ELISABETE APARECIDA CAMPOS

DETECÇÃO DO RNAm DOS ONCOGENES E6 E E7 DO HPV DE CÉLULAS DA CÉRVICE UTERINA E VERIFICAÇÃO DO EFEITO DO TEMPO DE ESTOCAGEM NA RECUPERAÇÃO DO mRNA ANTES E APÓS EXTRAÇÃO

Tese de Doutorado

ORIENTADOR: Prof. Dr. LUIS OTÁVIO ZANATTA SARIAN
CO-ORIENTADORA: Profª. Drª. SOPHIE FRANÇOISE M. DERCHAIN

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Banca examinadora:
Luiz Otávio Zanatta Sarian [Orientador]
Luiz Carlos Zeferino
Silvana Maria Quintana
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Lúcia Helena Simões da Costa Paiva

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Orientador: Prof. Dr. LUIS OTÁVIO ZANATTA SARIAN

Co-Orientadora: Profª. Drª. SOPHIE FRANÇOISE M. DERCHAIN

Membros:

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Dedico este trabalho...

Ao meu filho Gabriel,

Luz da minha vida.

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Ao prof. dr Luís Otávio Sarian por todo carinho, participação e dedicação neste projeto, tudo isso contribuiu para aumentar minha admiração e respeito por esse médico que antes de tudo é um ser humano maravilhoso.

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A minha família que é maravilhosa em todos os momentos.

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Dust in the wind

Kansas

I close my eyes
Only for a moment,
And the moment's gone.
All my dreams,
Pass before my eyes, a curiousity.
Dust in the wind,
All they are is dust in the wind.
Same old song,
Just a drop of water in an endless sea.
All we do
Crumbles to the ground,
Though we refuse to see.
Dust in the wind,
All we are is dust in the wind.
Now, don't hang on,
Nothing lasts forever
But the earth and sky.
It slips away,
And all your money
Won't another minute buy.
Dust in the wind,
All we are is dust in the wind.
Dust in the wind,
All we are is dust in the wind.

Sumário

Símbolos, Siglas e Abreviaturas

ASC – Células Escamosas Atípicas (*Atypical Squamous Cells*)

ASC H – Células Escamosas Atípicas de alto grau (*Atypical Squamous Cells - High-grade)*

CC – Câncer Cervical *(Cervical cancer)*

cDNA – **DNA complementar** (*complementary DNA*)

CEP – Comitê de Ética em Pesquisa

CH2/HC2 – Captura Híbrida 2 (*Hybrid Capture 2)*

IC/CI – Intervalo de Confiança (*Confidence interval)*

DNA – Ácido desoxirribonucleico (*Deoxyribonucleic acid)*

e.g. – Por exemplo

et al. – E outro(s); e outra(s)

FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo

H2O – DEPC – Água Milli-Q tratada com dietilpirocarbonato (*Diethylpyrocarbonate Milli-Q treated*)

HPV – Papilomavírus humano (*Human papillomavirus)*

HR – *Hazard ratios*

HR-HPV – HPV de alto risco *(High-risk HPV)*

HSIL – *Lesão intraepitelial escamosa de alto grau* (*High-grade squamous intraephitelial lesion)*

i.e. – Ou seja (*that is)*

LLETZ – Large Loop Excision of the Transformation Zone

LSIL – Lesão intraepitelial escamosa de baixo grau *(Low-grade squamous intraepithelial lesion)*

mRNA – Ácido ribonucleico mensageiro (*messenger Ribonucleic acid)*

NIC/CIN – Neoplasia intraepitelial cervical (*Cervical intraepithelial neoplasia)*

OR – *Odds ratio*

Pap – Papanicolaou

PCR – Reação em Cadeia da Polimerase (*Polymerase Chain Reaction)*

Ref – Referência *(Reference)*

UNICAMP – Universidade Estadual de Campinas

Resumo

Objetivo: Avaliar a detecção do mRNA dos oncogenes E6 e E7 do HPV através de técnica manual de baixo custo com reagente Trizol, e avaliar a recuperação do mRNA em amostras estocadas por diferentes intervalos de tempo antes e após a sua extração. **Sujeitos e métodos**: Estudo experimental, em duas fases, (1) transversal e (2) longitudinal, composto de 60 amostras de raspado cervical, sabidamente positivas para DNA dos HPV 16, 18, 31 e 45. As amostras foram obtidas de mulheres submetidas à conização cervical, em decorrência de lesões da cérvice uterina induzidas pelo HPV, atendidas pela Divisão de Oncologia Ginecológica da Universidade Estadual de Campinas (UNICAMP), São Paulo, Brasil, entre abril e dezembro de 2010. As amostras haviam sido coletadas imediatamente antes do procedimento de conização e a presença do DNA do HPV havia sido determinada por PCR (*primers* PGMY09/11). Para HPV- DNA tipo específico os *primers* foram desenhados especificamente para este estudo. A fase (1) do estudo objetivou avaliar a detecção de mRNA dos oncogenes E6 e E7 do HPV usando tecnologia manual com reagente Trizol. Para esta fase, foi realizada extração do mRNA, quantificado em espectrofotômetro e cuja qualidade foi avaliada através de eletroforese. A síntese do cDNA das amostras

positivas para mRNA foi realizada por transcriptase reversa, a fim de detectar os oncogenes E6 e E7 do HPV. Trinta e seis das 60 amostras foram consideradas positivas para ambos os oncogenes, e estes casos foram incluídos na fase (2). O objetivo desta fase foi avaliar a sobrevida do mRNA em função do tempo de estocagem das amostras a) antes da extração (imediata, 90 e 180 dias) e b) depois (imediata, 90 e 180 dias) da extração do mRNA. O grupo extração-imediata/PCR-imediata já havia sido avaliado na fase (1) do estudo e determinou a formação da amostra da fase (2). Foram então separadas 2 alíquotas de 200 microlitros do material pré-extração (a primeira alíquota já havia sido utilizada na fase (1) do estudo) para estocagem em freezer -80^{o} C por 90 e 180 dias. O produto de extração obtido da primeira alíquota pré-extração foi então separado em três outras alíquotas, a primeira delas havia sido utilizada na fase (1) para detecção dos oncogenes, e as duas restantes para estocagem por 90 e 180 dias em freezer -80^{o} C. Ao fim dos períodos de estocagem pós-extração, as alíquotas eram submetidas à transcrição e PCR para detecção dos oncogenes. Os mesmos procedimentos foram aplicados para as alíquotas arquivadas pré-extração por 90 e 180 dias. A detecção dos oncogenes E6 e E7 nos nove grupos assim formados foi utilizada para avaliação da sobrevida do mRNA, em função do tempo de estocagem antes e após a extração, através da técnica de maximização não-paramétrica de probabilidades (M-NPL). **Resultados**: O mRNA dos oncogenes E6 e E7 foi encontrado em 36/ 60 (60%) das amostras positivas para HPV dos genótipos de interesse. A presença do HPV 16 nas amostras foi positivamente associada com a expressão do mRNA (OR=9,08; 95%CI 1,26 para 65,32). A sobrevida do mRNA esteve negativamente associada ao tempo de estocagem pré-

extração maior de 180 dias (HR=2,63; IC95% 1,16 a 6,0), e positivamente associada à idade da mulher <30 anos (HR=0,93; IC95% 0,87 a 0,98) e à presença de HPV 31 na amostra (HR=0,39; IC95% 0,18 a 0,86). **Conclusões:** utilizando a técnica manual e de baixo custo Trizol, obteve-se mRNA dos oncogenes E6/E7 do HPV em quantidades semelhantes àquelas relatadas por outros pesquisadores utilizando técnicas automatizadas e de tecnologia proprietária, de custo substancialmente mais elevado que a técnica utilizada neste estudo. Concluiu-se também que o tempo de armazenamento pré-estocagem afeta negativamente a qualidade do mRNA, o que leva a sugerir que as amostras sejam estocadas após a extração do mRNA.

Palavras-chave: HPV-DNA, Expressão mRNA, cDNA, Transcritos E6/E7.

