COMPARATIVE ANALYSIS OF THE POLYMERASE CHAIN REACTION AND THE HYBRID CAPTURE ASSAY FOR THE DETECTION OF HUMAN PAPILLOMAVIRUS INFECTION

ANÁLISE COMPARATIVA DA REAÇÃO EM CADEIA DA POLIMERASE E DA CAPTURA DO HÍBRIDO PARA DETECÇÃO DE INFECÇÕES CAUSADAS POR PAPILOMAVÍRUS HUMANOS

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RESUMO

Introdução: o câncer da cérvice uterina é apontado como a segunda causa de morte entre mulheres em todo o mundo. A partir do ano 2000, a Organização Mundial de Saúde reconheceu os papilomavírus humanos (HPV) como agentes etiológicos do carcinoma cervical. Contudo, o diagnóstico destas infecções ainda representa um desafio. **Objetivo:** avaliar o emprego de métodos moleculares a fim de comprovar a presença dos HPV no trato genital para utilização em combinação com a citopatologia, método utilizado para rastreamento das lesões causadas pelos HPV. **Método:** a pesquisa foi realizada com material de pacientes atendidas no Laboratório Dr. Sérgio Franco, no ano de 2000. As amostras foram analisadas pelas técnicas de captura híbrida (HCA II) e de reação em cadeia da polimerase (PCR) com a utilização dos *primers* genéricos MY 09/MY 11. A nossa amostra foi composta de 40 pacientes com idades entre 17 e 41 anos. A análise comparativa da HCA II e da PCR foi realizada com o objetivo de determinar a sensibilidade e a especificidade da HCA II. **Resultados:** o HPV foi detectado em 75% dessas amostras quando analisadas pela HCA II enquanto pela PCR a detecção ocorreu em 70%. A citopatologia foi usada como método de referência para avaliar rastreamento das lesões. Os três testes demonstraram concordância absoluta quando a citopatologia apontou a amostra como normal, HPV e lesão de alto grau (HSIL). Casos discordantes ocorreram onde a citopatologia diagnosticou lesão de baixo grau (LSIL), onde a prevalência foi de 100% pela HCA II e de 85% pela PCR. Comparando as duas técnicas com os resultados da citopatologia, observamos que tanto a HCA II ($p = 0$) quanto a PCR ($p = 0,002$) demonstram associação positiva. **Conclusão:** apesar das diferenças observadas, a HCA II apresentou sensibilidade e especificidade adequadas para uso clínico, em combinação com a citopatologia. Além disso, a avaliação da medida de carga viral obtida pela HCA II parece relacionar-se com a severidade da lesão e merece estudos adicionais a fim de relacioná-la com o risco de progressão ao câncer.

Palavras-chave: HPV, SIL, câncer, captura do híbrido, PCR

ABSTRACT

Introduction: cervical cancer is the second major cause of death from cancer in female worldwide. From 2000, World Health Organization accepted the Human Papillomavirus (HPV) as the etiological agents of cervical cancer. Nevertheless, the diagnosis of such viral infections still needs improvements. **Objective:** to evaluate the use of molecular methodologies to access HPV DNA, in association to Cytopathology, the classical method of screening cervical lesions. **Method:** the research was conducted by using samples from cervical smears of women attended at Laboratórios Dr Sérgio Franco, at Rio de Janeiro, among 2000. They were analyzed by the hybrid capture assay II (HCA II) and the polymerase chain reaction (PCR) with generic primers MY 09/MY 11. The study was composed by 40 patients aging from 17 to 41 years old. The comparative analysis between HCA II and PCR was conducted with the objective to determine the sensibility and the specificity of HCA II. **Results:** HPV was detected in 75% of the samples, by HCA II, while PCR showed HPV in 70% of the patients. Cytopathology was used as a reference method of screening the lesions. The three tests showed absolute agreement when cytopathology referred the sample as NORMAL, HPV and High grade lesion (HSIL). Disagreements occurred when cytopathology pointed low squamous intraepithelial lesions (LSIL), in which HCA II showed 100% of HPV detection and PCR pointed only 85% of DNA detection. After comparing the two molecular techniques with cytopathology results, we observed that in HCA II ($p = 0$) as well as in PCR ($p = 0.002$) a positive association was demonstrated. **Conclusion:** hence, we concluded that, despite a few disagreements, HCA II showed to be sensitive and specific enough to be used in combination with cytopathology. Besides that, the evaluation of viral load measures obtained by HCA II showed to be related to the severity of the lesion and merits further studies to analyze possible association to risk of progression to malignancy.

