CERVICAL CYTOLGY, HPV DNA AND mRNA – A COMPARATIVE STUDY IN 162 PATIENTS

INTRODUCTION

In contrast to the general improvement of the socioeconomic status of the Brazilian population observed in the last decade, pathologies that are characteristic of poor health assistance persist. Among those, cervical cancer (CC) is emblematic; it still presents a persistently high incidence. Objective: to compare the performance of cervical cytology to HPV DNA and mRNA detection methods in 162 patients undergoing routine gynecological clinical practice. Methods: a total of 162 patients attended during routine gynecological examination in a private clinic in Florianópolis, Santa Catarina, Brazil, had cervical samples collected and processed for cytopathological and molecular tests, conventional PCR and NASBA. Positive samples positive for HPV DNA were submitted to Type-Specific PCR (TS-HPV PCR). Patients with altered smears were submitted to colposcopy and biopsy. Results: among the 162 samples, 19.8% (32/162) had altered smears, being 4/32 classified as ASC-H, 9/32 as ASC-US, 9/32 as LSIL and 10/32 as HSIL. Biopsies revealed nine cases of CIN I, nine CIN II and one CIN III, while seven were negative for cervical neoplasia. Overall, HPV DNA was detected in 38.3% (62/162) of the samples and HPV E6/E7 mRNA expression was found in 13.6% (22/162). Using TS-HPV PCR, HPV 16 was the most frequent type, found in 8% of the samples (5/62). Considering CIN2+ the gold-standard, cytology had 38.5% of specificity. Sensitivity and specificity of HPV-DNA PCR and NASBA were, respectively, 100% and 60%; 18.7% and 67.8%. Conclusion: mRNA E6/E7 expression was not a highly specific or sensitive marker for prevalent cervical disease while HPV DNA may be used for cervical cancer screening only in conjunction to more specific adjuvant tests.

Keywords: HPV, cervical carcinoma, PCR, E6/E7 mRNA, HPV types, NASBA

RESUMO

Introdução: em contraste com a melhora geral da situação socioeconômica da população brasileira, patologias que são características de uma deficiente assistência à saúde persistem. Entre elas, o câncer cervical (CC) é emblemático, ainda apresentando uma persistente alta incidência. Objetivo: avaliar o desempenho da citologia e de métodos de detecção de DNA e RNAm de HPV em 162 pacientes submetidas a prática clínica ginecológica de rotina. Métodos: cento e sessenta e duas pacientes atendidas em uma clínica particular de Florianópolis, Santa Catarina, Brasil, tiveram amostras cervicais coletadas e processadas para estudo citopatológico e molecular; PCR convencional e NASBA. Amostras positivas para o DNA do HPV foram submetidas a PCR tipo-específica (PCR HPV-TE). Resultados: entre as 162 amostras, 19,8% (32/162) apresentaram esfregaços alterados, sendo 4/32 classificados como ASC-H, 9/32 como ASC-US, 9/32 como LSIL e 10/32 como HSIL. Biópsias revelaram nove casos de NIC I, nove casos de NIC II e um caso de NIC III. O DNA do HPV foi detectado em 38,3% (62/162) das amostras. Expressão de E6/E7 (RNAm) foi encontrada em 13,6% (22/162) das amostras. Utilizando a PCR tipo-específica (HPV-TE), o HPV 16 foi o tipo mais frequente, encontrado em 8% (5/62) das amostras HPV+. Considerando NIC2+ o padrão-ouro, a especificidade da citologia foi de apenas 38,5%, enquanto a sensibilidade e a especificidade da PCR DNA e RNAm foram, respectivamente, 100% e 60%; 18,7% e 68,7%. Conclusão: a expressão de E6/E7 RNAm não se mostrou um marcador altamente específico ou sensível para doença cervical prevalente, enquanto o DNA HPV pode ser utilizado para rastreamento apenas em conjunto com testes adjuvantes mais específicos.

