Evaluation of the Combined use of Papanicolaou Screen Test and The Polymerase Chain Reaction for the Identification of Patients at Risk of Cervical Cancer

Avaliação do uso Combinado do Teste de Triagem de Papanicolaou e da Reação em Cadeia da Polimerase para Identificação de Pacientes em Risco de Câncer Cervical

Natalia Moysés[1], Daniela S Balthazar[2], Larissa A Afonso[1], Ledy HS Oliveira[3], Silvia Maria B Cavalcanti[4]

ABSTRACT

Introduction: in the last few years, the interest on Human Papillomavirus (HPV) has emerged due to the evidence of their carcinogenic potential, especially on the female genital tract. In the last fifty years, the screening diagnosis has been carried out by Papanicolaou test (Pap). Nevertheless, literature describes a high rate of false-negative and false-positive samples. **Objective:** since an early detection is crucial to diminish the risk of cervical cancer, we aimed to analyze the use of HPV DNA detection by PCR as complementary test to routine screening. **Methods:** nearly 450 female smears were obtained from the DNA Bank of the Virological Diagnosis Laboratory from UFF. HPV prevalence was evaluated by using MY09/11 consensus primers and was compared to Pap test. **Results:** our results showed that 67.5% of the studied samples were normal tissues, among them 85.6% were negative by PCR but 14.4% were HPV infected. The remaining 32.8% were altered by Pap test. Among them, HPV DNA detection by PCR revealed a prevalence of 56.7% in ASCUS, 87.5% in LSIL and 66,6% in carcinoma. Kappa index showed a good agreement between tests (0.80). The Positive Predictive Value was considered low (58%) pointing out that important cases may be misdiagnosed as false-negatives. On the other hand, the Negative Predictive Value was considered high (90,5%) indicating that PCR should not be used as a screening test, but as a complementary one, revealing true negatives when associated to a normal result in Pap test. HPV positive samples detected by MY PCR were typed and the prevalence obtained for the different types was: 48% HPV 16, 17% HPV 33, 13% HPV 18, 18% HPV 6 and 19% were undetermined because of non tested primers or technical problems. **Conclusion:** analyzing the results we concluded the combination of both tests is the best diagnostic procedure, allowing a more efficient evaluation of cancer risk and thus helping in prevention programs.

Keywords: HPV, PCR, citopathology, cancer, STD

RESUMO

Introdução: nos últimos anos, o interesse pelo estudo dos papilomavírus humanos (HPV) aumentou, uma vez demonstrada a evidência de seu potencial oncogênico, especialmente no trato genital feminino. A partir de 1950, o rastreamento das lesões genitais foi feito pelo teste de Papanicolaou (colpocitologia oncótica). Entretanto, a literatura o atribui altas taxas de falso-positivos e negativos. **Objetivo:** uma vez que a detecção precoce é crucial para prevenção do câncer cervical, analisamos o uso do PCR para detecção do DNA do HPV como teste complementar à atual rotina diagnóstica. **Métodos:** cerca de 450 esfregaços cervicais foram obtidos do banco de amostras do Laboratório de Diagnóstico Virológico da UFF. A prevalência do HPV foi avaliada pelo uso de *primers* consensuais MY09/11. **Resultados:** nossos resultados mostraram que 67,5% das amostras eram normais e dentre eles, 85,6% eram negativas pelo PCR, mas 14,4% estavam infectadas. As 32,5% amostras restantes tinham preventivo alterado e com alta prevalência de infecções por HPV, oscilando de 56,7% em ASCUS a 87,5% em LSIL. O índice Kappa apresentou boa concordância entre os testes (0,80). O valor preditivo positivo foi baixo (58%) indicando que casos relevantes podem ser subdiagnosticados como falso-negativos. Por outro lado, o valor preditivo positivo foi elevado (90,5%) revelando que o PCR, embora não deva ser usado como método de triagem, tem importante papel complementar ao preventivo, apontando os verdadeiros negativos, quando associado à colpocitologia oncótica normal. Amostras positivas para HPV pelo PCR-MY foram tipadas e a prevalência obtida revelou 48% HPV 16, 17% HPV 33, 13% HPV 18, 18% HPV 6 e 19% de HPV indeterminados. **Conclusão:** nossos resultados indicam que o uso combinado de ambos os testes parece ser a conduta diagnóstica mais adequada, permitindo avaliação eficiente do risco de câncer e assim colaborar em programas de prevenção.