Summary

Objective: To evaluate the detection of mRNA of HPV E6 and E7 oncogenes using a manual low-cost Trizol-based technique, and evaluate the recovery of mRNA in samples stored for different periods of time before and after extraction. **Subjects and methods:** experimental study in two phases, (a) cross-sectional and (2) prospective, comprising 60 samples of cervical scrapes, known to be positive for DNA of HPV 16, 18, 31 and/or 45. The samples were obtained from women undergoing cervical conization due to cervical lesions induced by HPV, assisted by the Division of Gynecologic Oncology at the University of Campinas (UNICAMP), Sao Paulo, Brazil, between April and December 2010. The samples were collected immediately prior to cervical conization, and the presence of HPV DNA was determined by PCR (primers PGMY09/11). Step (1) of the study was to evaluate mRNA detection of oncogenes E6 and E7 of HPV using Trizol reagent manual technology. For this phase, we performed mRNA extraction, quantified by spectrophotometer and the quality of the extracted mRNA was assessed by electrophoresis. The synthesis of cDNA from the mRNA of positive samples was performed by reverse transcriptase in order to detect oncogenes E6 and E7 of HPV. Thirty-six of the 60 samples were positive for both oncogenes,

and these patients were included in step (2). The objective of this phase was to evaluate the survival of the mRNA as a function of storage time of the samples a) before extraction (immediate, 90 and 180 days) and b) after (immediate, 90 and 180 days) the extraction of mRNA. The immediate extraction/immediate PCR group had been ascertained in step (1) and determined the formation of the sample for step (2). Two 200 microliters aliquots of the pre-extraction material (the first rate has already been used in step (a) of the study) were stored in a -80^{o}C freezer for 90 and 180 days. The product obtained by extraction of the pre-extraction first aliquot was then separated into three other aliquots, the first of these was used in step (a) to detect the oncogenes, and the two other were stored for 90 and 180 days. At the end of the post-extraction storage periods the aliquots were subjected to transcription and PCR for detection of the oncogenes. The same procedures were applied to the pre-extraction aliquots stored for 90 and 180 days. The detection of oncogenes E6 and E7 in the nine groups thus formed was used to evaluate the survival of the mRNA as a function of storage time before and after the extraction, through the technique of maximizing non-parametric likelihood (M-NPL). **Results:** The mRNA of oncogenes E6 and E7 was found in 36/60 (60%) samples positive for the HPV genotypes of interest. The presence of HPV 16 in these samples was positively associated with mRNA detection (OR = 9.08, 95% CI 1.26 to 65.32). The survival of the mRNA was negatively associated with pre-extraction storage time greater than 180 days (HR = 2.63, 95% CI 1.16 to 6.0), and positively associated with age <30 years (HR = 0 , 93, 95% CI 0.87 to 0.98) and the presence of HPV 31 in the sample (HR = 0.39, 95% CI 0.18 to 0.86). **Conclusions:** Using the low cost Trizol

manual technique, we obtained mRNA of HPV E6/E7 oncogenes in amounts similar to those reported by other researchers using automated proprietary technology, which are substantially costlier than the technique used in our study. We also conclude that the pre-extraction storage time negatively affects the quality of the mRNA, which leads us to suggest that the samples should be stored after mRNA extraction.

Keywords: HPV-DNA, mRNA expression, cDNA transcripts E6/E7.

1. Introdução

A infecção persistente pelo Papillomavírus humano (HPV) é um requisito para o desenvolvimento do câncer do colo do útero (CC) e suas lesões precursoras, as neoplasias intraepiteliais cervicais (NIC). Cerca de 40 tipos de HPV infectam o trato genital feminino, sendo 15 deles considerados de alto risco oncogênico (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 e 82) (1,2). Dentre os tipos de alto risco, os mais comumente encontrados nas lesões precursoras são os HPV tipos 16 e 18 e, dependendo da região geográfica estudada, o HPV 16 é o tipo que predomina em 60 a 80% dos casos (3,4).

Os testes para detecção do HPV e ou determinação do tipo viral, eram utilizados sobretudo em estudos epidemiológicos, a princípio visando estabelecer uma relação causal entre HPV e NIC ou CC. Com o aprimoramento das técnicas de biologia molecular, a determinação do tipo viral passou a ser usada em ampla escala em estudos clínicos e hoje começa a ter importância na prática clínica.O teste para DNA-HPV tem sido associado ao teste de Papanicolaou nos programas de *screening* nos Estados Unidos e em outros países, para algumas situações

clínicas específicas. Atualmente, a Captura de Híbridos 2 (CH2) QIAGEN e a Reação em Cadeia da Polimerase (PCR) são as técnicas mais comumente usadas para detecção do DNA-HPV. A CH2 detecta o DNA do HPV, de baixo e alto riscos oncogênicos sem identificar o tipo, enquanto a PCR, além da detecção, permite também a determinação do tipo viral (5,6).

O genoma do HPV é constituído aproximadamente por 8 *Open Read Frames (ORFs),* possuindo pelo menos 6 genes que se expressam precocemente e estão localizados na região denominada E *(Early),* dois que se expressam tardiamente, localizados na região denominada L *(Late)* e entre estas duas há uma região chamada de LCR que é de controle viral. A região E codifica as proteínas precoces (E1, E2, E4, E5, E6 e E7), que são responsáveis pela replicação, a transcrição viral e a transformação celular (7).

Dentre os eventos biológicos relacionados à infecção pelo HPV, estão àqueles decorrentes da ação das proteínas E6 e E7, que foram classificadas como genes oncogênicos, devido a sua capacidade de induzir alterações malignas em células infectadas. A oncoproteína viral E6 inicia a degradação da proteína do gene supressor de tumor p53, enquanto a oncoproteína E7 leva a inativação da proteína do gene supressor de tumor pRB. Hoje se sabe que esses são passos importantes na carcinogênese (8).

A verificação da expressão dos transcritos de E6 e E7 de tipos de HPV de alto risco tem sido sugerida como um teste que poderá servir como marcador de avaliação do risco de desenvolvimento da NIC (9,10). Estudos têm

sido realizados para verificar a expressão dessas oncoproteínas em amostras clínicas de pacientes infectadas com HPV de alto risco, com o intuito de analisar quais pacientes têm um risco aumentado para desenvolvimento de lesão de alto grau ou CC, separando-as daquelas que têm um menor risco para desenvolver a doença (11, 12, 13).

A utilização do mRNA é vantajosa para os fins já apontados, pois permite averiguar o processo de malignização em fases ainda incipientes, antes que anormalidades em proteínas (e, em um passo imediatamente seguinte, morfológicas) estejam presentes. Contudo, as moléculas de mRNA são instáveis, o que pode dificultar sua detecção em ensaios clínicos. Uma aplicação evidente é a detecção do mRNA dos oncogenes E6 e E7, a fim de se determinar se a infecção pelo HPV está produzindo os efeitos biológicos necessários para a carcinogênese (13). Para que a detecção do mRNA seja efetiva, após a coleta, os tecidos devem ser congelados imediatamente ou mantidos em uma solução que possa assegurar a integridade das ligações entre as bases nitrogenadas (14).

No último quinquênio, a questão da sobrevida de moléculas como o DNA e o próprio mRNA têm sido abordados intensamente, inclusive por este grupo de pesquisa. Autores referiram-se à recuperação de DNA HPV de esfregaços fixados e tecidos incluídos em parafina (15,16). O presente estudo, utilizando células de esfoliado cervical coletadas em a) meio líquido STM e b) Universal Collection Medium (UCM), já com reagente denaturante para realização de CH2, verificou em estudos anteriores que é possível recuperar 90% e 95%, respectivamente, de DNA- HPV viável para aplicação em técnicas moleculares

(17,18). Considerando as possíveis aplicações clínicas mencionadas anteriormente, e que várias metodologias estão atualmente disponíveis para a obtenção do mRNA e sua posterior amplificação, variando de alternativas de implementação fundamentalmente manual, baseadas na mistura de guanidina tiocinato, acetato de sódio, fenol clorofórmio, a *kits* específicos e totalmente automatizados. Contudo, os estudos comparativos da efetividade destas variantes técnicas são escassos e, eventualmente, sujeitos a conflitos de interesse (19,20,21).

A qualidade do mRNA obtido através de cada uma dessas técnicas anteriormente citadas pode influenciar a precisão dos resultados encontrados, e as condições de armazenamento das amostras são muito importantes para assegurar boas amostras de mRNA (14). Os fatores que mais influenciam na qualidade dos ácidos nucléicos extraídos de amostras fixadas são: o tipo de extração, o meio de coleta e o tempo de fixação do material. As amostras coletadas em meios de transporte para citologia líquida já são utilizadas para testes de extração de DNA e podem ser utilizadas para testes com mRNA se o rendimento e qualidade das extrações das amostras fossem melhorados (22).

A combinação entre uma boa técnica de extração e de detecção pode permitir a obtenção de mRNA de qualidade para uso em experimentos moleculares. O conhecimento atual sobre o tempo de preservação de DNA extraído de amostras de raspado cervical é valioso para a formação de bibliotecas. No entanto, o conhecimento em relação ao tempo em que amostras de raspado cervical poderiam ser preservadas, mantendo-se intacto o mRNA, ainda é falho. Sugere-se que o mRNA seja extremamente susceptível à deterioração em curto prazo, mas os

estudos sobre o tema são limitados na variável tempo de preservação (19, 23). Questiona-se qual seria o tempo máximo que as amostras poderiam ficar estocadas em meios para citologia líquida antes da extração do mRNA sem que houvesse perda de sua qualidade. Questiona-se também se amostras estocadas com mRNA extraído seriam passíveis de estocagem. E se a resposta for sim, questiona-se por quanto tempo.Outra pergunta é o que poderia causar menor degradação. Além disso, faltam dados na literatura sobre a efetividade que determinadas técnicas de extração possam ter sobre a qualidade do mRNA extraído (24).

As respostas às indagações mencionadas no parágrafo anterior permitiriam estabelecer a viabilidade da formação de bibliotecas de amostras para utilização em estudos posteriores ou quando for necessária a formação de coortes numerosas, ao longo de períodos de tempo extensos. Caso haja boa preservação do mRNA, o estudo também permitirá determinar o melhor momento (antes ou depois da extração) para a estocagem do material. Se, futuramente, os testes para detecção da super expressão dos oncogenes E6 e E7 se tornarem realmente um marcador primário no desenvolvimento das neoplasias cervicais e ou CC, este estudo terá contribuído para estabelecer um tempo de estocagem das amostras coletadas. Duas questões de interesse do laboratorista também serão abordadas neste estudo: 1) se técnicas de obtenção de mRNA debaixo custo, manuais, aplicáveis em circunstâncias de recursos econômicos limitados, teriam desempenho semelhante aos sistemas automatizados e de alto custo; 2) qual seria o melhor momento para a estocagem do material, se antes ou após a extraçãp do mRNA, e por quanto tempo estas fases de estocagem podem perdurar.