Palavras-chave: HPV, carcinoma cervical, PCR, E6/E7 RNAm, tipos de HPV, NASBA

ARTIGO

INTRODUÇÃO

No Brasil, o câncer cervical (CC) é emblemático; ainda apresentando uma persistente alta incidência. Objetivo: avaliar o desempenho da citologia e de métodos de detecção de DNA e RNAm (Real Time PCR) de HPV em 162 pacientes submetidas a prática clínica ginecológica de rotina. Métodos: cento e sessenta e duas pacientes atendidas em uma clínica particular de Florianópolis, Santa Catarina, Brasil, tiveram amostras cervicais coletadas e processadas para estudo citopatológico e molecular; PCR convencional e NASBA. Amostras positivas para o DNA do HPV foram submetidas a PCR tipo-específica (PCR HPV-TE). Resultados: entre as 162 amostras, 19,8% (32/162) apresentaram esfregaços alterados, sendo 4/32 classificados como ASC-H, 9/32 como ASC-US, 9/32 como LSIL e 10/32 como HSIL. Biópsias revelaram nove casos de NIC I, nove casos de NIC II e um caso de NIC III. O DNA do HPV foi detectado em 38,3% (62/162) das amostras. Expressão de E6/E7 (RNAm) foi encontrada em 13,6% (22/162) das amostras. Utilizando a PCR tipo-específica (HPV-TE), o HPV 16 foi o tipo mais frequente, encontrado em 8% (5/62) das amostras HPV+. Considerando NIC2+ o padrão-ouro, a especificidade da citologia foi de apenas 38,5%, enquanto a sensibilidade e a especificidade da PCR DNA e RNAm foram, respectivamente, 100% e 60%; 18,7% e 68,7%. Conclusão: a expressão de E6/E7 RNAm não se mostrou um marcador altamente específico ou sensível para doença cervical prevalente, enquanto o DNA HPV pode ser utilizado para rastreamento apenas em conjunto com testes adjuvantes mais específicos.

Palavras-chave: HPV, carcinoma cervical, PCR, E6/E7 RNAm, tipos de HPV, NASBA

Cervical cancer can be prevented; generally slow progression from precancerous lesions to invasive cancer provides ample opportunities for early detection and intervention. The incidence and mortality rates have declined over the past five decades in developed countries due to screening programs based on conventional cervical Papanicolaou (Pap) smears. A draw-back of cytology is its limited power to predict which low-grade cervical lesions will progress to high-grade lesions in addition to a false-negative rate ranging from 20 to 30%, demonstrated in several studies in which a number of high-grade cervical lesions were missed.

Scientific evidence, accumulated in the last 30 years from virological, molecular, clinical and epidemiological studies demonstrated that cervical cancer is a long term sequel of unresolved infection with certain HPV genotypes. Thus, HPV types are classified as high-risk (HR) or low-risk (LR) according to the risk of cancer in the infected hosts. The most common HR types are 16, 18, 31, 33, 45, which are detected in > 85% of the carcinomas worldwide.

On the premise that all cervical carcinomas contain HPV DNA, it has been discussed whether HPV testing may be used for primary screening of CC, while its use for triage of equivocal or low grade lesions and in the follow-up after treatment for CIN is firmly required...
established\(^9\). Encouraged by the current stage of development of molecular methods for HPV DNA detection in cervical cells, researchers have evaluated in large clinical trials, whether these technologies are potentially better alternatives for mass screening, in comparison to cytology\(^8,10\). These studies have shown that molecular testing for HPV DNA leads to a significant reduction in the number of advanced cervical cancer cases and deaths\(^11\) and that a single negative result in this test is associated to low development rates of CIN 3+ in six years of follow-up\(^9\).

Although it is clear that molecular testing for HPV offers much higher rates of detection of precursor lesions and invasive cervical cancer\(^12\), the positive predictive value is low, due to the common asymptomatic HPV infection. In order to enhance the specificity of the molecular approach, it has been postulated that testing for the transcripts (mRNAs) coding for the oncogenic proteins E6/E7 could provide a better correlation to disease, as only those lesions actively expressing these mRNAs, would be depicted\(^13\).

**OBJECTIVE**

This study aimed to evaluate the performance of molecular methods in comparison to conventional cytology in 162 patients undergoing routine gynecological clinical practice.

**METHODS**

**Patients and samples**

Patients, aging 14-69 years old (sd± 12), who agreed to sign the Informed Consent Statement approved by the institutional ethic committee from the Federal University of Santa Catarina (approval nº 072/2009) were invited to enroll in this prospective study during routine gynecological examination by the same medical gynecologist doctor, in a private clinic setting, from May to November, 2009, in Florianópolis, Santa Catarina, Brazil. One hundred and sixty-two participants had the cervical material collected with a cytobrush (endocervix) and spatula (ectocervix) by the same physician.