Palavras-chave: HPV, PCR, citopatologia, câncer, DST

INTRODUCTION

Cervical cancer is still the second cause of death from cancer in women worldwide. Each year, nearly 470,000 new cases of invasive cervical cancer are diagnosed and approximately 233,000 women die of this disease[1]. In Brazil, high rates of incidence and prevalence are recorded and the establishment of the disease has presented earlier in the female population in the last two decades[2].

Cervical cancer is preceded by a long period of pre-malignant lesions termed cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL), that are classified into several grades (CIN I or Low SIL; CIN II/III or High SIL). In principle, these lesions are reversible, although the more severe the lesion, the lower chance of spontaneous regression. Cervical cancer is considered a preventable disease because it can be precociously detected by exfoliative cytology and treated when necessary, with minor side effects. Currently, cervical screening is based on cytology only. However, cytology is subject to a substantial degree of false-negative and false-positive results, leading to missed cases

[1]Aluna de PIBIC da Biomedicina/Universidade Federal Fluminense.
[2]Biomédica/UFF.
[3]Professora titular da Disciplina de Virologia do Departamento de Microbiologia e Parasitologia/UFF.
[4]Professora associada da Disciplina de Virologia do Departamento de Microbiologia e Parasitologia/UFF.

of HSIL and cancer as well as to redundant follow-up and referrals to gynecologist[3].

OBJECTIVE

Since infection infection with high risk human papillomavirus types (hrHPV) is a necessary cause of cervical cancer, screening might become more efficient when it is based on combined cytology and hrHPV testing[4]. This study aimed to verify the veracity of such hypothesis.

METHODS

Studied samples

We studied 443 DNA cervical samples obtained from the DNA Bank of the Virological Diagnosis Laboratory from Universidade Federal Fluminense and were recorded from 2000 to 2007.

Cytologic Test

Papanicolaou test was developed in Saddy Diagnósticos Laboratory and files were gently given to our group. Smears were classified in normal for Normal epithelium, Inflammatory for minor alterations of cervical cells, low grade squamous intraepithelial lesions (LSIL) for low grade neoplasia, high grade squamous intraepithelial lesions (HSIL) for moderate and severe neoplasia, invasive cancer for malignant neoplasia and ASCUS for Atypical squamous cells of undetermined significance.

Polymerase chain reaction amplification of generic human papillomavirus

Consensus primers MY09/11, which amplify 450 bp DNA sequences within the L1 region of HPV, were used to detect generic HPV DNA[5]. Amplification was carried out in 50 µl reaction mixture (1X PCR buffer, 200 µM dNTPs, 1.5mM $MgCl_2$, 50 pmol of each primer, 0.25 U unit of Taq polymerase, and 5 µl of sample) with 35 cycles of amplification. Each cycle included a denaturation step at 94^0C for one minute, an annealing step at 55^0C for two minutes, and a chain elongation step at 72^0C for two minutes using DNA Thermal Cycler (Pekin Elmer, CETUS). The β-actine primers (0.1 pmol each), which amplify a 330 bp region of the human DNA was used as internal control. Polymerase chain reaction (PCR) products were analysed on 1.3% agarose gel with ethidium bromide staining for visualization of DNA under ultraviolet light and their PM determined by comparison with a 100bp DNA ladder.

HPV typing

HPV typing was done by PCR amplification with primers from the E6 gene DNA sequences of HPV 6, 11 (low risk to cervical cancer) and 16, 18, 31, 33, and 35 (high risk to cervical cancer)[5]. PCR reactions included a denaturating step at 94^0C for 30 seconds, 60^0C for one minute, and 72^0C for one minute. Negative controls for background contamination no added DNA template. The PCR run was completed by extension for ten minutes at 72^0C.

Statistical analysis

The statistical analyses were performed by using the Epi-Info® computer program package (Atlanta, USA). Cohen test, t-Student and Kappa indexes were determined. In all tests, the values of p<0.05 were regarded as statistically significant.

RESULTS

In our study, 443 DNA samples from cervical smears were evaluated. The average age of the studied women were 31.3, ranging from 16 to 83 years old. Regarding cytopathology, 67% of the cases presented normal/inflammatory results (**Table 1**). Twenty-two samples presented inconclusive results.

