and typing in tissue samples can be done using a simple and efficient protocol. The use of FFPE tissues allows the study of gene regulation in HPV positive lesions [38] and the identification of new survival markers to be used in the clinical practice [2,47].

Materials and methods

Study samples

Thirty-two blocks of FFPE cervical tissues stored for six years at the pathology laboratory in Liga Colombiana contra el Cáncer, Seccional Pereira were analyzed. Sixteen samples corresponded to cervical intraepithelial neoplasia (CIN3) and sixteen samples were evaluated as cervical squamous cell carcinomas (SCC). Serial sections were cut from each block (in each case, eight 10 μm thick slices). The first and last slices were stained with hematoxylin/eosin and evaluated histopathologically, whereas the remaining sections were used for DNA extraction and HPV detection. To prevent cross-contamination, a new microtome blade was used for each sample. Empty paraffin blocks were blindly included and analyzed as controls in the process for further quality control of laboratory results. The first histopathological diagnosis was reviewed and confirmed by a second pathologist in all cases.

DNA extraction from tissue sections

For DNA isolation from FFPE tissues, lesions were circled and the area of the lesion was microdissected from unstained slides using a sterile surgical scalpel. The material was transferred to Eppendorf tubes and submitted for DNA extraction using five different protocols (A–E).

For protocols A, B and E, tissues were dewaxed with 1 mL of xylene, washed with 1 mL of 100% ethanol and centrifuged at 14,000 rpm for 2 min to remove residual xylene. The pellets were dried overnight at room temperature.

In protocol A, tissues were suspended in 100 μL of digestion buffer (90 μL of 50 mM Tris, pH 8.0 and 10 μL of 20 mg/mL proteinase K). After overnight incubation at 37 °C, proteinase K was inactivated at 95 °C for 10 min, and the sample was centrifuged at 14,000 rpm for 2 min. The supernatant (90 μL) was transferred to a new Eppendorf tube and stored at −20 °C until needed.

For protocol B, tissues were suspended in digestion buffer (450 μL of 1× TE buffer, 70 μL of 10% SDS and 10 μL of 20 mg/mL proteinase K) and incubated overnight at 37 °C. After proteinase K inactivation at 95 °C for 10 min, the mixture was placed on ice for 5 min, 100 μL of 5 M NaCl was added and the mixture was incubated at room temperature for 5 min. The samples were then centrifuged with equal volumes of chloroform/isoamyl alcohol (24:1) at 13,000 rpm for 15 min. The aqueous phase was mixed with 0.6 volumes of isopropanol and centrifuged at 13,000 rpm for 15 min at 4 °C. The DNA pellet was washed with 70% ethanol and dried at room temperature. The DNA was resuspended in 50 μL of 1× TE buffer and stored at −20 °C until needed.

For protocol E, DNA extraction and purification were performed using the QIAamp DNA FFPE Tissue Kit (Qiagen), according to the manufacturer's instructions. The DNA was eluted with 50 μL of ATE buffer and stored at −20 °C until needed.

For protocols C and D, tissue sections were collected in 0.5 mL microcentrifuge tubes. In protocol C, each sample was dewaxed in 20 μL of 0.5% Tween-20 in a microwave for 45 s. After DNA extraction and purification using the QIAmp DNA mini Kit (Qiagen) according to the manufacturer's instructions, the samples were stored at −20 °C until needed. In protocol D, each sample was dewaxed in 20 μL of 0.5% Tween-20 (90 °C for 10 min) and incubated at 55 °C for 3 h with 30 μL of digestion buffer (22.5 μL of 1× TE, 1 μL of 20 mg/mL proteinase K and 6.5 μL of 10% SDS) using a thermal cycler. After incubation at 37 °C, the proteinase K was inactivated at 95 °C for 5 min. Finally, the samples were mixed with 480 μL of 1× TE and the DNA was extracted with chloroform/isoamyl alcohol as described in protocol B.

A total of 160 DNA extractions from 32 tissue blocks were performed using the five methods mentioned above.

Assessment of DNA purity and yield

The quality (OD260/OD280) and quantity (OD260) of DNA obtained from FFPE tissues was determined using the NANODROP 2000 (Thermo Scientific) equipment following the manufacturer's recommendations.