2. Objetivos

2.1. Objetivo Geral

Avaliar a detecção e a sobrevida do mRNA dos oncogenes E6 e E7 do HPV em amostras de raspado cervical sabidamente positivas para DNA dos HPV 16, 18, 31 e 45.

2.2. Objetivos Específicos

- **Artigo1:** Determinar a detecção de mRNA dos oncogenes E6 e E7 em amostras de raspado cervical sabidamente positivas para DNA de HPV tipos 16, 18, 31 e 45, utilizando extração manual, de baixo custo, com reagente TRizol.

- **Artigo 2:** Determinar a sobrevida do mRNA dos oncogenes E6 e E7, dos HPV 16, 18, 31 e 45 em função do tempo de: 1) estocagem das amostras em meio líquido antes da extração e 2) estocagem do mRNA extraído.

3. Publicações

Artigo 1 – Retrieval of HPV oncogenes E6 and E7 mRNA from cervical specimens using an manual open technology protocol

Artigo 2 – Effects of storage time on Human Papillomavirus oncogenes E6 and E7 mRNA viability in women with cervical intraepithelial neoplasia (CIN)

3.1. Artigo 1

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Retrieval of HPV oncogenes E6 and E7 mRNA from cervical specimens using an manual open technology protocol

Authors:

Elisabete Aparecida Campos, Biologist, PhD

Denise Pitta, Biologist, Master

Angela Maria de Assis, PhD

Sophie Derchain, M.D., PhD

Luis Otavio Sarian, M.D., PhD

1. Campinas State University, Department of Obstetrics and Gynecology, Campinas, Sao Paulo, Brazil

Correspondence to:

Prof. Luis Otávio Sarian

Campinas State University

Department of Obstetrics and Gynecology

Campinas, Sao Paulo, Brazil

e-mail: sarian@terra.com.br

Introduction

In the last two decades, the detection of Human papillomavirus (HPV) deoxyribonucleic acid (DNA) in cervical samples has been proven to be a good diagnostic and risk predictor tool for cervical intraepithelial neoplasia (CIN) and cervical cancer (CC) [1]. More recently, some studies have suggested that the levels of messenger ribonucleic acid (mRNA) of HPV oncogenes E6 and E7 in specimens collected from the uterine cervix may be a more specific early indicator of predisposition to carcinogenesis than DNA levels [2 - 4]. This may be related to the fact that the oncogenic potential of the high-risk HPV types (hr-HPV) lies in the actions of the oncoproteins coded by E6/E7, which bind to and modulate a number of different gene products, in particular, the tumor suppressors p53 and pRb [5, 6]. These interactions may lead to a disturbance of cell cycle control, and a deficiency in DNA repair, resulting in genomic instability and increased risk of malignant transformation [7, 8].

However, the potential use of HPV oncogenes mRNA detection in clinical practice is still under investigation, and robust confirmatory studies are pending. One of the major limiting factors for the development of such studies is the fact that in order to determine mRNA levels in cervical specimens, one must rely on a handful of detection assays, which in turn display different performance profiles. The totality of currently marketed assay options for mRNA detection makes use of proprietary technology, and features a prespecified set of target HPV types which is determined by the manufacturers. The PreTect HPV-Proofer (NorChip) and the NucliSENS Easy Q HPV (bioMérieux) are based on the same technology, and detect E6/E7 mRNA expression from the five most prevalent hrHPV types (16, 18, 31, 33 and 45). Other two tests aim at

an expanded set of HPV types: the TaqMan real-time PCR assay, which targets 12 high-risk (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59) and two low-risk (HPV6 and -11) types using E6/E7 region primers and probes in a duplex format [9, 10], and the APTIMA HPV Assay (Gen-Probe) targets mRNA expression of the 14 most carcinogenic hr-HPV types [11, 12]. However, the prevalence of the HPV types may vary between female populations, and the prespecified set of HPV types in a given test may prove to be inadequate in some epidemiological scenarios. Also importantly, the use of proprietary technology hinders the development of assay adaptations to laboratorial and economic local conditions. E.g., the costs of the currently available comercial assays may be not compatible with the resources available to many routine laboratories, especially in economically disadvantaged regions which, paradoxically, are heavily burdened by HPV-related disease.

The objective of this study was to assess the detection rate of HPV oncogenes E6 and E7 mRNA from cervical specimens, using a manual open technology protocol, and determine whether the key epidemiological factors related to HPV viral load may affect the mRNA retrieval yielded by this technique.

Subjects and methods

Sample and data collection

The study was carried out at the colposcopy clinics of Campinas State University (Unicamp), Brazil, a public health institution dedicated to comprehensive care for women, and was approved by the local Ethics Committee (Protocol: N° CEP 723/2009). The sample consisted of cervical specimens, collected from women who underwent cervical diathermic conization due to HPV-induced squamous lesions at Unicamp's

cervical pathology clinics. Women were invited to participate while awaiting the conization procedure. After signing the informed consent, an interview concerning clinical and epidemiological aspects was performed. After that, and immediately before the conization procedure, a cervical specimen was obtained with an endocervical brush, and stored in a 1-mL tube containing Specimen Transport Medium (STM, Qiagen Inc.).

In order to obtain the sample for these experiments we examined 117 specimens between April and December 2010. We first performed PCR reactions in order to ascertain the presence of HPV DNA using standard protocols [13]. Of the 117 original cases 74 were positive for HPV DNA (pgmy 9/11). We next performed PCR reactions in order to ascertain the presence of DNA of the following HPV types: 16, 18, 31 and/or 45. Samples not harboring at least one of the HPV types of interest were discarded, and the patient excluded from the study. After completing 60 samples positive for at least one of those HPV types, we closed the accrual phase of the study (the estimated sample size, considering 95% confidence intervals, and 80% Beta power, for an estimated difference in mRNA levels of 20% between women harboring different HPV types, was 58 women). We then proceeded to the next phase of the study: detection of the mRNA in the sample.

mRNA extraction

As mentioned earlier, only samples positive for DNA of HPV types 16, 18, 31 and 45 were tested for mRNA. For mRNA extraction, an aliquot of 200 ul of STM was sampled (Qiagen) and centrifuged at 13,000 *g* for 10 min. The supernatant was removed and 1 mL of the TRizol™ reagent (Invitrogen, Carlsbad, USA) was added at cellular pellet and the cells were lysed by repeat pipetting and stand at room temperature for 5

min. After that, 200 ul of chloroform was added and shaken vigorously, stand at room temperature for 3 min before centrifugation at 12,000 *g* for 15 min. The resultant aqueous layer was transferred to a new tube. To the aqueous solution, a volume of 500 µl of isoamyl alcohol was added and mixed by vortex. The whole mixture was stand at -80° C for 2 hours and 10 min at room temperature and centrifuged at 12,000 *g* for 10 min. The supernatant was removed and 1 mL of iced ethanol was added and shaken vigorously. The mixture was centrifuged at 7,500 *g* for 5 min, the supernatant was removed and after the pellet of mRNA was dry, mRNA was eluted in 30µl of mRNAase- free water (DEPC) and stored at−80 ◦C pending analysis. The mRNA purity and concentration were determined by the absorbance at 260 nm (A260) and 280 nm (A280) using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA).

Type-specific PCR for HPV 16, 18, 31 and 45 oncogenes E6 and E7 mRNA

The reactions were done separately according the reagent mixture: five µl of DNA were added to a PCR mix containing 0.5pmol each primers (Table 1), specifically designed by the investigators for the present study, 4.0mM MgCl2, 0.25mM dNTPs and 5 units of Taq platinum in a final volume of 50µl. All the steps of the PCR reactions were similar, except annealing temperature that was different for each type HPV. During each PCR run, all samples were tested together with one negative control (water) and one positive control (obtained in priori studies). A 9-min denaturation step at 95°C was followed by 40 cycles of amplification. Each cycle included a denaturation step at 95°C for 1 min, a primer annealing step at according each type HPV for 1 min, and a chain elongation step at 72°C for 1 minute. The final elongation step was prolonged by 7 min to ensure a complete extension of the amplified DNA.

cDNA transcription

The mRNA was reverse-transcribed in first strand cDNA (SuperScript III First Strand Synthesis System, Invitrogen), according to the manufacturer's guidelines. The PCR product of cDNA was amplified as described in the type-specific PCR section, however with a final volume of 25µl and 2µl of cDNA.

Statistical analyses

All statistical analyses were performed with the R Environment for Statistical Computing (R Project). Significance was set at 95% (p=0.05) and 95% confidence intervals were used (95%CI). We constructed a multivariate regression model assessing the HPV oncogenes E6 and E7 mRNA retrieval according to the clinical features of the women and the presence of HPV-DNA of types 16, 18, 31 and/or45. Odds ratios were obtained by exponentiation of the regression coefficients.