Keywords: HPV, SIL, cancer, hybrid capture, PCR

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Introduction

Human Papillomavirus (HPV) infection is the major cause of most cervical cancer and cervical intraepithelial neoplasia (CIN) worldwide[1]. Nearly 500.000 cases of cervical cancer arise each year and 200.000 women eventually die. In Brazil, uterine cancer is the third cause of death due to neoplasias[2]. Consequently, there is strong motivation to evaluate the use of HPV testing in cervical cancer screening, what requires further improvements in standardization of testing methods. HPV detection has generally been conducted by Hybridization and the Polymerase Chain Reaction (PCR) methods. But neither research assays nor commercial kits (dot blot or in situ hybridization) have shown to be adequate for clinical use. An assay for routine clinical use requires reliable and accurate detection of the broad range of pathogenic HPV types infecting the genital tract [3]. The diagnosis of cervical disease, pointing the presence of abnormal cervical epithelial cells, is usually obtained by microscopic examination of Papanicolaou stained smears. This has been the method of choice since the 1950s, proving valuable for mass screening and enabling detection of lesions early enough to be treated effectively. The Pap smear, however, has limited sensitivity in detecting cancer precursors,giving false-negative rate ranging from 20% to 30%[4]. Hence, complementary methods that may enable the improvement of the diagnosis of cervical disease have been studied for the past two decades.

Recently developed, the second generation of Hybrid Capture System HPV DNA detection test (HCA II) from Digene Diagnostics (Silver Spring, Md.), is a non-radioactive, relatively rapid, liquid hybridization assay designed to detect eighteen HPV types divided into high-risk and low-risk groups. As a possible unique advantage compared with other available HPV test kits, the hybrid capture test is also designed to provide quantitative estimates of viral load, which may correlate with the grade and the natural history of cervical pathology.[5,6]

Given the fact that diverse clinical laboratories are currently using this method, the present investigation was conducted to compare HCA II with PCR, with the aim to give tools to the interpretation of this new available procedure, with the objective to contribute to cancer prevention.

METHODS

Study population and collection of specimen

The study population included 40 women attending at Laboratórios Sérgio Franco, Rio de Janeiro, Brazil, from January to December, 2000. Women were referred to routine exam. The cervical smears were collected by using a cervical cytobrush, and transported in Digene Specimen Transport Medium (Digene Diag, Md).

Cytologic Test

Papanicolaou test was developed and smears were classified in grade I (PAP I) as normal epithelium, grade II (PAP II) for minor alterations of cervical cells, grade III (PAP III) for low grade squamous intraepithelial lesions (LSIL) and grade IV (PAP IV) for high grade squamous intraepithelial lesions (HSIL), in situ carcinoma and invasive cancer.

HPV testing by Hybrid Capture

The assay kit detects low and high-risk. The high-risk types 16,18,31,33,35,39,45,51,52,56,58,59 and 68. The low-risk group detects the types more commonly associated with condyloma acuminatum: HPV types 6,11,42,43 and 44. According to the kit protocol, specimens were treated with sodium hydroxide to hydrolyze specimen RNA and to denature the DNA. The liberated single strand DNA was hybridized in solution with a RNA probe mix consisting of the high-risk or the low-risk HPV types. Each reaction mixture, containing any RNA-DNA hybrids that formed, was transferred to a capture tube coated with antibodies to the hybrids, immobilizing them. Bound RNA-DNA hybrids were then reacted with an alkaline phosphatase-conjugated antibody directed against the hybrids. Unreacted material was removed by washing, and a dioxetane-based chemiluminescent compound, Lumi-Phos 530, was added as a substrate for alkaline phosphatase. The light produced by ensuing reaction was measured by a Luminometer. Light measurements were expressed as relative light units (RLUs). As a negative control, sonicated herring sperm DNA in Digene transporting medium (100 g/ml) was used. Triplicate specimens of HPV 16 or HPV 11 DNAs at 10pg/ml served as the positive controls for high-risk and low-risk probes, respectively.

All RLU measurements for specimens were divided by the mean RLU of the three appropriate positive controls (PCs) to give a ratio of specimen RLU/PC. A ratio of 1.0 or greater was regarded as positive for HPV DNA, and a ratio of less than 1.0 was regarded as negative.

Since the amount of the light produced by the hybrid capture assay is theoretically proportional to the amount of target HPV DNA, HCA II can be analysed as a quantitative method.

HPV testing by Polimerase Chain Reaction (PCR)

The PCR assay was achieved from the clinical samples initially collected for the HCA II. The specimens were stored at – 20°C in sodic azidum until required.

First, the specimens were neutralized by using HCl 1N. The DNA extraction was performed as follows: 500µl of the neutralized specimen were precipitated on 50µl of the sodium acetate and 1250µl of the 100% ethanol. The specimens were incubated at -20°C overnight. The day after, these samples were centrifuged for 30min at 14000rpm. After centrifugation, the supernatant was discarded and the remaining precipitated sample was washed in 70% ethanol and centrifuged at 10 min at 14000rpm. The precipitate was left to dry at room temperature and ressuspended at 50µl of distilled water and stored at –20ºC.