PCR amplification

To evaluate the quality of the DNA extracted from the tissue sections, PCR amplification of the β-globin gene was performed in two separate reactions using primers PCO3 (5′-ACA CAA CTG TGT TCA CTA GC-3′)/PCO5 (5′-GAA ACC CAA GAG TCT TCT CCT-3′) to generate a fragment of 209 bp [14] and PCO3/PCO4 (5′-CAA CTT CAT CCA CGT TCA CC-3′) to generate a fragment of 110 bp [48]. The PCR conditions have been previously reported [14].

HPV DNA was detected using the generic primer sets GP5+ (5′-TTT GTT ACT GTG GTA GAT ACT AC-3′)/GP6+ (5′-GAA AAA TAA ACT GTA AAT CAT ATT C-3′) to generate a fragment of 142 bp [15] and the new TS 16 primer set (5′-GGT CGG TGG ACC GGT CGA TG-3′)/5′-GCA ATG TAG GTG TAT CTC CA-3′) to generate a fragment of 96 bp [1] for the detection of HPV-16, the most frequent HPV genotype in the world. The conditions for PCR amplification were previously reported for each primer set [1,15].

Positive (DNA from SiHa cell line) and negative (deionized water) controls were used in each assay. The amplification products were run on 2.5% to 3.5% agarose gels stained with SYBR safe (Invitrogen, CA, USA) and visualized on a gel documentation system (Gel-Doc XR system, Bio-Rad, California, EU).

Ethical issues

The study was approved by the Ethics Committee of Universidad Tecnológica de Pereira.

Statistical analysis

The differences between the percentages of PCR positive samples detected by each extraction method or between each PCR reaction used, were analyzed by applying the Mann Whitney test. The difference in terms of quality (OD260/OD280) and quantity of DNA obtained for each method was analyzed by applying the Kruskal Wallis test. The hypotheses were tested at a 5% significance level. The GraphPad Prism Software Version 6.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis.

Table 1
Comparison of five protocols for DNA extraction in 32 FFPE tissues in terms of β-globin and HPV DNA amplification.

Protocol	β-Globin amplification[a]						HPV–DNA amplification[b]					
	110 bp (PCO3/PCO4)			209 bp (PCO3/PCO5)			96 bp (new TS16)			142 bp (GP5+/GP6+)		
	n	(%)	[95% CI]	*n*	(%)	[95% CI]	*n*	(%)	[95% CI]	*n*	(%)	[95% CI]
A	29	(90.6)	[79.9–100]	4	(12.5)	[0.38–24.6]	10	(31.2)	[14.3–48.2]	3	(9.3)	[−1.3–20]
B	29	(90.6)	[79.9–100]	8	(25.0)	[9.0–41.0]	4	(12.5)	[0.38–24.6]	0	(0.0)	[0.0]
C	25	(78.1)	[63.0–93.2]	5	(15.6)	[2.3–29.0]	5	(15.6)	[2.3–29.0]	0	(0.0)	[0.0]
D	23	(71.8)	[55.4–88.3]	8	(25.0)	[9.0–40.9]	1	(3.1)	[−3.2–9.4]	0	(0.0)	[0.0]
E	28	(87.5)	[75.3–93.6]	12	(37.5)	[19.8–55.2]	7	(21.8)	[6.0–37.0]	3	(9.3)	[−1.3–20]

CI: Confidence interval.
[a] Differences between PCO3/PCO4 and PCO3/O5 according to the DNA extraction protocol ($p < 0.0001$).
[b] Differences between GP5+/GP6+ and New TS16 according to the DNA extraction protocol ($p = 0.0317$ for protocol A, $p = 0.042$ for protocol B and $p = 0.021$ for protocol C).

Results

Five different DNA extraction protocols were carried out to evaluate the DNA obtained from FFPE tissues by PCR. The PCR amplified the human β-globin gene and the L1 and E6 HPV genes. As shown in Table 1, the five DNA extraction protocols allowed a better amplification of the 110 bp fragment of β-globin compared with the 209 bp fragment ($p < 0.0001$); however, we did not find significant differences between the five different DNA extraction protocols in terms of β-globin amplification. From the 32 samples evaluated for the presence of HPV DNA using the primers GP5+/GP6+, only three samples extracted with protocols A and E were positive, whereas samples tested with the new TS 16 primers allowed a higher amplification in all five extraction protocols used, without significant differences between them (Table 1). However, significant differences were found when we compared HPV DNA amplification between the GP5+/GP6+ PCR and the new TS 16 PCR for protocols A, B and C ($p = 0.0317$, 0.042 and 0.021, respectively; Table 1). The GP5+/GP6+ and the new TS 16 primers allowed the detection by PCR of HPV in DNA obtained from SiHa cells (data not shown).