Results

We retrieved oncogenes E6 and E7 mRNA from 36 (60%) of the 60 specimens previously found to be positive for the DNA of the HPV genotypes of interest. None of the clinical and epidemiological features of the women classically linked to viral load, was significantly associated with mRNA retrieval. The presence of HPV 16 and 18 in the sample was positively associated with mRNA retrieval (OR=9.08; 95%CI 1.26 to 65.32 for HPV 16; and 18.2; IC95% 1.86 to 391.44 for HPV 18). Presence of other HPV genotypes was not associated to mRNA retrieval at the same rate, and the associations were not significant at the 95% threshold (Table 2).

Table 3 lists the key findings of the major studies addressing the mRNA detection rate in HPV-DNA positive cervical specimens. Our detection rate of 60% is comparable to that reported by Halfon & cols. [5] (68.5%) and Ratnam & cols. [3] (68%), who used the automated NucliSens Easy Q HPV Assay BioMerieux and Aptima HPV detection assays, respectively. Those detection assays are targeted to HPV types 16, 18, 31, 33 and 45, which is basically the same set of HPV types addressed in our experiments, except for HPV 33. Dockter and cols. [12], compare the performance of the fully automated Aptima HPV Assay DST with that of the semi automated Aptima HPV Assay Tigris DST System. In samples previously tested for HPV-DNA with Hybrid Capture 2, they achieved 92.6% and 91.9% E6/E7 mRNA detection rates with those techniques, respectively. However, those assays target 14 HPV types. Molden and col. [14] and Benevolo & col. [2] using the Pre-Tec HPV Proofer NASBA report 22.8% and 36.0% mRNA detection rates, respectively.

Discussion

In our study, using open technology protocols, which thanks to their relatively low-cost are very appropriate for low-resource settings, we retrieved viable mRNA of the oncogenes E6 and E7 in almost 60% of the HPV-DNA positive cases. To our knowledge, ours is the only study in which a completely manual extraction technique has been used so far. The other studies on this subject used automated our semi-automated detection techniques, with manufacturer prespecified sets of HPV types included in the assay. Our mRNA detection rate was comparable to that reported by most authors using automated mRNA detection techniques [2, 7]. With proprietary technology protocols, such as the PreTect HPV-Proofer (NorChip) and the NucliSENS

Easy Q HPV (bioMérieux), studies similar to ours achieved overall mRNA retrieval rates ranging from 36% to 68% [2, 3, 5, 7, 12, 14, 15). Therefore, our results clearly suggest that open technology protocols for mRNA detection may be used safely in the clinical and laboratorial practice. Also of note, open technology manual techniques such as the one used by us may allow tailoring the experiments to the HPV epidemiological profile (i.e. the HPV types most prevalent) of the population under scrutiny.

The scientific evidence accumulated from virological, molecular, clinical and epidemiological studies has demonstrated unequivocally that cervical cancer is in fact a sequel to a long term unresolved infection by certain genotypes of the HPV [16]. In the largest multinational studies performed so far, HPV types 16, 18, 31, 33 and 45 were shown to be the most prevalent types associated with cervical carcinomas with HPV-16 alone found in more than 50% of the cases [6, 17]. In North India, HPV-16 was by far the commonest single type in all histological categories. Similar results have been reported in other studies [18]. Studies using quantitative type-specific PCR for high-risk HPV-16, -18, -31, -33, and -45 and low-risk HPV-6 and -11 have shown that HPV-16 can reach much higher viral loads than the other types and that only for HPV-16 does increased viral load correlate with increased severity of cervical disease [19]. All these previous studies leave unattended the question as to whether better oncogene mRNA transcription rates may favor the carcinogenic potential of some of the HPV types, and whether individual patient characteristics may also be involved in the DNA to mRNA transcription rates.

For the reasons mentioned in the last paragraph, the potential for clinical applicability of mRNA detection in low-resource backgrounds is large. Despite the substantial resources spent in cytology screening and follow-up, cervical cancer is still

the 10th most common cause of cancer death in European women. In Brazil, by contrast, although rapidly declining, cervical cancer is one of the leading causes of mortality among women, and the cervical cancer burden is even heavier in less developed countries. Because cervical cancer is the only cancer that is almost completely preventable through regular screening and thus early treatment, improvement and expansion of existing screening strategies and technologies constitutes a main target of the European Council Recommendation on Cancer Screening [20]. However, many of the proposed screening strategies, due to resource constraints, may not be applicable worldwide. A test that can reliably differentiate between transient and persistent infections would allow additional effective targeting and prevention of cervical cancer [11] is very welcomed, and probably mRNA detection in cervical samples may serve this purpose, for the reasons mentioned above.

Current lines of evidence reinforce the biological reasons for detecting oncogenes E6 and E7 mRNA. HPV screening cohort studies have shown that HPVDNA testing has a higher sensitivity than cytological testing for the detection of cervical lesions, although it has slightly lower specificity [5]. The sensitivity for moderate dysplasia or worse is lower, but the specificity is higher [7, 21, 22]. It is well-known that many cervical lesions with moderate or severe dysplasia will regress spontaneously. Only 31% of colposcopically visible lesions with severe dysplasia will progress to invasive cancer within 30 years [23]. Changes such as integration of viral DNA (which stabilizes the expression of E6 and E7) are virus specific, and are associated with the malignant progression of the tumor. Other changes include the alteration of cellular genes, leading to down regulation of tumor suppressor genes and proapoptotic genes or upregulation of proto-oncogenes or antiapoptotic genes. These alterations reflect the

effects of prolonged viral gene expression, in particular viral proteins E6 and E7 [24]. In productive HPV infections, which appear cytologically as LSIL and histologically as CIN1, the expression of the viral E6 and E7 oncogenes is tightly regulated, with high-level expression only in suprabasal postmitotic cells. On the other hand, in high-grade CIN and cancer, E6 and E7 are expressed throughout the thickness of the cervical epithelium [25].

Given the fact that sustained viral oncogene E6 and E7 expression is essential for the initiation and maintenance of the transformed phenotype induced by mucosal high-risk HPVs, several assays have been developed for the specific detection of HPV E6⁄E7 mRNA [26], whereas, the test positivity rates of the HPV mRNA test are one third of the HPV DNA tests. Increased knowledge concerning the molecular biology of cervical carcinogenesis raises expectations that biomarkers will result in more accurate diagnosis of cervical cancer [27]. Moreover, the emergence of molecular medicine has resulted in the increased use of RNA in clinical diagnostics [28]. The identification of persistent infections has become a primary target of HPV mRNA testing because infections that do not regress are more likely to lead to cellular changes of the cervix, pre cancer lesions and/or invasive cervical cancer.

DNA and mRNA testing may be employed together for screening to take advantage of the combined higher sensitivity and specificity, respectively, of the tests; patients would e.g. then be referred for a biopsy when both tests are positive [5]. Although the combined use of DNA and mRNA is a promising alternative in order to improve the performance of molecular-biology based screening of cervical abnormalities, proprietary technology costs may preclude the implementation of the technology in low-resource settings, and the use of assays targeted at a prespecified set

of HPV types may be a waste of resources in regions with an HPV type distribution that does not match that of the target HPV types of the assay.

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Table 1 – Primers designed for E6/E7 mRNA detection

HPV	Region	Size	Sequence
16	E6	± 659 pb	5’ TAAAACTAAGGGCGTAACCG 3’ 5’ TCTATTTCATCCTCCTCCTCTG 3’
16	E7	± 491 pb	5’ ACTGTGTCCTGAAGAAAAGCAA 3’ 5’AACCATCCATTACATCCCGT 3’
18	E6	± 608 pb	5’ TAGGTTGGGCAGCACATACT 3’ 5’ ATACTTGTGTTTCTCTGCGTCG 3’
	E7	± 358 pb	5’ CGACGCAGAGAAACACAAGTAT 3’ 5’ ATTGTTGCTTACTGCTGGGAT 3’
31	E6	± 606 pb	5’ AAGTAGGGAGTGACCGAAAGT 3’ 5’ ACAGTGGAGGTCAGTTGCC 3’
	E7	± 437 pb	5’ AGGCACGGCAAGAAAGACT 3’ 5’ AAAGAACCAGCCATTACACC 3’
45	E6	± 659 pb	5’ ATACTACATAAAAAAGGGTG 3’ 5’TCGTAACACAACAGGTCAACA 3’
	E7	± 439 pb	5’ AGGCACGGCAAGAAAGACT 3’ 5’ AAAGAACCAGCCATTACACC 3’

Table 2 – Recovery of viable E6/E7 mRNA according to key clinical features of the women and cyto-histological characteristics of the samples

Characteristic	mRNA E6 or E7 neg (%)	mRNA E6 or E7 pos (%)	OR	(95% CI)	p
Age					
<30	14 (58.3)	16 (44.4)	4.18	(0.87 to 20.05)	0.073
>30	10 (41.7)	20 (55.6)	1.00		
First intercourse					
<16	14 (58.3)	19 (52.8)	0.73	(0.20 to 2.72)	0.637
>16	10 (41.7)	17 (47.2)	1.00		
Lifetime sex partners					
>2	5 (20.8)	9 (25.0)	0.55	(0.11 to 2.69)	0.461
1-2	19 (79.2)	27 (75.0)	1.00		
Current smoker					
Yes	8 (33.3)	11 (30.6)	0.80	(0.20 to 3.18)	0.751
No	16 (66.7)	25 (69.4)	1.00		
Citology					
ASC-H or HSIL	19 (79.2)	27 (75.0)	0.36	(0.06 to 2.02)	0.245
ASC-US or LSIL	5 (20.8)	9 (25.0)	1.00		
Histology					
CIN2-CIN3	21 (87.5)	35 (97.2)	5.43	(0.24 to 124.79)	0.290
Cervivitis-CIN1	3 (12.5)	1 (2.8)	1.00		
HPV Type-specific mRNA transcription*, **					
DNA-HPV 16					
positive	17 (40.4)	25 (59.6)	9.08	(1.26 to 65.32)	0.028
DNA HPV 18					
positive	2 (28.5)	5 (71.5)	18.27	(0.85 to 392.44)	0.033
DNA HPV 31					
positive	14 (54.4)	12 (45.6)	3.18	(0.68 to 14.89)	0.142
DNA HPV 45					
positive	5 (50.0)	5 (50.0)	13.45	(0.73 to 246.64)	0.080