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PCR amplification of generic HPV

Consensus primers MY09 / MY11 which amplify 450 bp DNA sequences within the L1 region of HPV were used to detect generic HPV DNA. Amplification was carried out in 50 µl reaction mixture (1X PCR buffer, 200 µM dNTPs, 1,5mM $MgCl_2$, 50 pmol of each primer, 0,25U unit of Taq polymerase, and 5 µl of sample) with 35 cycles of amplification. Each cycle included a denaturation step at 94^0C for 1minute, an annealing step at 55^0C for 2 minutes, and a chain elongation step at 72^0C for 2 minutes using DNA Thermal Cycler (Perkin Elmer, CETUS). The actine primers (0,1 pmol each), which amplify a 360 bp region of the human DNA was used as internal control.

PCR PRODUCTS WERE ANALYSED ON 1,3% AGAROSE GEL WITH ETHIDIUM BROMIDE STAINING FOR VISUALISATION OF DNA UNDER ULTRAVIOLET LIGHT AND THEIR PM DETERMINED BY COMPARISON WITH A 100BP DNA LADDER.

Statistical analysis

The statistical significance of the results has been analyzed by using SPSS-8 computer program (2002-USA).

RESULTS

Our study compared the results achieved by PCR and Hybrid Capture HCA II tests for diagnosing genital infection by HPV. Fifty-two samples of cervical smears were collected and tested by HCA II and PCR: 40 were cervical smears from attended patients, 6 were positives controls and 6 negative controls.

The medium age presented for the patients was 29,5 ranging from 17 to 41 years old (**Table 1**). The prevalent interval for HPV infection was from 21-30 years old. Samples were classified according to Bethesda system in Normal (3/40), ASCUS (8/40), HPV(8), LSIL(20) and HSIL(1).

Table 1 presents the results obtained by either HCA II and PCR. From the 40 studied patients, 30 (75%) were infected by HPV, as shown by HCA II. When samples were tested by PCR, 28 (70%) women were HPV-positive. Concordant results were observed for 36 samples (90%) while discordant results were found for 4 samples (10%): one case of ASCUS positive for PCR and negative at HCA II and 3 cases of LSIL positive for HCA II but negative at PCR. The two tests presented a good association index according to Fisher Exact Test (p=0.0002) (Fisher Test) and a kappa coefficient k=0.76 (95% confidence interval), pointing to an excellent agreement index for diagnosing HPV infection.

The prevalence of high and low risk types of HPV by the Hybrid Capture assay in comparison to the results of polymerase chain reaction is presented on the **Table 1**. High risk group B was the most prevalent group detected by HCA II, counting for 13 cases alone or mixed with group A in 14 cases. Hence, 27 women out of 40 studied ones showed high-risk infections (67.5%). Sensibility and specificity rates are presented in **Table 2** and were obtained by using colpocytology as a reference for screening patients. Sensibility were better for HCA II (96.5%) than PCR (86.2%) and detected all referred cases of LSIL but specificity rate was 100% for both techniques.

The medium values of the viral load measured by the Hybrid Capture Assay can be observed on **Table 3**. There was an increase in the values according to the severity of the cytologic test for both benign viruses (Group A: 15,28 to 370,78) and oncogenic HPV (Group B: 43,32 to 337,45). For the HSIL patient, a decrease in viral load was associated to integration and loss of E2 gene expression (data not shown).

We compared the viral load measurements obtained by HCA II with PCR and results can be observed on the **Table 4**. For the patients infected by low risk HPVs (Group A) we have seen that for higher viral loads (RLU/PC >3.0), there was a complete correlation with PCR positivity and 100% of tests gave concordant results (coefficient φ =1.0, p=0.008) (Fisher exact Test, p value=0.02). For the patients infected by high risk HPVs, alone

Table 1 - Results and Agreement rate obtained by the PCR and the Hybrid Capture Assay (HCA II) according to the Colpocytology.

Colpocytology Diagnosis	Number of Patients	Hybrid Capture			PCR	Agreement
		A	B	A/B		
NORMAL	3	-	-	-	-	100%
ASCUS	8	-	2	-	3	66%
HPV	8	2	3	2	7	100%
LSIL	20	1	7	12	17	85%
HSIL	1	-	1	-	1	100%
Total	40	3	13	14	28	85.7%

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Table 2 - Evaluation of statistic parameters of Sensibility and Specificity of PCR and HCA II using Colpocytology as a Reference for screening patients.

	Hybrid Capture II	PCR
Sensibility	96.5%	86,2%
Especificity	100%	100%

Table 3 - Media of viral load measurements (RLU/PC) obtained by HCA II according to Cytology.

