Utilization of human papillomavirus testing for cervical cancer prevention in a university hospital

Utilização de método de biologia molecular para papilomavírus humano na prevenção do câncer de colo uterino em um hospital universitário

Abstract

This study aimed to evaluate the performance and cost of using polymerase chain reaction (PCR) and hybrid capture in the detection of cervical intraepithelial neoplasia (CIN) in patients with cytological abnormalities (ASCUS/low-grade squamous intraepithelial lesion – LSIL), and the feasibility of implementing these methods in Brazil's Unified National Health System (SUS). Colposcopy gave a negative predictive value of 92.86% and efficiency of 87.8% for diagnosing CIN. The sensitivity of PCR and hybrid capture for detecting CIN was 83.33% and 66.67%, respectively, and the negative predictive value for diagnosing CIN2/CIN3 was 100% and 94.74%, respectively. The annual cost for 80 patients was lower when all patients with ASCUS/LSIL were referred for colposcopy than when HPV testing was performed and those with positive results were referred for colposcopy. Therefore, at present, it is financially unfeasible for the National Health System to implement HPV testing to screen patients with cytological abnormalities (ASCUS/LSIL). However, considering that large-scale use might make such methods cheaper, PCR should be the chosen method, since it is less expensive, more sensitive, and has a high negative predictive value.

Cervical Intraepithelial Neoplasia; Polymerase Chain Reaction; Colposcopy; Health Care Costs

Introduction

Human papillomavirus (HPV) causes approximately 30 million new infections every year and is present in the female genital epithelium in some 10% of women. This family of viruses infects a wide variety of organisms in a species-specific manner, and around 100 subtypes have been described in humans. Although the majority induces benign papillomas, some are classified as high-risk and contribute to the development of anogenital cancer. For example, HPV 16, 18, 45, and 56 can lead to malignant transformation when they infect epithelial cells. The majority of HPV genital infections are asymptomatic and self-limiting. Studies have shown that among women with normal oncological cytology, prevalence of HPV infection declines with age. Among patients with only cytological alterations due to HPV, regression reaches some 85% of cases.

HPV infection is more frequent in young women with early sexual debut, smokers, and those with multiple sex partners. Smoking in particular is an independent factor related to precursor cervical lesions and invasive cancer. Despite the impact of Pap smears on cervical cancer screening programs, the method has many limitations: high false-negative rates; the test’s subjective nature; the need for frequent retesting; and wide inter-laboratory variation in sensitivity and specificity. Some explanations for diagnostic errors related to cervical neoplas-
sia and precursor lesions by cytological methods are: lack of safe morphological criteria for micro-invasion; lack of sampling the squamous-columnar junction; and scarcity of neoplastic cells in the sample. There have thus been attempts to find alternatives to cytology as the primary screening test, as an adjunct for cytology to manage cases of atypical squamous cells of unknown significance (ASCUS), and for post-treatment follow-up.

Among patients with ASCUS cytology, management is a subject for discussion because of the occurrence of cervical intraepithelial neoplasia (CIN) during follow-up, ranging from 5% to 60% 10,11. High-grade lesions are found in 10 to 15% of patients with ASCUS, while high-risk HPV is found in 40 to 60% of patients with ASCUS 12.

As for management of patients with ASCUS and LSIL (low-grade squamous intraepithelial lesion), the ALTS study (ASCUS/LSIL Triage Study) 13 was a large randomized trial designed to evaluate different methods for managing patients with cytological findings of ASCUS or LSIL. The study considered three strategies: immediate colposcopy, repetition of the cervical smear, or HPV testing. The study concluded that HPV testing was worthless in women with LSIL, and the ASCCP (American Society for Colposcopy and Cervical Pathology) recommended that all patients with LSIL should be referred to colposcopy. For management of cytological findings of ASCUS, the ASCCP recommended that any of the three steps could be taken 14,15.