Samples were immediately preserved in PreservCyt® solution (Hologic, USA) and referred to the Molecular Biology and Mycobacteria Laboratory (LBMM), Hospital Universitário Polydoro Ernani de São Tiago at Federal University of Santa Catarina for molecular tests. The Pap smears were referred to a cytopathology laboratory where cytological diagnosis was made by two pathologists, and when there was disagreement, a third cytopathologist reviewed the smear. Pap smears were analyzed and classified according to the 2001 Bethesda system\(^14\).

Samples with atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells that cannot exclude HSIL (ASC-H) or squamous intraepithelial lesion (SIL) were considered positive (ASC-US+); the normal pattern and inflammatory or reactive changes were classified as negative (NILM – Negative for Intraepithelial Lesion or Malignancy). According to the current guidelines only women with altered cytology (ASC-US+) were referred to colposcopy-directed biopsy.

Cervical biopsy specimens were histologically examined and classified according to the cervical intraepithelial neoplasia (CIN) system. Molecular tests, targeting HPV DNA and messenger RNA of the viral oncogenic proteins (E6 and E7) were performed without prior knowledge of cytopathology results. Positive samples for the HPV DNA test were genotyped by type-specific PCR (TS-HPV PCR). Results of the molecular tests didn’t influence clinical management.

**DNA/RNA extraction from cervical cells**

After collection, samples were stored at ~70°C in PreservCyt® solution (Hologic, USA) for further extraction. Upon thawing, 5 mL was removed to a 15 mL conical tube with cap, which was centrifuged for 12 minutes at 400 x g. The supernatant was discarded, leaving 1 mL in which the pellet was re-suspended. Extraction was performed on the NucliSENS® MiniMAG device (bioMérieux, France) according to the manufacturer instructions.

The cell suspensions were transferred to another tube containing 2 mL of lysis buffer (NucliSENS Lisys Buffer), the mixture was homogenized and allowed to rest for 10 minutes at room temperature. Afterwards, magnetic silica was added and the homogenization done by vortexing. Then, the suspension was centrifuged for 2 minutes at 400 x g and the supernatant was discarded. This was followed by washing steps, and the final RNA/DNA was re-suspended in 50 μL of elution buffer and stored at ~20°C until used.

**Human β-globin**

As an internal control to verify the extracted DNA integrity and quality, all samples were submitted to a PCR reaction with PC03/PC04 primers that amplify a 110 bp fragment of the human β-globin gene\(^15\).

PCR was performed in a final reaction volume of 20 μL containing 1.5 mM MgCl₂, 0.2 mM dNTP (Invitrogen® – USA), 10 pmoles each primer; 1X Taq polymerase buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1 U of recombinant Taq DNA polymerase (Invitrogen® – USA) and 1 to 3 μL of DNA template. The amplification conditions held in a thermocycler (Eppendorf® – Germany) were: 95°C for 1 min, 56°C for 1 min, 72°C for 1 minute at 39 cycles and a final incubation at 72°C for 7 minutes.

**HPV DNA (HPV L1 PCR)**

Extracted DNA was amplified with consensus primers MY09 and MY11\(^16\) spanning a fragment of 450 bp from the L1 gene. PCR was held in a final reaction volume of 20 μL containing 1.5 mM MgCl₂, 0.2 mM dNTP (Invitrogen® – USA), 10 pmoles each primer, 1X Taq polymerase buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1 U of recombinant Taq DNA polymerase (Invitrogen® – USA) and 1 μL to 3 μL of DNA template. The amplification conditions carried in a thermocycler (Eppendorf® – Germany) were: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min at 39 cycles and a final step at 72°C for 5 min. Samples that did not amplify with primers MY09 and MY11 were submitted to a nested PCR reaction using consensus primers GP5+ and GP6+ that generate a 150 bp product\(^17\).