Colpocytology Diagnosis	Viral Load Medium (RLU/PC)		
	A	B	A/B
NORMAL	-	-	-
ASCUS	-	43,32	-
HPV	15,28	309,60	870,50/237,48
LSIL	370,78	337,45	911,81/908,05
HSIL	-	63,44	-

Table 4 - Association of viral load obtained by HCA II and PCR results.

PCR	Viral Load (RLU/PC)		
	A	B	A/B
POSITIVE	199,68	291,77	915,87/1095,62
NEGATIVE	1,98	115,79	891,48/470,19

or in multiple infections, viral load measurements showed a slight upward trend but with no statistic significance, showing a poor correlation to PCR positivity. Results of the two tests were weakly related (Fisher Exact Test, p value = 0,05).

DISCUSSION

In Brazil, cervical cancer is the third most frequent cause of death from cancer and the establishment of the disease has presented earlier in the female population in the last two decades[7]. Regarding these data, some authors have questioned the reliability of screening for cervical neoplasia using Papanicolaou smear alone and thus have suggested the use of additional methods to improve the accuracy of cervical lesions routine diagnosis. Investigators suggested combining routine cytology and HPV DNA testing in order to improve the detection of HPV-caused disease.[3]

Hybrid capture assay is currently being used for testing HPV DNA in Brazil as well as worldwide, but no studies were conducted to test its usefulness in our country. Hence, our investigation was conducted to compare HCA II with PCR, with the aim to give tools to the interpretation of this new available procedure. For that purpose we studied cervical samples by using both HCA II and PCR assays. The tests were concordant for 36 out of 40 samples (90%). HCA II pointed out HPV infection in 75% (30/40) of the patients while PCR detected HPV DNA in 70% (28/40) of the cases. Discrepancies in the results were noticed: PCR failed to detect three samples classified as LSIL by the cytology and positive for high risk types of HPV in HCA II. Muñoz [8] have already described false negatives when using exclusively MY primers to screening HPV infection and suggested the use of an additional pair of primers (GP5/6) to reduce these events. We suggest that screening by MY primers is still useful but samples might also be tested for high risk HPVs, specially HPV 16 and 18, the two most oncogenic and prevalent types detected in Rio de Janeiro, Brazil [9]. It is interesting to notice that these problems were concentrated in samples that were high-risk infections by HCA II. Thus, could be associated to HPV integration that leads to L1 deletion. Hence MY primer amplification could in fact turn to be negative.[10]

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The other discrepancy was observed for an ASCUS sample: HCA II was positive while PCR were negative. ASCUS diagnosis is of exclusion for HPV infection and the HPV infection here described might represent a false-positive by HCA II. Previous studies have already suggested false positives and authors associated the cases to low viral loads measured by HCA, suggesting that they could be solved by changing cut off of the reaction from 1.0 to 3.0 RLU/PC[11,12]. Nevertheless, the diagnosis of ASCUS presents problems and definition of criteria are still in study since misdiagnosing is often seen and an ASCUS observation might even represent a HSIL[13]. Manos *et al*[14] have already proposed that ASCUS diagnosis would indicate the testing for HPV DNA as well as of colposcopy exam in order to discard the possibility of a lost high grade lesion.

In our study we describe statistical parameters of sensibility and specificity for HCA II and PCR, using the Cytopathologyc test as a refference method of screening Brazilian patients. Sensibility rates of 96.5% and 86.5% were described for HCA and PCR respectively (**Table 2**) and show that both PCR and HCA II are excelent tests for screening patients infected by HPV and that can be at a higher risk of cancer.

In **Table 3**, data points to an increase in the viral load measurements according to the severity of the lesions. It is interesting to observe that in the HSIL case, a low viral load was obtained. This might indicate integration of HPV genome that leads to a non-productive cycle of replication. In fact we detected integration of HPV in this sample, with the lost of E2 gene (data not shown). The usefulness of measuring viral load by HCA II has been the object of several studies. Clavel *et al*[15] did not find significant relation of measures and lesions, but, like us also noted the low values associated to high grade lesions. Their study described that it was due to the small amount of HPV genome, due to integration and loose of productive replication. Further studies will be necessary to amplify our knowlegde and help in elucidating the meaning of HCA viral load.

In spite of being an easily preventable and treatable disease, the cervical cancer is still presenting high rates of morbidity and mortality among Brazilian female. Molecular techniques able of detecting HPV infection are expensive and can not be used in routine diagnosis but would complement cervical screening, being applied subsequently to Pap test, in order to reduce the risk of cancer establishment. As already pointed out by Lopes *et al* [2], female patients would be tested when presenting progressive or recurrent lesions, since these represent an increased risk for cancer, associated to HPV persistence in the cervix.

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