*As per study design, samples negative for a given HPV type were not tested for that type-specific mRNA; **percentages in rows

Table 3 – Studies assessing mRNA retrieval from HPV-DNA positive cervical samples using currently available detection assays

Author	Year	Detection technique	Automation	HPV types included in assay	E6/E7 mRNA detection rate
Molden	2005	Pre-Tec HPV Proofer NASBA	Automated	16, 18, 31, 33, 45	98/429 (22,8%)
Catani	2009	NucliSens Easy Q HPV Assay BioMerieux	Automated	16, 18, 31, 33, 45	81/180 (45%)
Dockter	2009	Aptima HPV Assay Tigris DST System	Semi-automated	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68	407/443 (91,9%)
Dockter	2009	Aptima HPV Assay DST	Automated	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68	410/443 (92,6%)
Halfon	2010	NucliSens Easy Q HPV Assay BioMerieux	Automated	16, 18, 31, 33, 45	61/89 (68.5%)
Monsonego	2010	Aptima HPV Assay	Automated	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68	456/4429 (10.3%)
Benevolo	2011	Pre-Tec HPV Proofer NASBA	Automated	16, 18, 31, 33, 45	162/464 (36%)
Ratnam	2011	Aptima HPV Assay	Automated	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68	964/1418 (68%)
Our study	**2012**	**Homebrew low-cost TRizol protocol**	**Manual**	**16, 18, 31, 45**	**36/60 (60%)**

3.2. Artigo 2

----- Mensagem encaminhada -----

09-Mar-2012

Effects of storage time before and after extraction on the quality of HPV oncogenes E6 and E7 mRNA obtained from cervical specimens
Campos, Elisabete; Pitta, Denise; Assis, Angela; Derchain, Sophie; Sarian, Luís
NAR-00669-2012

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This letter was generated automatically at the time of submission.

Effects of storage time on Human Papillomavirus oncogenes E6 and E7 mRNA viability in women with cervical intraepithelial neoplasia (CIN)

Authors:

Elisabete Aparecida Campos, Biologist, PhD

Denise Pitta, Biologist, Master

Angela Maria de Assis, PhD

Sophie Derchain, M.D., PhD

Luis Otavio Sarian, M.D., PhD

1. Campinas State University, Department of Obstetrics and Gynecology, Campinas, Sao Paulo, Brazil

Correspondence to:

Prof. Luis Otávio Sarian

Campinas State University

Department of Obstetrics and Gynecology

Campinas, Sao Paulo, Brazil

e-mail: sarian@terra.com.br

Introduction

Research and laboratory logistics are heavily dependent on the lifespan of stored biological samples. It is widely accepted that RNA products are fairly less resistant to the effects of time than DNA specimens and this fact has important technical implications (1). The urge in processing samples timely may lead to suboptimal utilization of laboratory resources due to losses in the processing scale. However the true effects of time on mRNA degradation in e.g. human papillomavirus (HPV) samples have never been solidly ascertained in controlled laboratorial conditions and in theory improper awareness of effects of time on mRNA survival may lead to premature disposal of samples and thus to loss of invaluable clinical and research data (2).

In HPV research oncogenes E6/7 mRNA detection has become a potential target for routine clinical practices because recent evidence shows that mRNA detection may improve reproducibility and specificity of HPV testing compared to that yield by DNA detection. However the less stable moiety of mRNA compared to that of DNA could affect the suitability of mRNA based testing in clinical samples (3). Many authors have been concerned about the storage of HPV RNA in different liquid media such as PreservCyt ThinPrep SurePath RNALater (2-5). Due to the presumed instability of mRNA most studies recommend that the mRNA be used immediately after sampling or that tissue sample be frozen in liquid nitrogen for future testing (5). We know that several liquid media can be used for subsequent mRNA extraction but few studies refer to how long after the sample has been collected the mRNA can be extracted. Also it remains unknown how long after extraction the mRNA can be considered suitable for instance cDNA production. All these information may prove useful in laboratory routine planning.

In this prospective study we designed an experiment in which we stored aliquots of mRNA samples for different amounts of time and assessed the survival of viable material for HPV E6/E7 cDNA production. We thus obtained important information on the lifespan of mRNA in preserved HPV samples which we presume would be of value to research and clinical laboratories keeping libraries of stored specimens.

Materials and Methods

In this experimental study we addressed the survival of viable mRNA of HPV oncogenes according to two time effects: 1) storage time before mRNA extraction and 2) storage time of extracted mRNA samples. The study was carried out at the colposcopy clinics of Campinas State University (UNICAMP), Brazil, a public health institution dedicated to comprehensive care for women, and was approved by the local Ethics Committee (Protocol: N° CEP 723/2009). The sample consisted of cervical specimens, collected from women who underwent cervical diathermic conization due to HPV-induced squamous lesions at Unicamp's cervical pathology clinics. Women were invited to participate while awaiting the conization procedure. After signing the informed consent, an interview concerning clinical and epidemiological aspects was performed. After that, and immediately before the conization procedure, a cervical specimen was obtained with an endocervical brush, and stored in a 1-mL tube containing Specimen Transport Medium (STM, Qiagen Inc.).

In order to obtain the sample for these experiments we examined 117 specimens between April and December 2010. The experimental phase of the study, as described below, lasted through November 2011. We first performed PCR reactions in order to ascertain the presence of HPV DNA using standard protocols (6). Of the 117 original

cases 74 were positive for HPV DNA (PGMY 9/11). We next performed PCR reactions in order to ascertain the presence of DNA of the following HPV types: 16, 18, 31 and/or 45. Samples not harboring at least one of the HPV types of interest were discarded, and the patient excluded from the study. After completing 60 samples positive for at least one of those HPV types, we closed the accrual phase of the study. The sample size calculation was based on the presumed differences of the rate of decay of mRNA quality among study groups over time. We estimated a mean difference of 15% in mRNA recovery between groups 5% confidence intervals 80% power and 60 days of accrual time. These parameters resulted in a sample size of 30 in each of the three study groups. Next we divided the samples intro three aliquots of equal volume forming the three study groups, each one with 36 samples: a) immediate mRNA extraction; b) mRNA extraction 90 days after collection and c) mRNA extraction 180 days after collection. We then performed mRNA extraction (see technical details below) on group **a**: we obtained viable mRNA from 36 samples; the remaining 24 cases were discontinued (and the samples collected from these patients and allotted to groups **b** and **c** were discarded). After mRNA extraction from group **a** we divided the mRNA aliquots of the group **a** 36 positive samples into two other samples of equal volume: one for cDNA testing of mRNA viability (mRNA survival) at 90 days after extraction and the other for the same procedure after 180 days. Samples of group **b (**only the original 36 women positive for mRNA at immediate extraction in group **a)** were stored before extraction for 90 days and then mRNA extraction was performed and mRNA samples were stored for 90 and 180 days for cDNA testing. Samples of group **c** were stored for 180 days before extraction and then mRNA extraction was performed and the same routine used for

groups **a** and **b** was followed. The timetable for the entire set of experiments is shown in Figure 1.

mRNA extraction

As mentioned earlier, only samples positive for DNA of HPV types 16, 18, 31 and 45 were tested for mRNA. For mRNA extraction, an aliquot of 200 ul of STM was sampled (Qiagen) and centrifuged at 13,000 *g* for 10 min. The supernatant was removed and 1 mL of the TRizol™ reagent (Invitrogen, Carlsbad, USA) was added at cellular pellet and the cells were lysed by repeat pipetting and stand at room temperature for 5 min. After that, 200 ul of chloroform was added and shaken vigorously, stand at room temperature for 3 min before centrifugation at 12,000 *g* for 15 min. The resultant aqueous layer was transferred to a new tube. To the aqueous solution, a volume of 500 µl of isoamyl alcohol was added and mixed by vortex. The whole mixture was stand at -80° C for 2 hours and 10 min at room temperature and centrifuged at 12,000 *g* for 10 min. The supernatant was removed and 1 mL of iced ethanol was added and shaken vigorously. The mixture was centrifuged at 7,500 *g* for 5 min, the supernatant was removed and after the pellet of mRNA was dry, mRNA was eluted in 30µl of mRNAase- free water (DEPC) and stored at−80 ◦C pending analysis. The mRNA purity and concentration were determined by the absorbance at 260 nm (A260) and 280 nm (A280) using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA).