Manos et al. 16 studied a cohort of 46,009 women, of whom 995 had a cytological finding of ASCUS. In 6.7% of cases, CIN2, CIN3, or cancer was found by histology, and 39.5% of patients had high-risk HPV (HPV test). The sensitivity of HPV testing for high-grade dysplasia was 89.2%, and specificity was 64.1%. The percentage of patients referred to colposcopy was 39%.

The present study is one component of the State Healthcare Agenda: Control of Priority Diseases and Complaints, in the context of testing women at an age when they are at risk of cervical cancer (Program for Cancer Prevention and Control and Oncological Care). This is a relevant public health issue, given that reduction in the transmission rate and especially in the female mortality rate resulting from this infection is a key goal on Municipal, State, and Federal healthcare agendas, as well as a World Health Organization (WHO) target.

The present study aimed to: verify the concordance between polymerase chain reaction (PCR) and hybrid capture for diagnosing HPV; compare the performance and cost of PCR and hybrid capture in the detection of CIN confirmed by biopsy in patients with cervical smear showing ASCUS and LSIL; and assess the feasibility of implementing the biomolecular method in the Unified National Health System (Sistema Único de Saúde – SUS) for the prevention of cervical cancer in patients with cytological findings of ASCUS and LSIL.

Material and methods

A prospective study was carried out at Universidade Federal do Triângulo Mineiro (UFTM) from December 2003 to July 2005. The study group consisted of patients (n = 80) aged 15 to 75 years with a diagnosis of ASCUS or LSIL based on cervical smears (inclusion criteria). The exclusion criteria were: previous ASCUS or LSIL, conization, or Chlamydia infection.

Routine annual cytological smears were collected during gynecological examination (Gynecological Department, UFTM). Patients with a diagnosis of ASCUS or LSIL were referred to the Colposcopy Service (UFTM). All patients agreed to participate in the study. Data were collected on patient age, habits (smoking, drug use, and number of sex partners), living conditions, contraceptive methods, history of sexually transmitted diseases (STD), and use of hormone replacement therapy.

Patients who presented abnormalities in the colposcopic examination then underwent further procedures after having provided free and informed consent. These consisted of collecting the following: smears for cytology from the endocervix, ectocervix, and vagina; smears from the endocervix and ectocervix for hybrid capture and PCR; and biopsy of the lesion (when visible) for anatomopathological diagnosis.

The Rome Classification was used, dividing the colposcopic findings into normal (original squamous epithelium, columnar epithelium, and normal transformation zone) and abnormal (acetowhite, stippled, and mosaic-pattern epithelium, leukoplakia, iodine negative zone, and atypical vessels). These were then subdivided into greater or lesser alterations, depending on the degree to which the tissue was compromised 17.

Cytological smears were evaluated according to morphological criteria: amphophilia, perinuclear halo, dyskeratosis, anisocytosis, nuclear criteria (binucleation or multinucleation), increased nucleus/cytoplasm ratio, anisokaryosis, hyperchromasia, nuclear atypia, and karyorrhexis. 18,19. The universally familiar Pap smear was also performed. Patients with cytological smears showing ASCUS/LSIL by any method (morphological criteria or Pap smear) and after being reviewed were included in the study.
For PCR, samples were stored in Trizol at -20°C and thawed when the DNA extraction was performed, at which time 200 μl of chloroform was added for every 1.0 ml of Trizol collected. The DNA was added to an amplification solution according to the manufacturer’s suggested protocol (Invitrogen, Carlsbad, USA). Table 1 shows the characteristics of the indicators synthesized for amplifying specific DNA fragments 20,21. After performing the PCR reaction, the amplification products were subjected to electrophoresis on 14% polyacrylamide gels and stained using silver. The Trackit 1 kB DNA ladder (Invitrogen, Carlsbad, USA) was used as a positive control pattern. 10.0 μl of amplified sample and 3.0 μl of buffer were homogenized and placed in each opening of the 14% polyacrylamide gel. The gel was run at 90 volts for approximately one hour, and then placed in a fixing solution for 15 minutes. This solution was discarded and silver solution was added for 15 minutes, followed by washing in Milli-Q H2O and incubation in a developing solution for approximately 15 minutes. The gel was returned to the fixing solution for 15 minutes, after which the resulting bands were observed.