PCR was performed in 20 μL final volume containing 1.5 mM MgCl₂, 0.2 mM dNTP (Invitrogen® – USA), 10 pmoles each primer, 1X Taq polymerase buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1 U of Taq DNA polymerase recombinant (Invitrogen® – USA) and 1 μL of the PCR product amplified with primers MY09 and MY11 as a template. The amplification conditions were: 94°C for 1 min, 40°C for 2 min, 72°C for 1 minute at 39 cycles and a final step at 72°C for 5 minutes.
TS-HPV PCR

DNAs from positive samples in the L1 PCR were amplified with specific primers for the following HPV types: 6, 11, 16, 18, 31, 33 and 45 in independent reactions\(^1\). PCR was held in 20 μL or 50 μL final volume containing 1.5 mM MgCl\(_2\), 0.2 mM dNTP (Invitrogen\(^*\) – USA), 10 pmoles each primer, 1X Taq polymerase buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1 U of Taq DNA polymerase recombinant (Invitrogen\(^*\) – USA) and 1 μL to 5 μL of template DNA. The amplification conditions were the same as those for the β-globin gene, except for annealing temperatures, as following: for HPV 6 and 16: 2 min at 62ºC; for HPV 11: 2 min at 64ºC; for HPV 18: 2 min at 65ºC; for HPV 31: 2 min at 63ºC; for HPV 33: 2 min at 61ºC and for HPV 45 2 min at 66ºC.

All PCR products were submitted to electrophoresis in 2% agarose gel stained with ethidium bromide and analyzed under UV light. In all PCR reactions a positive control was included which consisted of a previously characterized HPV positive sample. A negative control was also included which contained all the reagents except DNA. Positive controls were kindly provided by Dr. José Eduardo Levi (Institute of Tropical Medicine – University of São Paulo) and Dr. Jaqueline Germano de Oliveira (Virus Laboratory – Federal University of Minas Gerais), consisting of positive samples containing HPVs 6, 11, 16, 18, 31, 33 and 45.

NASBA (Nucleic acid-sequence based amplification assay, BioMérieux, France)

The mRNA amplification tests for E6 and E7 oncocgenes were held strictly according to the instructions of the kit NuclisENS EasyQ\® HPV (bioMérieux, France). In short, 5 μL of DNA/RNA extract was submitted to NASBA. Six different molecular probes able to detect HPV 16, 18, 31, 33, 45 types and U1A ribonucleoprotein (internal reaction control) were used\(^9\). The multiplex detection assay was developed with two fluorescent dyes: 6-carboxy-fluorescein (6-FAM) for 16, 31 and 33, 6-carboxy-X-rhodamine (6-ROX) HPV types for internal control and HPV 18 and 45 types. The kinetics reaction analysis was performed by measuring the fluorescent signal that revealed the signal from the internal control and of each of the five types of HPV mRNA targets, expressed as graphs.

Sensitivity and specificity of cytology, HPV DNA and E6/E7 mRNA methods were calculated by SPSS 16.0\(^*\) considering histological results of CIN2+ as gold standard.

RESULTS

Among the 162 samples, 19.8% (32/162) had an altered cytology. HPV DNA was detected in 90.6% (29/32) of the positive cytology cases and it was found in 100% of the samples with ASC-H, LSIL and HSIL diagnosis. E6/E7 mRNA expression was found in 50% of HSIL and 6.9% of negative cytology cases (Table 1). Overall, 62 samples were HPV-DNA positive by L1-PCR (38.3%), 35 of them amplified with primers MY09 and MY11 and 27 after nested PCR with GP5+/6+. The E6/E7 mRNA expression was found in 13.6% (22/162) of the samples (Table 1). Using TS-HPV PCR for distinct seven types, it was possible to genotype 37.1% (23/62) of the HPV-DNA+ samples. HPV 16 was found in 8% (5/62), HPV 18 in 6.5% (4/62), HPV 33 in 6.5% (4/62), HPV 6 in 6.5% (4/62), HPV 31 in 3.2% (2/62) and HPV 11 in 1.6% (1/62). Among these 62, coinfections were observed in three cases, two HPV 16/33 (2/62) and one HPV 33/45.

Expression of E6/E7 detected by the presence of mRNA was found for HPV 16 in 22.7% (5/22), 18 in 22.7% (5/22), 33 in 18.2% (4/22), 31 in 13.6% (3/22) and 45 in (2/22). Coinfection was also found by this test, revealing HPV 16/33 in 9.1% (2/22) and 33/45 in 4.6% (1/22) of the samples. HPV 16 was the most frequent type found in both negative cytology cases and HSIL, present in 3.1% (4/130) and 30% (3/10), respectively.