PCR results using consensus MY09/11 revealed that 151 samples were HPV infected (35.3%). **Table 2** presents DNA prevalence according to citopathology. From the 421 samples diagnosed in cytopathology, 138 had HPV DNA (32.8%). From the 22 inconclusive Pap tests, 13 (59.1%) samples were positive at MY PCR.

According to Pap test, 284 samples were classified as normal/inflammatory and among them, 14.4% were positive by PCR. Regarding ASCUS results, 30 samples were recorded and 56.7% were HPV infected. In samples classified by cytology as suggestive of HPV, 84% were confirmed by PCR. Within the forty cases of LSIL, 87.5% were HPV infected and the 36 HSIL samples presented 55.6% of HPV DNA prevalence. Finally, 6 samples were classified as invasive carcinoma and 66.6% were positive by PCR.

Regarding the 283 cases showing altered cytology 10% were negative in MY PCR, suggesting false-negative in molecular detection of HPV, more probably false-positives in cytology.

After proceeding to MY PCR for HPV screening, 141 positive samples were tested in a second PCR test using specific primers for the most prevalent benign and malignant HPV types. Among them, 89 were high risk types and 11 were low risk types, 13 were multiple infections (**Table 3**). From the 100 typed samples, HPV 16 was the most prevalent type (48%) followed by HPV 33 (17.0%) and HPV 18 (13.0%). Benign HPV 6 prevailed in 11.0% and HPV 11 was only observed in mixed infections, associated to HPV 6. High risk HPV 45 and 58 were detected in 4.0% of the positive samples and HPV 35 in 3%, while HPV 31 was not found at all. Nearly 20% of the samples remained untyped (**Figure 1**). Multiple infections were diagnosed in 13 samples, with a higher prevalence of HPV 16 and 18 (46,2%), followed by HPV 6 and 11 (38,8%) (**Table 3**).

Regarding the 80 samples positive by PCR presenting altered cytology, 77 were typed and 72.7% were high risk HPV, HPV 16 prevailing among the others (48.2%).

From the 41 PCR positive presenting normal cytology 35 cases were typed and 18 were high risk, with a prevalence of 50% for HPV 16. **Table 5** describes the association of HPV types with de cytological results. HPV 16 were detected in 34% of the samples, for all the classified samples.

The evaluation of statistical parameters pointed out that PCR presented a sensibility of 74.8% (80/107) with a Positive predictive value of 58% (80/138). A high specificity of 81.5% (256/314) was detected, with a Negative predictive value of

Table 1 . Distribution of cases according to Papanicolaou test results

Samples (n)	Normal	ASCUS	HPV	LSIL	HSIL	Ca
	284	30	25	40	36	6
Prevalence	67,5 %	7,1 %	5,9 %	9,5 %	8,6 %	1,4 %

Table 2. HPV DNA detection by PCR according to cytological diagnosis

Cytopathology	HPV prevalence (%)		HPV negative (%)		TOTAL
Normal	41	(14,4)	243	(85,6)	284
ASCUS	17	(56,7)	13	(43,3)	30
HPV	21	(84,0)	4	(16,0)	25
LSIL	35	(87,5)	5	(12,5)	40
HSIL	20	(55,6)	16	(44,4)	36
Ca	4	(66,6)	2	(33,3)	6
Total	138	(32,7)	283	(67,2)	421

Table 3. Prevalence of HPV types detected by PCR according to citopathology.

Citology	Typing by PCR												Untyped	Total
	Types							Multiple Infections						
	6	16	18	33	35	45	58	6 e 11	16 e 18	16 e 33	33 e 45	33 e 58		
Normal	2	9	4	3	2			1					14	35
ASCUS		10	1	1					1			1	2	16
HPV	3	8	2	2			1	2	1				2	21
LSIL	6	12	3	3	1		2	1	2	1			4	35
HSIL		5	3	4					2		1		2	17
Carcinoma		2			1								1	4
Inconclusive		2		4	3	1							3	13
Total	11	48	13	17	3	4	4	4	6	1	1	1	28	141

Table 4. PCR sensibility and specificity according to cytology

PCR		Citology		
		Positive (Altered)	Negative (Normal)	Total
	Positive	80	58	138
	Negative	27	256	283
	Total	107	314	421

90,5% (256/283). Cohen test was developed in order to analyze the tests agreement rates and the resulting Kappa index was considered good (0.80, 336/421) (**Table 4**).