For hybrid capture, the Hybrid Capture II apparatus was used together with the DML 2000 microplate system, with signal amplification by chemical luminescence according to the technique performed by Silva et al. 22. For HPV detection, the kit contains 18 viral types grouped in two probe pools. The probes for low-risk viruses included types 6, 11, 42, 43, and 44, representing approximately 70% of this group of viruses. For the high-risk viruses, the system contained probes for types 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59, and 68, representing approximately 99% of this group of viruses. HPV sensitivity in the microplates was 1 pg/ml.

The results were analyzed statistically. Tables were built with the results from HPV testing and colposcopy in relation to histology. Test performance included calculation of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency (accuracy). Efficiency determined the percentage of correct diagnostic exams related to correct diagnosis. Women with negative colposcopy did not undergo biopsy, and these, together with women whose biopsies showed squamous metaplasia, alterations suggestive of HPV, or cervicitis, were defined as diagnostically negative. Women with a diagnosis of CIN1, CIN2, or CIN3 on biopsy were defined as diagnostically positive. Concordance between hybrid capture and PCR was calculated according to the following classification: kappa < 0.4: weak concordance; 0.4 ≤ kappa < 0.8: moderate concordance; 0.8 ≤ kappa < 1: strong concordance; kappa > 1: perfect concordance 23.

The study was approved by the UFTM Research Ethics Committee.

Results

Eighty patients were evaluated. Mean age was 33±12.21 years (range: 15 to 74). Twenty-three patients (28.75%) were smokers. Eight women (10%) used hormonal contraceptives and one (1.25%) was on hormone replacement therapy. With regard to previous sexually transmitted disease, two patients (2.5%) reported a history of syphilis. Mean number of sex partners was 2±0.76 (range: 1 to 5). Mean age at sexual debut was 15.79±2.01 years (range: 13 to 26), and mean parity was 2.22±1.96 (range: 0 to 12).

As for frequency of HPV diagnoses by PCR for each age group, the incidence decreased with ad-

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Size of the amplified product</th>
<th>Annealing temperature</th>
<th>Reference</th>
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<td>HPV-16</td>
<td>5’=5’...ACC GAA ACC GGT TAG TAT AAA AGC...3’ 3’=5’...ATA ACT GTG GTA ACT TTC TGG GTC...3’</td>
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<td>56°C</td>
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<td>HPV-18</td>
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<td>422 bp</td>
<td>56°C</td>
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<tr>
<td>β-actin</td>
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<td>295 bp</td>
<td>50°C</td>
<td>Tamim et al. 21</td>
</tr>
</tbody>
</table>

Table 1

Characteristics of the synthesized indicators for amplifying specific DNA fragments.
Advancing age, and 45.71% of cases were in patients aged 30 years or less.

Hybrid capture diagnosed 38 patients with high-risk HPV (47.5%), while PCR diagnosed 70 positive cases (87.5%). With regard to case distribution according to type of high-risk HPV diagnosed by PCR, 14 (17.5%) of patients were only positive for HPV 16, while 56 patients (70%) presented two types simultaneously.

Among the 80 patients with abnormal cytology, colposcopy was normal in 31 (38.75%), inconclusive in 8 (10%), and abnormal in 41 (51.25%). Of the latter, there were stronger colposcopic findings in 13 (16.25%) and weaker findings in 28 (35%).

With regard to type of cytological diagnosis in the 80 patients, 36 patients (45%) had ASCUS that was probably viral or neoplastic, 29 (36.25%) had ASCUS that was probably reactive, and 15 (18.75%) had LSIL (alterations suggestive of HPV in 12 women and CIN1 in three).

All patients with LSIL cytology presented positive PCR for high-risk HPV. With hybrid capture, eight patients (53.33%) with LSIL according to cytology were positive for high-risk HPV, and 30 (46.15%) with ASCUS on cytology were positive for high-risk HPV.