With regard to DNA concentration and quality, the amount of DNA obtained with protocol A was higher than that obtained by the other methods ($p < 0.0001$), although its quality was the worst ($p = 0.0035$, Table 2). It is important to note that lower amounts of DNA were obtained with protocols B and E, whereas the DNA quality was similar for protocols B to E (Table 2).

The five protocols were compared in terms of time spent on the procedure, price, DNA quality and DNA efficiency. Quality was calculated as the number of β-globin positive samples divided by the total number of tested samples, whereas the efficiency of HPV DNA detection was calculated by the number of GP5+/6+ PCR positive samples divided by the number of β-globin positive samples (Table 3). The time spent on the procedures ranged from 90 to 150 min, with protocol A taking the shortest amount of time. The price (calculated for 50 samples) ranged from 100 to 150 US dollars and the cheapest procedure was protocol A.

Table 2
DNA concentration (average) and DNA quality (260/280 ratio) obtained from FFPE tissues using five different DNA extraction protocols.

Protocol	Concentration (ng/μL) ± SD[a]	Ratio 260/280 ± SD[b]
A	101.5 ± 20.90	0.6 ± 0.04
B	6.7 ± 1.97	1.1 ± 0.47
C	23.1 ± 7.16	1.1 ± 0.63
D	35.5 ± 16.35	1.2 ± 0.54
E	13.5 ± 6.10	1.3 ± 0.64

SD: Standard deviation.
[a] DNA concentration obtained with Protocol A was higher than with the other methods ($p < 0.0001$).
[b] DNA quality was the worst with Protocol A ($p = 0.0035$).

The quality of DNA detection ranged from 71% to 90.6% if an amplicon of 110 bp in the β-globin gene was detected, whereas the percentage decreased (from 37.5% to 12.5%) when an amplicon of 209 bp was detected. The efficiency of HPV DNA detection ranged from 0% to 10% in samples positive for the 110 bp amplicon and from 0% to 75% in samples that amplified the 209 bp amplicon (Table 3). These results show that the best methodology in terms of time, price, DNA quality and efficiency is protocol A

Discussion

It has become very important to have protocols for DNA extraction from FFPE tissues that are efficient and reproducible and that also yield DNA of high molecular weight with low levels of fragmentation and high quality. The isolation of high-quality DNA from FFPE tissue can be difficult because only minimal quantities of intact DNA could be present in the sample. While routine formalin fixation preserves tissue morphology, the process can cause the formation of protein-DNA crosslinks, limiting the analysis of nucleic acids by reducing the quantity and size of amplified products compared to those obtained from fresh or frozen tissues.

The success of PCR amplification from preserved tissues can vary with the type of fixative, fixation protocol, storage time, temperature and PCR conditions. Furthermore, PCR inhibitors could be present and the DNA could be degraded [17,23,25,28,34,51,52,55].

Paraffin-embedded material represents a useful resource for retrospective studies depending on the facility to collect tissues from already existing archives in comparison to frozen and fresh tissues [18]. In this study, DNA was obtained from FFPE cervix tissues using different methods and downstream applications such as PCR were performed in order to evaluate the quality of DNA and the efficiency of HPV DNA detection.

All the evaluated methods have advantages and disadvantages. Proteinase K and chloroform/isoamyl alcohol extractions are the fastest and cheapest but more laborious, and they use dangerous chemicals for DNA extraction. The commercial DNA extraction protocols were the simplest to perform but the most expensive. Many of these protocols have been used by other researchers with varying results [4,7,9,14,17,18,23].

With the procedures described here, DNA amplification was obtained from archival paraffin blocks stored for six years. However, the amplification products were obtained more efficiently with primers that amplified a shorter fragment. This is shown by the failure to amplify the larger DNA fragments (142 bp and 209 bp) than the smaller ones (96 and 110 bp), as described by other authors [1,9,15,17,27,33,34,45].

HPV DNA was only detected in three samples after PCR using GP5+/GP6+ primers with protocols A and E, while the new TS 16 primers detected HPV DNA in 10 and 7 samples with protocols A and E, respectively. This is possibly explained because the B, C and D protocols are not able to remove DNA-tissue protein cross-links,

Table 3
Comparison of five protocols for DNA extraction in 32 FFPE tissues in terms of DNA quality, efficiency of HPV DNA detection, method cost and time required for sample processing.