PCR HPV-cDNA type-specific for 16, 18, 31, and 45

The reactions were done separately according the reagent mixture: five µl of DNA were added to a PCR mix containing 0.5pmol each primers, specifically designed

by the investigators for the present study, 4.0mM MgCl2, 0.25mM dNTPs and 5 units of Taq platinum in a final volume of 50µl. All the steps of the PCR reactions were similar, except annealing temperature that was different for each type HPV. During each PCR run, all samples were tested together with one negative control (water) and one positive control (obtained in priori studies). A 9-min denaturation step at 95°C was followed by 40 cycles of amplification. Each cycle included a denaturation step at 95°C for 1 min, a primer annealing step at according each type HPV for 1 min, and a chain elongation step at 72°C for 1 minute. The final elongation step was prolonged by 7 min to ensure a complete extension of the amplified DNA.

cDNA transcription

The mRNA was reverse-transcribed in first strand cDNA (SuperScript III First Strand Synthesis System, Invitrogen), according to the manufacturer's guidelines. The PCR product of cDNA was amplified as described in the type-specific PCR section, however with a final volume of 25µl and 2µl of cDNA.

Statistical analysis

The cumulative mRNA failure was estimated by maximizing the cox-proportional non-parametric likelihood (M-NPL), which adjusts for interval censoring between the dates of successive visits. If the maximum likelihood estimate of a cumulative risk was not unique the largest risk estimate was reported. 95% confidence intervals (CIs) were calculated with the score test. Differences in cumulative failures between subgroups were assessed by exact log-rank tests for interval-censored data with

Sun's scores measured from baseline to the date of the experiment in which the mRNA detection failed.

In order to perform a multivariate analysis of the mRNA survival after extraction, taking into account the pre-extraction storage time (groups A, B and C) and the patients age, smoking status, referral cytology result and the HPV DNA types in the sample, we used Cox regression to calculate hazard ratios (HRs) and 95% CIs. Time to mRNA failure was set as the midpoint of the time between the first successful mRNA detection and the first unsuccessful mRNA detection attempt. The tests were done with the *interval R* package within the statistical software program R (version 2.14.1).

Results

Table 1 shows the failure rates of mRNA retrieval over time in the three study groups: specimens subjected to A) immediate mRNA extraction B) extraction after 90 days and C) extraction after 180 days. Group A defines the sample that was tested in 90 and 180 days after extraction. In that group we observed 8/36 failures 90 days after the first extraction and another after 180 days, totaling 9/36 failures. In group B, which was stored for 90 days before extraction, seven failures occurred at the first mRNA detection attempt and another two failures were detected after 90 and 180 days, totaling 9/36 failures. In group C, which was stored for 180 days before extraction, we observed a significantly higher failure rate at the first mRNA detection attempt (10 samples) and detection failures continued to mount after 90 days (3 failures) and 180 days (another 4 failures) totaling 17/36 failures. Comparing the three groups the final survival

probability for group C, of 52.8% (95%CI 38.7 to 61.9%), was significantly lower than that for groups A and B (75.0%; 95%CI 62.1 to 90.6%).

Table 2 shows the hazard ratios derived from multivariate survival analysis for mRNA according to the pre-extraction storage time and the characteristics of the subjects and samples. Pre-extraction storage time of 180 days was significantly related to a worse mRNA survival (HR=2.63; 95%CI 1.16 to 6.0; p=0.02). The mRNA survival was higher in patients with age <30 years (HR=0.93; 95% CI 0.87 to 0.98; p=0.01) and with presence of DNA of HPV 31 in the sample (HR = 0.39; 95%CI 0.18 to 0.86; p=0.01).

Discussion

We evaluated the yield of viable HPV oncogenes E6 and E7 mRNA in cervical samples stored for different times before and after mRNA extraction. Our results demonstrate that pre-extraction storage time in -80^{o}C in for 90 days does not affect mRNA quality or quantity but we detected a decline in mRNA yield in samples stored for 180 days before extraction. We also detected that after extraction there is a sharp decrease in mRNA availability which tends to stabilize after 30 days of storage in -80^{o}C. Our results have also shown that the decline in mRNA after extraction availability is sharper in samples stored before extraction for 180 days.

The interest in preservation of mRNA for exploratory research and clinical analyses is increasing as accumulated evidence shows that HPV mRNA levels may be a more sensitive early indicator of predisposition to carcinogenesis than deoxyribonucleic (DNA) levels (7-9). This happens because among the biological events related HPV

infection more likely to yield clinical applications are those arising from the action of oncoproteins E6 and E7 whose functions are relevant to malignant cells. The oncogenic potential of the high-risk HPV types (16 18 31 and 45) lies in the actions of these oncoproteins which bind to and modulate a number of different gene products in particular the tumor suppressors p53 and pRb (10,11). These interactions may lead to a disturbance of cell cycle control and a deficiency in DNA repair resulting in genomic instability and an increased risk of malignant transformation (12,13). The detection of E6 and E7 mRNA may be technically challenging and very specific devices and techniques were devised in order to maximize detection accuracy. The totality of currently marketed assay options for mRNA detection makes use of proprietary technology, and features a prespecified set of target HPV types which is determined by the manufacturers. The PreTect HPV-Proofer (NorChip) and the NucliSENS Easy Q HPV (bioMérieux) are based on the same technology, and detect E6/E7 mRNA expression from the five most prevalent hrHPV types (16, 18, 31, 33 and 45). Other two tests aim at an expanded set of HPV types: the TaqMan real-time PCR assay, which targets 12 high-risk (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59) and two low-risk (HPV6 and -11) types using E6/E7 region primers and probes in a duplex format (14, 15), and the APTIMA HPV Assay (Gen-Probe) targets mRNA expression of the 14 most carcinogenic hr-HPV types (16, 17). However, the prevalence of the HPV types may vary between female populations, and the prespecified set of HPV types in a given test may prove to be inadequate in some epidemiological scenarios. Also importantly, the use of proprietary technology hinders the development of assay adaptations to laboratorial and economic local conditions. E.g., the costs of the currently available comercial assays may be not compatible with the resources available to many

routine laboratories, especially in economically disadvantaged regions which, paradoxically, are heavily burdened by HPV-related disease.

The concern as to whether can we work with mRNA in preserved material dates back to the last two decades. Dimulescu et al. (1998) (18) examined the RNA yield of samples stored in PreservCyt at 4°C for one month and denaturing gel electrophoresis revealed that intact 28S and 18S RNA from CaSki cells was easily obtained Fixation and storage of the experimental and clinical samples in PreservCyt solution did not appear to affect the ability to reverse transcribe and amplify messenger RNA. Other authors worked with RNAlater assays in preserved tissue samples and found that this method retained the amplification products for up to one year if stored in liquid nitrogen or -80 ° and for only 2 hours at room temperature (4, 19). Three methods have so far been used to test the preservation of mRNA in tissue samples. The first method snap-freezing the tissue is difficult due to the absence of liquid nitrogen tanks in most operating rooms. Freezing the specimen at a later time point is not an option because several studies have reported the effects of ischemia on gene expression levels. A second method is formalin fixation and paraffin embedding of the tissue. However this method results in degradation of the RNA. Reverse transcriptase-polymerase chain reaction- based assays can generate data of sufficient quality using RNA isolated from formalin-fixed paraffin-embedded tissues but use of degraded RNA in microarray based assays requires further protocol optimization. A third method is the use of preservatives such as RNAlater preservative (20). However RNAlater was found previously to be suitable for preservation of biopsy material (21).

Experience with Papanicolaou (Pap) smear screening demonstrates that exfoliated cervical cells reflect cervical neoplasia. The molecular quality of this sample is clearly adequate for DNA-based molecular testing such as PCR and hybrid capture. Many pre-analytical factors such as the time of collection sampling tubes preservatives transportation condition storage condition and purification methods affect the quality of mRNA (22). As fixation of cells can interfere with mRNA quality it would be favorable that fixatives used for routine collection and processing of cervical specimens maintain RNA integrity (23, 24).

In a 2003 study using samples preserved in Preservy Cyt the authors found that the integrity of RNA for RT PCR was not dependent on storage temperature because when kept at 4 ° C or - 20 for up to nine months the amplification products were detected but for samples stored at room temperature PCR efficiency rapidly declines with increasing storage time (25). The RNA that is used for RT-PCR that can be analyzed using the protein truncation test; direct sequencing; or indirect mutation screening techniques such as single-stranded conformation polymorphism denaturant gradient gel electrophoresis or denaturing high-performance liquid chromatography (26). These assays typically use PCR products that range in size from 300 to 600 bp which were the primers used in our study.

At this point when it comes to scraping cervical cells our study may have contributed to elucidate the best timing for extraction and conservation of mRNA for the detection of HPV oncogenes E6 and E7. Our data clearly indicates that pre-extraction storage time is pivotal to mRNA quality and that extracted mRNA quality declines faster for samples stored before extraction for 180 days or more. This information would

probably be of value for research and clinical laboratories as mRNA detection gains importance in cervical cancer screening.