DISCUSSION

Cervical cancer is a long multiple-step process that eventually leads to invasion. The natural history shows that 10 to 20 years are required to complete tissue transformation. Hence the detection of precursor lesions can determine efficient treatment and control of this neoplasia. Nevertheless, Brazil presents high cancer incidences[6] and cancer progression can occur in nearly 30% of cases associated to hrHPV[7]. Only precoce diagnosis extended to whole female population showed to be efficient worldwide[8]. This early diagnosis pointing abnormal cervical cells is obtained by Pap test: a simple, easy and inexpensive procedure. This method was elected the screening test for general population and in countries where it was properly applied; it resulted in a 75% reduction in cancer incidence rates[9,10].

But the test presents limitations: inadequate samples constitute 5% to 10% of the material received by cytopathology laboratories. The low sensibility to detect premalignant lesions as well as the subjectivity of results interpretation are also relevant questions. High rates of false-positive and false-negative results, ranging from 20 t0 30%, have been described[8]. Hence patients can eventually develop cancer and reach death[11].

Hence, complementary tests have been developed in order to increase efficiency of screening programs for cervical prevention, identify women presenting or in risk of developing high grade lesions (HSIL and Ca), diagnose truly negative results, improve ASCUS diagnosis and check therapy response[12].
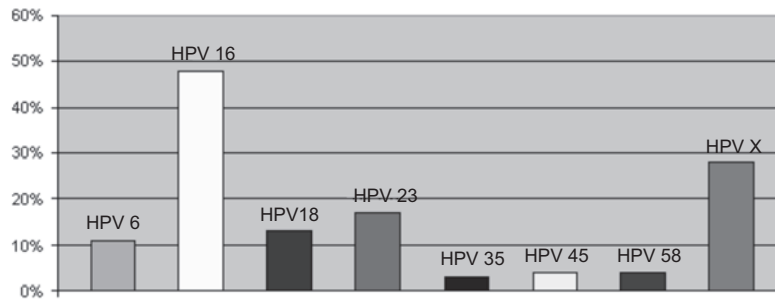
Figure 1. Prevalence of HPV types.

According to Brink[4], PCR is the best novel procedure to identify patients at risk for HPV cervical cancer. The other available technique, the hybrid capture assay presents the disadvantage of cross-reactions between low and high risk virus. Regarding the consensus PCR results obtained in this study, we observed a HPV prevalence of 34.1% (151/443), pointing out a high prevalence of infection in the studied population. These were already expected results, since part of the group were clinically directed to the search of HPV on previous detected cervical lesions. By relating cytology and PCR results, we observed the increase in the severity of the lesions related to the increase in the hrHPV DNA detection. In **Table 2**, we can observe this positive relation. It is interesting to notice that normal cytology was related to 85.6% of PCR negative results. However, the other 14.4% were positive by molecular testing. We can argue if they were false negative by cytology but we know PCR is a highly sensitive technique that can detect latent virus and thus may be showing clinically irrelevant infections. According to previous studies, 10 to 15% of the cytological normal women can present HPV DNA.

Regarding HPV prevalence in ASCUS cases, 56.7% of positive MY were detected, and literature describes ASCUS as possible HSIL in 10% of the women[8].

Among smears suggestive of HPV, viral DNA was detected in 84.0% of the cases by PCR. This is an expected result, since patognomonic alterations are described by cytology, as koylocytosis and dysceratosis. On the other hand, 16% of the samples classified as HPV were negative by PCR MY. These results might be due either to low celularity of the sample used to DNA extraction or to DNA degradation. We can also suggest cytology false-positive results.

In LSIL cases HPV DNA was detected in 87.5% of the samples while in HSIL, detection felt to 55.6% (**Table 3**). Molecular testing of cancer samples can be difficult once these are lesions difficult to detect, such as microcarcinoma or microinvasive cancer. DNA integration can also result in genomic loss and false-negative MY PCR[14].

Among the 6 squamous cell carcinoma, 4 (66.6%) presented viral DNA. Again, integration, as well as sample contaminants as blood as cell debris can result in poor amplification in consensus PCR. Finally, PCR although specific and sensible depends on sample collection, proper conservation and transport, contaminants that can interfere and reduce viral detection.