From comparative analysis of the 80 cases, using both hybrid capture and PCR for diagnosing HPV, 30 cases (37.5%) were true positives and two (2.5%) were true negatives. Hybrid capture sensitivity in relation to PCR was 42.8%, and specificity was 20%. Concordance of 40% (32 of 80) was observed between hybrid capture and PCR. The kappa coefficient was less than 0.4, thus showing weak concordance.

Prevalence of CIN diagnosed by biopsy was 15% (NIC in 12 of 80 patients). Even when hybrid capture gave a negative result, four (33.33%) of 12 cases of CIN were positive on biopsy. When PCR gave a negative result, this number dropped to two (16.67%). Of the 12 cases of CIN, 10 (83.33%) were positive for HPV using PCR. Of the seven cases of CIN2 and CIN3, PCR was HPV-positive for all seven: six cases of HPV 16 and 18 and one case of HPV 16 (Table 2).

PCR performance for CIN detection was as follows: sensitivity 83.33%; specificity 13.33%; PPV 16.13%; NPV 80%; and efficiency 25%. Hybrid capture performance for CIN detection was: sensitivity 66.67%; specificity 56.67%; PPV 23.53%; NPV 89.47%; and efficiency 58.33%. When only CIN2 and CIN3 were considered to be positive diagnoses, the NPV increased to 100% and 94.74% for PCR and hybrid capture, respectively.

The Brazilian National Health System (SUS) spends the following amounts on each diagnostic method for HPV and uterine cervical lesions: gynecological consultation: R$2.04; cervicovaginal cytological tests: R$5.37; colposcopy: R$1.69; biopsy: R$9.26 (information obtained from official SUS tables). The costs for PCR and hybrid capture (total materials used) are R$27.13 and R$104.30, respectively (currency exchange rate on November 11, 2005: U$1.00 = R$2.162).

The annual cost to the SUS for these 80 patients would be R$869.76 using a protocol whereby all patients with ASCUS/LSIL cytology were referred to colposcopy (Figure 1). The annual cost would be R$2,931.30 using a protocol whereby patients with ASCUS/LSIL cytology were only referred to colposcopy if they were PCR-positive for HPV 16 and 18 (Figure 2). The annual cost would be R$8,757.70 using a protocol whereby patients with ASCUS/LSIL cytology were only referred to colposcopy if they were positive for high-risk HPV according to hybrid capture (Figure 3).

Table 2

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>PCR</th>
<th>Hybrid capture</th>
<th>Cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
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</tr>
<tr>
<td>HPV</td>
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<td>7.1</td>
<td>2</td>
</tr>
<tr>
<td>CIN1</td>
<td>3</td>
<td>4.3</td>
<td>2</td>
</tr>
<tr>
<td>CIN2</td>
<td>4</td>
<td>5.7</td>
<td>0</td>
</tr>
<tr>
<td>CIN3</td>
<td>3</td>
<td>4.3</td>
<td>0</td>
</tr>
<tr>
<td>Not performed</td>
<td>35</td>
<td>50.0</td>
<td>4</td>
</tr>
</tbody>
</table>
Discussion

According to the literature, HPV infection occurs most frequently in sexually active young women who smoke. Our study showed a high prevalence of high-risk HPV. Of the 12 cases of CIN, 10 (83.33%) were positive for HPV according to PCR. The literature has shown that HPV is detected in approximately 80 to 100% of CIN cases. Cases that test negative may be due to the presence of new viral types (not included in the tests) or because the virus was not the etiological agent. In the case of hybrid capture, the lesions may present few viral copies, not yet detectable by this method.

Many HPV infections are transitory, eliminated by the immune system within a few months, without detectable residual lesions. HPV infection in young women generally tends to regress spontaneously, while among older women the virus is more prone to persist. Frequency of infection thus decreases with increasing age, while the HPV clearance rate in younger patients is high. This agrees with our findings, demonstrating peak frequency in the third decade of life and a subsequent decline. The positive predictive value of the HPV test thus increases with age, because the ratio of true positives (positive tests among women with high-grade lesions) to false positives increases among older women.