Histological results were obtained from 81.2% (26/32) of these women while six patients did not return for the biopsy. Three samples classified as ASC-US on cytology were negative in molecular analysis. Among the 162 HPV-DNA positive samples, coinfections were observed in three cases, two HPV 16/33 (2/62) and one HPV 33/45.

DISCUSSION

During the past 40 years, cytology has been the primary tool for cervical cancer prevention, greatly reducing invasive cervical...
cancer incidence in countries where these screening programs have been successfully implemented\(^4\). Following cytological diagnosis by repeated Pap testing at short intervals, patients are referred for abnormality confirmations by colposcopy and histology in order to treat cancer precursor lesions. This kind of program is highly expensive and not affordable by many developing countries, what explains why CC remains as the most incident neoplasia among women in these nations. In Brazil, as stated in the 2011 guideline\(^5\), screening will continue to rely exclusively on cytology with no perspective of inclusion of molecular testing. In despite of the increase in coverage verified in the last years, CC incidence in Brazil is still high in certain areas, as estimated for the Mato Grosso do Sul state (35.13/100,000)\(^6\) which is among the highest incidences worldwide\(^7\). Evidence of the deficiencies of the national CC screening program is illustrated by a study in Southern Brazil with 5,485 women in whom previous cytology was reported by 100% of the women diagnosed with carcinoma, while Pap smear in a previous period of less than 3 years was referred by, respectively, 86.5% and 92.8% of women with abnormal cytology and histology\(^8\).

In the present study, HPV DNA was detected in 100% of LSIL and HSIL cases, similar to that reported by Freitas et al.\(^9\), who detected HPV in 93.3% of LSIL. Possibly, the use of MY/GP+ in PCR nested system has increased the sensitivity, as reflected by the additional 27 samples (in 162) found to be HPV reactive. However, nested-PCR is not suitable for routine clinical diagnosis, as it is time consuming and prone to false-positive results, stemming from the need to open tubes and manipulate amplicons during the process. Our 100% sensitivity obtained by L1 PCR must be balanced against a low specificity, of 18.7% for biopsy-proven CIN2+. In routine practice, such value would require a triage test that could be played by cytology.

If we had used this algorithm (PCR screening followed by cytology of HPV+ samples) from the 162 samples, we would have performed 62 cytological smears, avoiding 100 procedures. However, our study didn’t investigate thoroughly those women with negative smears and/or HPV negative to rule out the presence of cervical neoplasia, so we cannot really assure that the sensitivity of PCR for biopsy-proven CIN2+ was 100% and for the same reason we are not able to estimate the sensitivity of cytology, since women cytologically normal but HPV positive were not submitted to colposcopy/biopsy.

In Brazil, a systematic review reported a variation of 16.8% to 28.6% in the prevalence of cervical HPV infection\(^10\). Our data showed an HPV prevalence of 39.5%, similar to the 39.6% prevalence obtained using consensus primers PCR findings in a population from Minas Gerais\(^11\), Brazil, but higher than observed in a regular screening high-risk population in São Paulo\(^12\), in whom 13.8% of the subjects were HPV positive by consensus primers PCR. Off course the variation in the prevalence rates, when controlled by the HPV determination method, derives from the characteristics of the population, mainly age and sexual activity. In this study, HPV DNA was detected in 25.4% of the cytological smears classified as NILM, almost the same prevalence verified in women from Rio Grande do Norte, Brazil, with normal cytology\(^13\), but twice that obtained in a global survey, in which 11.7% of the cytologically normal women harbored HPV DNA\(^14\). It has to be considered that the positivity rate of cytology results does not represent the epidemiological reality, but a number of selected patients with and without evident clinical suspicion for cervical disease to evaluate the molecular methods in this sampling compared to cytology.

As the majority of HPV infections are transient events, many women testing HPV DNA positive do not have clinical lesions, 

Table 2 – Results of molecular methodologies on cytology positive samples (ASCUS+) N = 32.