From the 141 HPV typed samples, 69.5% (98/141) had oncogenic viruses alone or in multiple infections. Hence, such women can be regarded as a risk group for cancer development, requiring medical surveillance and close examination, including colposcopic evaluation and reduced follow-up intervals.

HPV 16 was the prevalent type (34%, 48/141). In 28 samples, HPV was not typed and perhaps represent new circulating types that are not composing our pool of testing primers. Camara et al[2] have already chosen that nearly 10% of HPV cases are undetermined even by RFLP of sequencing methods.

It is noticeable that HPV 31 was not found in our sample is in agreement with studies from Carestiato et al[13], that confirms the low circulation rate among our patients. On the other hand, nearly 10% of HPV 31 infection are detected in samples from Brazilian central region[2] and in Europe[14].

PCR typing of normal cytology smears revealed 18 cases of oncogenic HPV infections, 2 benign HPV 6 and 1 multiple benign infection harboring HPV 6 and 11. Among oncogenic types, HPV 16 was the most prevalent (50%), followed by HPV 18 (22,2%), 33 (16,6%) and 35 (11,1%) (**Figure 1**). As reported by Brinks et al[4], such infections may represent a relative risk of development of neoplastic lesions, requiring precoce follow-up. Women over 35 years old, presenting oncogenic HPV infection are considered the high risk group for cancer, even in the absence of cervical lesions, and must be clinically followed at 4 month intervals, as proposed by CDC Working Group[15].

Analyzing ASCUS samples, we observed that only oncogenic virus were detected and HPV16 was the most prevalent (62.5%). Although not related to HPV infection, an ASCUS result can be represent a HSIL (specially the difficult detecting microcarcinoma) misdiagnosed in 10% of the cases and are described in literature as ASC-H (high risk ASC). Molecular testing can help revealing the risk and thus redirect the patient to a new test or a colposcopy.

Cytology classified as suggestive of HPV revealed high risk HPV 16 in 38% of the cases, similar to the one found in LSIL (34.3%). As described by Brink[4], the negative predictive value of molecular testing is of 99%, for both HPV and LSIL results, indicating that there are no high grade lesions when they are negative to high risk HPV. On the other hand, women positive for high risk types should be immediately directed to colposcopic biopsy and retesting after 6 months. Hence the combined use of Pap test

and HPV detection would allow a closed diagnosis, helping clinic management of patients.

Regarding HSIL and Ca patients, only hr HPV were found, an expected result, due to its hole in cancer development. Once more, 16 was the most common type (29.4%), followed by 33 (23.5%) and 18 (17.6%) in HSIL and by HPV 45 (25%) in Ca cases (**Table 3**). HPV detection in cancer cases has an impact in research data since clinical conduct will not be altered by PCR result.

It is worth noting that the 13 samples presenting inconclusive results but showing HPV infection, presented hr HPV in 76.9% of the samples, indicating the need of early repetition of the Pap test.

We might point out that new molecular methods to HPV detection might have an unbalance between analytical and clinical evaluation. PCR have a high analytical power but can reveal latent or transient infections clinically irrelevant. On the other hand, misdiagnosis common in Pap test can result in loss of important pathologies that can eventually result in cancer[4].

Our results showed that the sensibility of the PCR test was low (74.8%), with an unsatisfactory PPV (58.0%). These data point to the fact that PCR should not be used alone but combined to other screening methods, once it can loose important lesions (low clinical evaluation level) although presenting a high analytical power. This important losses observed might be due to degradation of genetic material, deletion of HPV genes due to integration, leading to false negative in PCR-MY[13].

But our data also points out a high specificity in PCR testing (81.5%) as well as the NPV (90.5%), reassuring its use to elucidate Pap test controversial results. In conclusion, although a few cases were negative by PCR when Pap test showed a SIL, when PCR is positive, the chance of presenting clinical relevant lesions is high, mainly in women older than 35 years old.

In our study, Cohen test to determine agreement index between PCR and Pap test were 0.80: a good result, certifying PCR as a good complement to cytology in order to elucidate inconclusive smears (5%), ASCUS and associated risk of HPV and LSIL.