Olaniyan et al. performed a meta-analysis to compare colposcopic findings with histological diagnosis and found 89% accuracy, with 61% exact correlation with histology. The sensitivity of colposcopy ranges from 87% to 99%, with specificity of 26% to 87%. In our study, colposcopy showed 83.33% sensitivity, 89.66% specificity, 76.92% positive predictive value, 92.86% negative predictive value, and 87.8% efficiency.

Comparing hybrid capture to PCR, Cuzick et al. demonstrated 95% sensitivity, with a 2.3% false positive rate for hybrid capture. Venturoli et al. showed 91.7% sensitivity and 95.4% specificity for this method. Most authors have demonstrated high concordance between hybrid capture and PCR, ranging from 76.5% to 90%. Our study showed 40% concordance between PCR and hybrid capture. According to Cope et al., PCR showed higher sensitivity for detecting the virus. In an analysis of sensitivity for detecting high-grade lesions, Nindl et al. showed that hybrid capture failed to diagnose a large proportion of these lesions, as compared to PCR. Meanwhile, Kulmala et al. showed 85% concordance between the two methods for diagnosing such lesions, and also a low positive predictive value.
Potential annual cost to the SUS for 80 patients using a protocol whereby patients with ASCUS/LSIL cytology were only referred to colposcopy if they were positive for HPV 16 and 18 by PCR.

and a negative predictive value of nearly 100%. Lonky et al. demonstrated that hybrid capture gave a negative result in 25% of cases in which PCR tested positive for high-risk HPV in patients with ASCUS, thereby limiting its use.

ALTS obtained a negative predictive value of 99.50% in women with ASCUS, in relation to detection of CIN3. This finding agrees with our study, which showed a negative predictive value of 94.74% using hybrid capture and 100% using PCR for detecting CIN2 and CIN3. Most studies have shown negative predictive values ranging from 97% to 100%.

Thus, when a molecular test for HPV is negative, even if the cytological tests show ASCUS, the possibility of a high-grade lesion is very small. The specificity of hybrid cap-
Figure 3

Potential annual cost to the SUS for 80 patients using a protocol whereby patients with ASCUS/LSIL cytology were only referred to colposcopy if they were positive for high-risk HPV by hybrid capture.

- **ASCUS/LSIL**
  - Hybrid capture
    - 80 x 104.30 = R$8,344.00
  - Positive
  - Negative
    - Colposcopy
      - 38 x 1.69 = R$64.22
      - Negative
    - Annual cervical smear
    - Cytology + colposcopy in 6 months
      - 15(5.37+2.04+1.69) = R$136.50
      - Negative
      - Positive
    - Biopsy
      - 23 x 9.26 = R$212.98
- **Total:** R$8,757.70

In our study, all cases of LSIL cytology were HPV-positive by PCR, while 53.33% of cases were positive by hybrid capture; 84.61% and 46.15% of ASCUS cases were positive for high-risk HPV by PCR and hybrid capture, respectively. This very high result, particularly for PCR, can be explained by the fact that most of the women were less than 30 years old: in this age group, transitory and sub-clinical HPV infections are common. ALTs found that 83% of patients with LSIL had detectable HPV DNA, and since such positivity is so common, the authors suggested that the test should be limited to management of this group. In contrast, 50.6% of women with ASCUS were
positive for high-risk HPV, and among these patients, 5.10% had underlying CIN3 15. Zerbini et al. 39, using PCR to detect HPV 6, 11, 16, 18, 31, 33, and 45, demonstrated 27.3% and 53.55% detection rates for ASCUS and LSIL, respectively.

Negative HPV testing in women with ASCUS or LSIL cytology could reduce the number of referrals for colposcopy. However, the identification of many transitory infections, which occur mainly before 30 years of age, greatly reduces the test’s specificity for high-grade lesions 40. Adding to cytology, biomolecular tests increase the detection rate for high-grade CIN and can prevent more cases of cancer than cytology alone. However, given the high rate of positive cases of high-risk HPV found by PCR in our study, for both LSIL and ASCUS, PCR would be more expensive than to refer patients directly to colposcopy. In Brazil, HPV testing is more expensive than cervical smear and colposcopy, although the cost could decrease if HPV testing were implemented on a large scale. Moreover, costs tend to level off or decrease if a test can reliably allow screening intervals to be extended.