<table>
<thead>
<tr>
<th>Cytology</th>
<th>Histology</th>
<th>E6/E7 mRNA</th>
<th>HPV-DNA</th>
<th>TS-HPV PCR</th>
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<tr>
<td>ASC-US</td>
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<td>NR</td>
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<td>VAIN I</td>
<td>HPV 33</td>
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<td>HPV 33</td>
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<td>HPV 18</td>
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Not identified (NI); no return (NR); ASC-US: atypical squamous cells of undetermined significance; ASC-H: atypical squamous cells cannot exclude HSIL; LSIL: low grade squamous intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion; CIN I: cervical intraepithelial neoplasia grade I; CIN II: cervical intraepithelial neoplasia grade II, CIN III: cervical intraepithelial neoplasia grade III; VAIN I: vaginal intraepithelial neoplasia grade I.
leading to the low specificity of these tests. We aimed to evaluate whether HPV mRNA E6/E7, whose levels have been found to increase with lesion severity \(^{(29)}\), may be of higher prognostic value, thus, improving the specificity value compared to HPV DNA testing. In this study, HPV DNA results were positive in about three times more samples than E6/E7 mRNA expression. Studying women under the age of 30 years, Molden et al. \(^{(30)}\) observed HPV E6/E7 mRNA expression in approximately two out of three HPV DNA positive women, being similar to the findings observed for women older than 30 years \(^{(31)}\).

In our study, 60% of the CIN2+ samples were positive for E6/E7 mRNA. This apparently low positivity level may be attributed to high-risk HPV types not included in the assay, which explains the remaining 40% positive for HPV DNA PCR but HPV negative on the TS-HPV PCR for the five types (16, 18, 31, 33 and 45). Longitudinal follow-up of these patients may allow a better assessment of the test by investigating whether these lesions regressed or progressed to CIN3+.

Samples that were negative in cytology showing positive mRNA may represent cytology false negatives, or the spurious amplification of viral double-stranded DNA \(^{(32)}\), since they were also L1 PCR positive.

Eighty per cent of the cases diagnosed as HSIL by cytology (8/10) were confirmed by histology. The two discordant samples were diagnosed as squamous metaplasia, in one of these only HPV DNA has been detected and in the other it was also verified the expression of HPV 33 E6/E7 mRNA, which may represent a failure in biopsy, resulting from either non-representative sampled material or misdiagnosis.

Among the samples with normal cytology that we could genotype, HPV 16 was the most prevalent (3.1%). It has to be considered that despite normal cytology, women positive for high-risk HPV genotypes have much higher risk of developing CIN3+ in the future \(^{(33)}\). It has been recently shown among cytologically normal women, those testing E6/E7 mRNA positive were at a much higher risk for CIN2+ development compared to mRNA negative, on a short follow-up period within 3 years \(^{(34)}\).

NILM subjects harboring HPV 16 DNA may represent, in a subset, limitations of the cytological diagnosis. In the current casuistic, three out of four were also positive for E6/E7 mRNA. More studies are needed to investigate whether this method can be used for monitoring ASC-US, LSIL and to triage HPV DNA positive individuals, possibly reducing the number of colposcopy referrals.

It should be emphasized that, as for DNA tests, not all mRNA tests are expected to show the same performance and the reliability of other tests that detect mRNA from larger subset of genotypes, should be also evaluated. As recently highlighted in a compilation of HPV E6/E7 mRNA data, the sensitivity and specificity values obtained by different kits were quite variable \(^{(35)}\). Future cohort studies with larger populations and cost-benefit evaluation of screening using HPV mRNA and DNA detection and/or cytological analysis are warranted.

**CONCLUSION**

The mRNA E6/E7 expression was not a highly specific or sensitive marker for prevalent cervical disease while HPV DNA may be used for cervical cancer screening only in conjunction to more specific adjuvant tests.

**Conflict of interest**

The Authors declare that there is no conflict of interest.

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Address to correspondence:
TONI RICARDO MARTINS
Instituto de Medicina Tropical de São Paulo – IMT USP Laboratório de Virologia – LIM 52 Rua: Dr. Enés Carvalho de Aguiar, 470, 2º andar, São Paulo – SP. CEP: 05403-000.
E-mail: tr.martins@yahoo.com.br
Phone: +55 (11) 3061-7020

Institution address:
Universidade Federal de Santa Catarina – UFSC Hospital Universitário Polydoro Ernani de São Thiago Laboratório de Biologia Molecular e Micobactérias – LBMM Campus Universitário, s/n – Trindade Florianópolis – SC – Caixa Postal 5199 – CEP: 88040-900
Phone: +55 (48) 3721-8148

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