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Table 1 – Survival of the extracted mRNA according to the pre-extraction storage time

Time stored after extraction	Sample (n)	Number of failures (n)**	Survival probability	95% confidence interval	Total (before +after extraction) storage time
		Group A) Immediate extraction*			
Immediate	36	Defines the sample for next experiments			Immediate
90 days	36	8	77.8%	65.3 to 92.6%	90 days
180 days	28	1	75.0%	62.1 to 90.6%***	180 days
		Final number of samples with viable mRNA = 27			
		Group B) stored for 90 days before extraction*			
Immediate	36	7	80.6%	68.6 to 94.6%	90 days
90 days	29	1	77.9%	65.3 to 92.6%	180 days
180 days	28	1	75.0%	62.1 to 90.6%***	270 days
		Final number of samples with viable mRNA = 27			
		Group C) stored for 180 days before extraction*			
Immediate	36	10	72.2%	59.0 to 88.4%	180 days
90 days	26	3	63.9%	50.0 to 81.7%	270 days
180 days	23	4	52.8%	38.7 to 61.9%***	360 days
		Final number of samples with viable mRNA = 17			

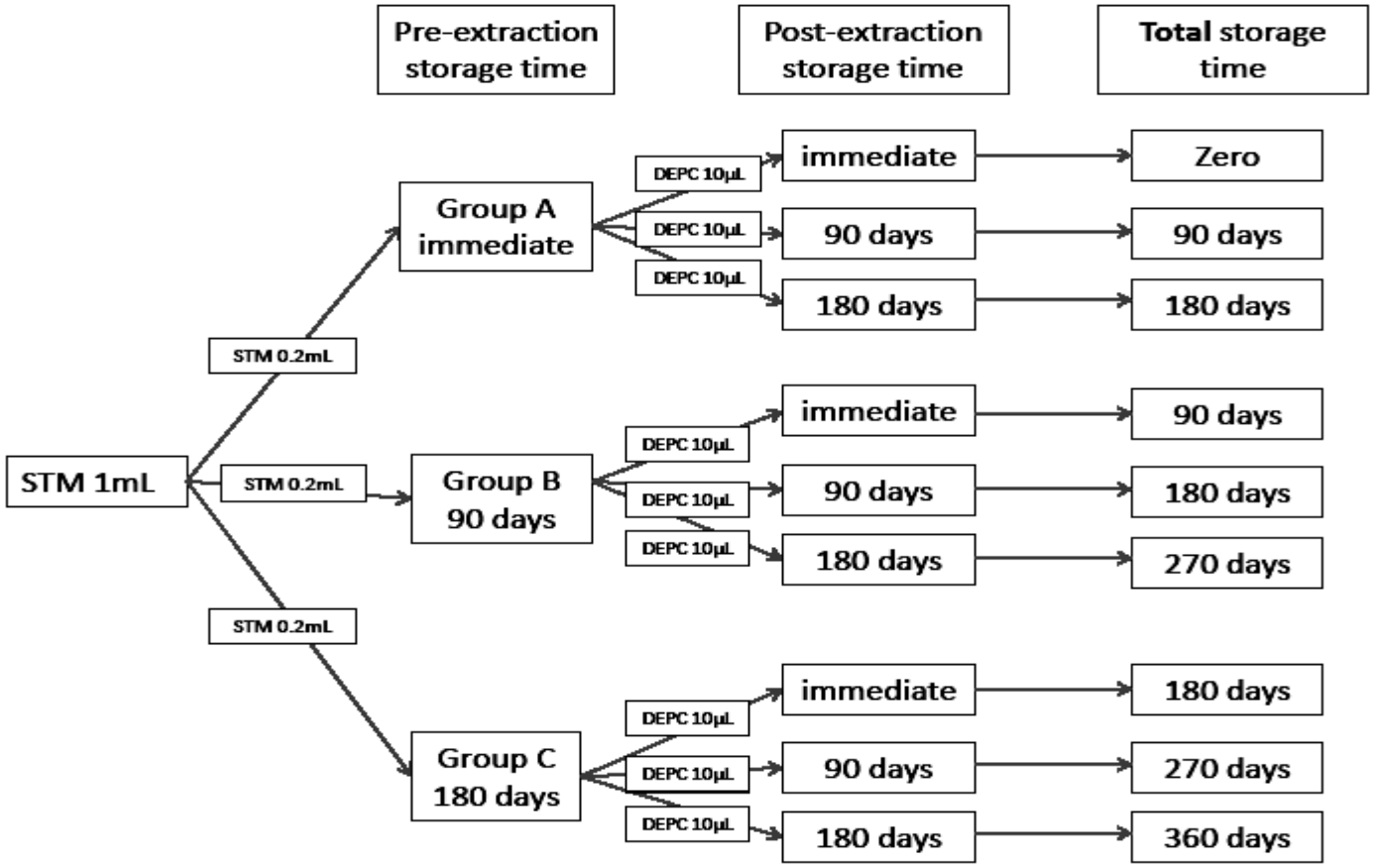
*Please refer to Figure 1 for clarification

**Number of samples from which we were unable to retrieve HPV 16, 18, 31 and/or 45 E6/E7 oncogenes mRNA

*** Adjusted likelihood cox-proportional hazards: Group C vs A (p=0.02) and group C vs B (p=0.02)

Table 2 – Multivariate survival analysis of the extracted mRNA according to the pre-extraction storage time and the key epidemiological and clinical features of the women

	mRNA retrieval failure		
Characteristic	**Hazard Ratio**	**(95% CI)**	**P**
Pre-extraction storage time			
Immediate	1.00		
90 days	1.19	(0.47 to 3.01)	0.71
180 days	**2.63**	**(1.16 to 6.0)**	**0.02**
Age			
>30	1.00		
<30	**0.93**	**(0.87to 0.98)**	**0.01**
Current smoker			
No	1.00		
Yes	0.62	(0.26to 1.47)	0.28
Citology			
ASC-US or LSIL	1.00		
ASC-H or HSIL	1.27	(0.78to 3.13)	0.59
DNA-HPV 16			
negative	1.00		
positive	0.34	(0.11to 1.04)	0.28
DNA HPV 18			
negative	1.00		
positive	0.47	(0.13to 1.64)	0.24
DNA HPV 31			
negative	1.00		
positive	**0.39**	**(0.18to 0.86)**	**0.01**
DNA HPV 45			
negative	1.00		
positive	1.89	(0.77to 4.63)	0.15



STM = Specimen transport medium; DEPC = Diethylpyrocarbonate water

Figure 1: schematic overview of the study design.

4. Discussão

O propósito fundamental deste estudo foi testar a adequabilidade ao longo do tempo do mRNA de oncogenes do HPV, usando para sua obtenção técnicas de biologia molecular cuja tecnologia não fosse de uso ou retenção exclusivas de determinados fornecedores. Embora a comparação das taxas de obtenção do mRNA entre as técnicas manuais (abertas) e automatizadas de tecnologia proprietária (fechada) não seja essencialmente inédita, a avaliação da estabilidade do mRNA ao longo do tempo o é. Ademais, testamos os efeitos do tempo em duas fases distintas do processo de obtenção do mRNA, antes e após a extração. Esta abordagem em muito interessa ao laboratorista, que se vê em dúvida sempre que deseja estocar materias biológicos para futuras extrações do mRNA.

Os protocolos de tecnologia aberta que utilizamos neste estudo são muito apropriados para locais de poucos recursos, graças ao seu custo relativamente baixo. O orçamento global de custeio do presente estudo atingiu aproximadamente R$33.000,00, embora essas tecnologias demandem uso intensivo de mão-de-obra. Foram recuperados por este estudo HPV-DNA viável em 81,8% das

amostras e os oncogenes E6 e E7 mRNA em quase 60% do casos. A taxa de detecção de mRNA neste estudo foi semelhante ao esperado, considerando-se dados de estudos recentes (12, 25). Também digno de nota, a presença de DNA dos HPV 16 e 18 favoreceram a detecção de oncogenes E6 e E7 mRNA nas amostras. Usando protocolos de tecnologia fechada e “kits” de detecção como o HPV-PreTect Proofer (NorChip) eo NucliSENS Fácil Q HPV (bioMérieux), estudos semelhantes aos nossos alcançaram taxas de obtenção de mRNA, que varia de 36% a 68% (11, 12, 25, 26, 27, 28, 29). Portanto, os resultados do presente estudo sugerem claramente que os protocolos de tecnologia aberta para a detecção de mRNA podem ser usados com segurança nas práticas clínica e laboratorial, o que favorece a aplicabilidade da detecção de mRNA em ensaios clínicos futuros a serem desenvolvidos em locais com recursos econômicos relativamente limitados.

Outra vantagem das técnicas de protocolo aberto, manual, é a possibilidade de escolha dos tipos de HPV a serem testados. Essa possibilidade permite o uso mais adequado dos recursos disponíveis, visto que permite dirigir os experimentos aos tipos de HPV mais prevalentes em uma dada população. A escolha dos HPV 16, 18, 31 e 45 como alvo deste estudo deveu-se a evidências recentes que atribuem a estes virus a maior (mais de 90%) parte das lesões cervicais (5, 30, 31) em populações urbanas semelhantes à da região em que as amostras foram coletadas.

Muitos estudos anteriores deixam de questionar se as taxas de transcrição de mRNA podem favorecer os efeitos cancerígenos potenciais de alguns dos

tipos de HPV, e, se as características individuais do doente também podem estar envolvidos no processo de transcrição de DNA para mRNA. Desta forma, o potencial de estudos que avaliam os fatores associados aos processos de transcrição é muito alto, e provavelmente pesquisadores precisarão de subsídios para recorrer com segurança a bibliotecas de amostras em estoque. Daí, a relevância deste estudo que permite antever o que se pode esperar de materiais estocados e, não obstante, sugerir aos pesquisadores o melhor momento para se fazer a estocagem das amostras. Este estudo, em comunhão com futuras investigações de testes baseados na detecção do mRNA dos oncogenes dos HPV de maior potencial carcinogênico, poderão ajudar na distinção entre infecções transitórias e persistentes, permitindo o direcionamento eficaz dos recursos de prevenção do câncer cervical (13).