Although molecular testing is an expensive and laborious procedure, preliminary analysis recommends the combined use for specific cases, pointing to a 100% of efficiency increase[16]. Especially, in cases with ASCUS result and altered cytology in older women[4]. Hence combined use can be an important tool for improving cervical cancer screening.

CONCLUSION

We concluded the combination of both tests is the best diagnostic procedure, allowing a more efficient evaluation of cancer risk and thus helping in prevention programs.

REFERENCES

1. Bosch FX & Sanjosé S. Chapter 1: Human papillomavirus and cervical cancer – Burden and assessment of causality. J Nat Can Inst Mongr 2003; 31: 3-13.
2. Camara GNL, Cerqueira DM, Oliveira APG, Silva EO, Carvalho LGS, Martins CRF. Prevalence of human papillomavirus types in women with preneoplastic and neoplastic cervical lesions in the Federal District of Brazil. Mem Inst Oswaldo Cruz 2003; 98: 879-883.
3. Sherman, M.E. Future directions in cervical pathology. J Nat Cancer Inst Monogr 2003; 31: 72-79.
4. Brink, A.A.T.P.; Zielinsk, G. D.; Steenbergen, R.D.M.; Snijders, P. J. F.; Meijer, C. J. L. M. Clinical relevance of Human papillomavirus testing in cytopathology. Cytopathology 2005; 16: 7-12.
5. Oliveira, L.H.S.; Rodrigues, E.V.; Salles, L.A.P; Fernandez, A.P; Cavalcanti, S.M.B. HPV 16 detection in cervical lesions, physical state of viral DNA and changes in p53 gene. São Paulo Med J 2003; 121 (2):67-71.
6. INCA. Câncer cervical, Ministério da Saúde, INCA, 2006. Disponível em http://www.inca.gov.br
7. Cavalcanti, S.M.; Zardo, L.; Passos, M.R.L.; Oliveira, L.H.S. Epidemiological aspects of Human papillomavirus infection and cervical cancer in Brazil. J Infect 2000; 40 (1): 80-87.
8. Burd, E. M. Human papillomavirus and Cervical Cancer. Clin Microbiol. Rev. 2003; 16 (1): 1-17.
9. Schiffman, M. H.; Brinton, L. A.; The epidemiology of cervical carcinogenesis. Cancer 1995; 76:1888-901.
10. Jemal, A.; Thomas, A.; Murray, T.; et al. Cancer Statistics 2002, CA. Cancer J Clin 2002; 52: 23-47.
11. Morrison, H. Human papillomavirus absence predicts normal cervical histopathologic findings with abnormal papanicolaou smears. J Hum Virol 1993; 4: 283-287.
12. Molijn, A.; Kleth, B; Quint, W.; van Doorn, L.J. Molecular diagnosis of human papillomavirus infection. J Clin Virology 2005; 23S:43-51.
13. Carestiato, F.N.; Carvalho, M.O.O.; Ribeiro, M.O.; Barbosa, F.M.; Silva, L.E.; Dimetz, T.; Oliveira, L.H.S. & Cavalcanti, S.M.B. Estudo das infecções causadas por papilomavírus humanos em pacientes do sexo feminino detectadas pela técnica de captura do híbrido: levantamento de casos. J Bras Doenças Sex Transm 2002; 14: 9-12.
14. Clifford GM, Gallus S, Herrero R, Muñoz N, Snidjers PFJ, Anh PTH, Ferrecio C, Hieu NT, Franceschi S et al for IARC group. Worldwide distribution of human papillomavirus types in cytologically normal women in IARC: a pooled-analysis. Lancet 366: 991-998, 2005.
15. CDC - Centers for Disease Control and Prevention - Prevention of Genital Human papiliomavirus Infection. Gerberding, J.L. (Director) Department of Health and Human Services. Report to Congress 2004.
16. Bosch, F.X.; Roham, T.; Schneider, A.; et al. Papillomavirus research update: highlights of the Barcelona HPV 2000 International Papillomavirus Conference. J Clin Pathol 2001; 54: 163-175.

Endereço para correspondência:
SILVIA MARIA BAETA CAVALCANTI
Laboratório de diagnóstico Virológico
Departamento de Microbiologia e Parasitologia da
Universidade Federal Fluminense
E-mail: silviacavalcanti@vm.uff.br