Furthermore, HPV infection in young patients fluctuates greatly, with a high regression rate. Regression is difficult to evaluate, given the possibility of reactivation or reinfection. Thus, one must ask whether it would truly be safe to increase the screening interval in the face of a negative biomolecular test for HPV, if the possibility of reinfection exists. Evander et al. 41 demonstrated that in 7.2% of their patients, a new type of virus was detected during follow-up. HPV infection was mainly associated with acquiring a new sex partner and having positive cytology. Schneider & Koutsky 42 and Schneider et al. 43 also showed that the presence of HPV fluctuated during the period studied.

Developing countries need to assess the role of new technologies for cervical cancer prevention that can be useful in the healthcare context. However, a positive HPV test does not necessarily mean that the woman has CIN or cancer, but that she is at risk of developing these lesions. Any combination of tests will increase the sensitivity, while decreasing the specificity and increasing the cost. Despite the promise, there are insufficient studies to define the best combination.

Thus, despite the high negative predictive value of biomolecular methods for high-grade HPV infection, the possibility of reinfection, the high cost of the tests, and the better efficiency and lower cost of colposcopy are factors that demonstrate the unfeasibility of implementing biomolecular methods for HPV testing within the SUS, for patients with cytological findings of ASCUS or LSIL. However, with the possible cost reduction through large-scale use of the method, PCR should be the biomolecular method of choice, because of its high negative predictive value and lower cost as compared to hybrid capture.

In conclusion, PCR detected more high-risk HPV cases than hybrid capture; PCR and hybrid capture have a high negative predictive value for high-grade lesions, but the sensitivity of PCR is better; and at present, for cervical cancer prevention it is financially unfeasible for the SUS to implement molecular biology methods to screen patients with ASCUS and LSIL detected by cervical smear. Nonetheless, considering that large-scale use could make such methods cheaper, PCR should be the method of choice, is less expensive, and shows better sensitivity and a high negative predictive value.

Resumo

O trabalho objetivou avaliar o desempenho e o custo do PCR e captura híbrida na detecção de neoplasia intra-epitelial cervical (NIC) em pacientes com anormalidades citológicas (ASCUS/leões de baixo grau – LBG), e a viabilidade da implantação destes métodos no serviço público de saúde. Observou-se valor preditivo negativo de 92,86% e eficiência de 87,8% da colposcopia para diagnóstico de NIC. A sensibilidade do PCR e captura híbrida na detecção de NIC foi de 83,33% e 66,67%, respectivamente. O valor preditivo negativo no diagnóstico de NIC II e III do PCR e captura híbrida foi de 100% e 94,74%, respectivamente. O custo anual de oitenta pacientes foi menor encaminhando-se todas as pacientes com ASCUS/ LBG à colposcopia do que se fossem realizados testes biomoleculares e encaminhando-se aquelas com resultados positivos à colposcopia. Portanto, por enquanto, é inviável financeiramente para o Sistema Único de Saúde a implantação de métodos de biologia molecular para pacientes com ASCUS e LBG à citologia cérvico-vaginal, mas considerando que o uso em grande escala poderia torná-los mais baratos, o PCR deveria ser o método escolhido, por ter menor custo, alto valor preditivo negativo e melhor sensibilidade.

Neoplasia Intra-Epitelial Cervical; Reação em Cadeia da Polimerase; Colposcopia; Custos de Cuidados de Saúde
Contributors

R. S. Nomelini participated in data collection and analysis and in preparing the article. A. C. M. Barcelos collaborated in collecting the material and performing the colposcopy. M. A. Michelin collaborated in performing the PCR and hybrid capture. S. J. Adad participated in reading the slides and performing the hybrid capture. E. F. C. Murta contributed by orienting the research, performing the PCR and hybrid capture, conducting the statistical analysis, and preparing the article.

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