Por essas razões, o potencial de aplicabilidade clínica da detecção de mRNA em locais de recursos escassos é muito grande, pois é nelas que se concentra o maior número de lesões induzidas pelo HPV. Apesar dos recursos gastos na triagem citológica e no seguimento de mulheres com anormalidades cervicais serem substanciais, o câncer do colo do útero ainda é décima causa mais comum de morte por câncer em mulheres europeias, e no Brasil o câncer cervical é uma das principais causas de mortalidade feminina. A melhoria e ampliação das estratégias de triagem e tecnologias existentes constitui um alvo central da Recomendação do Conselho Europeu sobre o rastreio do câncer (24). No entanto, muitas das estratégias de triagem propostas, devido a limitações de recursos, podem não ser aplicáveis em todo o mundo. As taxas de positividade

para mRNA são um terço daquelas obtidas em testes para DNA do HPV e, como mencionado anteriormente, a detecção do mRNA é mais específica para lesões cervicais do que a detecção do DNA. Daí, testes focados na detecção de mRNA podem, em tese, reduzir a sobrecarga de serviços de saúde, caso seu uso venha a ser associado, por exemplo, com outras técnicas de triagem. Concluímos, assim, que este estudo está em plena conformidade com as mais recentes recomendações para estudos sobre técnicas de rastreio do câncer do colo (24).

Especificamente em relação à preservação do mRNA, uma vez que as amostras tenham sido estocadas, os resultados deste estudo demonstram que a pré-extração em amostras armazenadas em -80 º C por até 90 dias é segura, não havendo perdas significativas de qualidade e de quantidade do mRNA. Contudo, detectamos uma diminuição no rendimento de mRNA nas amostras armazenadas por 180 dias antes da extração. Também detectamos que após a extração há uma acentuada diminuição na disponibilidade de mRNA, o que tende a se estabilizar após 30 dias de armazenamento em -80°C. Os resultados deste estudo também mostraram que o declínio da extração de mRNA é mais nítido nas amostras armazenadas antes da extração por mais de 180 dias, ou seja, o tempo de armazenamento pré-extração diminui a durabilidade do mRNA após a sua extração.

A preocupação quanto a trabalhar com mRNA em material preservado remonta às duas últimas décadas do século XX. Dimulescu et al. (32), examinaram o rendimento de mRNA de amostras armazenadas em PreservCyt a 4°C por um mês, e a eletroforese em gel de agarose revelou que as frações

28S e 18S de mRNA de células CaSki foi facilmente obtida. A fixação e armazenamento das amostras em solução apropriada também não parecem afetar a capacidade de transcrição reversa e amplificação do mRNA. Outros autores trabalharam com amostras de tecido preservado e descobriram que a manutenção de produtos de amplificação pode se dar por até um ano, se o material for armazenado em nitrogênio líquido ou -80° C e exposto à temperatura ambiente por não mais de duas horas (33,34). Contudo, esses são estudos em tecidos, o que em muito difere das condições de preservação e exposição celular das amostras de raspados citológicos. Muitos fatores pré-analíticos, como o momento da coleta, os tubos de amostragem, conservantes, a condição de transporte, condições de armazenamento e métodos de purificação podem afetar a qualidade do mRNA em raspados cervicais (14). Os dados do presente estudo indicam claramente que o tempo de armazenamento pré-extração é fundamental para a obtenção de mRNA de qualidade, e que a qualidade do mRNA extraído diminui mais rápido para amostras armazenadas antes da extração por 180 dias ou mais. Esses dados têm grande aplicabilidade laboratorial, pois sugerem que a pronta extração do mRNA pode favorecer a sobrevida do material transcrito de amostras em estoque, uma vez que as perdas após a extração são poucas e o mRNA pode ser obtido em qualidade e quantidade, mesmo após seis meses de armazenamento.

5. Conclusões

- **Artigo 1** A recuperação do mRNA dos oncogenes de E6 e E7 do HPV de amostras de raspado cervical usando tecnologias de protocolo aberto foi de aproximadamente 60% e compatível com aquela obtida por outros pesquisadores, utilizando técnicas cuja tecnologia é fechada. Os fatores associados com maior detecção do mRNA foram a presença de DNA de HPV dos tipos 16 e 18.

- **Artigo 2: A recuperação do mRNA** dos oncogenes de E6 e E7 do HPV foi semelhante em amostras armazenadas, antes da extração, por até 90 dias e inferior naquelas armazenadas por 180 dias. Após a extração, a sobrevida do mRNA foi semelhante nos grupos armazenados antes da extração por até 90 dias, e significativamente inferior nas amostras que haviam sido armazenadas por 180 dias.

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7. Anexos

7.1. Anexo 1 – Termo de Consentimento Livre e Esclarecido

Determinação do efeito do tempo de conservação sobre a recuperação de RNA de células da cérvice uterina para verificação da expressão dos oncogenes E6 e E7 do HPV

Eu, Sra _____, atendida no Centro de Atenção Integral à Saúde da Mulher (CAISM) no ambulatório de patologia do trato genital inferior e colposcopia fui convidada a participar desta pesquisa porque o resultado do meu exame de prevenção (colpocitologia oncológica) mostrou alterações das células do colo do útero. Sei que responderei a um questionário com informações pessoais que serão avaliadas somente pelo médico que me atendeu e que as fichas ficarão de posse do(s) Doutor(es) responsáveis pela pesquisa, Dr. Luis Otávio Sarian, Dra. Sophie Derchain, e as biólogas Elisabete Campos e Denise Moraes, que manterão o sigilo destas informações.

Fui informada que a pesquisa trata da avaliação da recuperação de material genético do vírus HPV em amostras retiradas de meu colo uterino e estocadas por diferentes períodos de tempo. A mesma não interferirá em meu tratamento e não implicará em manipulação adicional de partes do meu corpo, além daquelas já necessárias para o tratamento. Também fui informada de que os resultados obtidos através desta pesquisa têm finalidade apenas para avaliação de técnicas laboratoriais, ou seja, não têm valor clínico imediato para mim. Sei que ao voltar ao ambulatório para

receber os resultados dos meus exames serei tratada de acordo com a necessidade da minha doença. Sei também que deverei voltar ao ambulatório nos retornos marcados após o tratamento para novamente realizar uma nova coleta de Papanicolaou e colposcopia (ver o colo com lente de aumento) e para seguir adequadamente o meu caso de maneira cuidar da minha saúde. Autorizo o Dr. Luis Otávio a realizar uma cópia dos resultados dos exames de laboratório para que sejam anexados às fichas de pesquisa. Estou ciente de que o material biológico referente a esta pesquisa ficará sob a guarda do Laboratório Clínico Especializado do CAISM, sob responsabilidade direta do Dr. Luís Otávio Zanatta Sarian.

FUI ESCLARECIDA QUANTO AO MEU DIREITO DE NÃO PARTICIPAR DA PESQUISA E DE SER ATENDIDA NO AMBULATÓRIO SEMPRE QUE NECESSÁRIO. A NÃO ACEITAÇÃO NA PARTICIPAÇÃO NO ESTUDO NÃO IMPLICARÁ PERDA DOS DIREITOS INICIAIS ROTINEIRAMENTE OFERECIDOS PELO AMBULATÓRIO. EM CASO DE DÚVIDAS OU ESCLARECIMENTO, TENHO O DIREITO DE TELEFONAR PARA A DR LUIS OTÁVIO NO NÚMERO (19) 3521-9305 OU PARA O COMITÊ DE ÉTICA DA UNICAMP NO NÚMERO (19) 3521-8936. SEI QUE NÃO SEREI PAGA PARA PARTICIPAR DESTE ESTUDO.

Paciente _____

Campinas,_____de_____de 2009/10

Profissional que atendeu _____

7.2. Anexo 2 – Ficha para coleta de dados

I. Identificação **Ficha:** I__I__I__I
HC: I__I__I__I__I__I__I__I__I__I__I__I__I

Iniciais:_____ Data da Coleta: I__I__I__I__I__I__I

Idade: I__I__I anos Data de Nascimento ___I___I_____I

Idade na primeira relação sexual I__I__I

Nº de parceiros: I__I__I

Estado menstrual: menacme I__I menopausada I__I

HIV: I__I positiva I__I negativa I__I NI I__I NS

Tabagismo: I__I sim I__I não
Há quanto tempo:_____ Cigarros ao dia I__I__I
Fumou por quanto tempo:_____
Contato com fumante em casa: I__I sim I__I não

NI = Não informado NS = Não sabe

Teste para HPV:	I__I positivo	I__I negativo		
Extração de RNA:		**Expressão de E6 e E7**		
até 30 dias	**|**___**|**ng/μl	Até 30 dias	I__I sim	I__I não
		Após 90 dias	I__I sim	I__I não
		Após 180 dias	I__I sim	I__I não
90 dias	**|**___**|**ng/μl	Até 30 dias	I__I sim	I__I não
		Após 90 dias	I__I sim	I__I não
		Após 180 dias	I__I sim	I__I não
180 dias	**|**___**|**ng/μl	Até 30 dias	I__I sim	I__I não
		Após 90 dias	I__I sim	I__I não
		Após 180 dias	I__I sim	I__I não