Protocol	DNA quality[a]				DNA efficiency[b]				Price[c]	Time[d]
	110 bp (PCO3/PCO4)		209 bp (PCO3/PCO5)		110 bp (PCO3/PCO4)		209 bp (PCO3/PCO5)		(US Dollars)	(Min)
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)		
A	29	90.6	4	12.5	3	10.3	3	75.0	100	90
B	29	90.6	8	25.0	0	0.0	0	0.0	130	150
C	25	78.1	5	15.6	0	0.0	0	0.0	150	115
D	23	71.8	8	25.0	0	0.0	0	0.0	130	115
E	28	87.5	12	37.5	3	10.7	3	25.0	150	130

[a] Number of β-globin positive samples (*n*) divided by the total number of tested samples (32).
[b] Number of GP5+/6+ PCR positive samples (*n*) divided by the number of β-globin positive samples (PCO3/PCO4: 29 for protocol A and B, 25 for protocol C, 23 for protocol D and 28 for protocol E. PCO3/PCO5: 4 for protocol A, 8 for protocol B, 5 for protocol C, 8 for protocol D, 12 for protocol E).
[c] Calculated for 50 samples.
[d] Overnight incubations were not considered.

resulting in DNA loss during the washing steps, DNA fragmentation during DNA extraction or PCR inhibition. DNA fragmentation was evident by the fact that a higher number of HPV positive samples were found using the HPV16 specific PCR (96 bp amplicon) compared with the PCR using primers GP5+/GP6+ (142 bp amplicon). The DNA obtained from the FFPE tissues evaluated in this study is highly fragmented, indicating that this is a major factor limiting DNA amplification and HPV detection. This DNA fragmentation differs between samples due to several factors such as type of fixative solution, time of fixation before embedding [21] and the use of unbuffered formalin [7,30]. All paraffin blocks used in this study were fixed with unbuffered formalin. DNA degradation in FFPE tissues precludes testing with the larger fragments (450 bp) generated with primers MY09/11 [24,33,45]. We decided to use the new TS 16 primers because in Pereira, HPV16 is the most prevalent genotype in both, CIN2/3 and SSC (Álvarez-Aldana et al. Unpublished results). HPV16 is also associated with the development of oropharyngeal squamous cell carcinoma [59], however, high risk HPV DNA detection is low in malignant and dysplastic lesions of the oral cavity [58].

It is important to note based on the DNA quality results and the efficiency to amplify HPV that even in the presence of high quality DNA (as in the DNA extracted with protocol E), the efficiency of HPV detection can be low, indicating that, depending on the extraction method, a positive PCR amplification of a human gene such us β-globin does not necessarily guarantee the successful extraction of the target DNA. The protocol A yielded high DNA quantity and was the most efficient method to detect HPV. This is the method commonly used to extract DNA in order to detect HPV [40,41,50,53,54].

The detection of HPV DNA in FFPE tissues using PCR has several limitations in terms of sensitivity even with the combination of type-specific and consensus primer sets [1,3,29,43]. Therefore, PCR techniques with high performance in terms of sensitivity, reproducibility and coverage of HPV types, such as the SPF10 LiPA, have been developed to use with FFPE tissues [6,13,26,41,49], and the results are comparable with those obtained with fresh tissues [39]. This method is based in the amplification of a 65 bp region of the L1 open reading frame [31]; however, the use of this methodology is expensive.

HPV typing using FFPE tissues is used to evaluate the impact of the HPV vaccination [35]; however, the amount of DNA obtained from these tissues and its quality can negatively affect the results.

The results of the present study show that a crude extract from FFPE tissues can be used to detect HPV DNA even if the DNA quality is not appropriate. Also, the results suggest that amplification of the β-globin gen cannot assure viral DNA detection. It is also noteworthy to mention that the tissues used in this study were microdissected to extract DNA only from abnormal tissues.

The method used to obtain DNA from FFPE tissues significantly influences downstream molecular analysis, as others have described [28,46]. The PCR technique is a robust, simple and sensitive method for HPV detection in FFPE tissues, making this technique applicable to routine HPV testing. However, it is necessary to use primers with the ability to generate small amplification products. In addition, it is very important to carefully choose the DNA extraction method from FFPE tissues depending on the specific requirements of the study being carried out [44]. Recently, RNA/DNA coextraction from FFPE tissues was reported [22].

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