NESTED PCR ON HPV SCREENING IN CERVICAL SMEARS FROM ASYMPTOMATIC PATIENTS

Fernanda Santos Nascimento¹, Gentil Arthur L. B. Vasconcelos¹, Maria Luiza Garcia Rosa², Maria Diva Paes de Lima Ferreira²; Ledy do Horto dos Santos Oliveira¹

¹Instituto Biomédico, Universidade federal Fluminense, Niterói, Brazil
²Instituto da Saúde da Comunidade, Niterói, Brazil
³Hospital Universitário Antonio Pedro, Niterói, Brazil

Corresponding author:
L.H.S. Oliveira, Departamento de Microbiologia e Parasitologia, Instituto Biomédico, Rua Prof. Ernani Melo 101, 24210-130, Niterói, RJ, Brasil
e-mail: mipledy@centroin.com.br

Key words: Nested PCR, HPV, young female, GP5+/6+
ABSTRACT

This study evaluated the nested-PCR technique in HPV cervical infection from 19 women who presented negative result for HPV when they were tested by single PCR assay. The outcome indicated a high sensibility of this approach to detect HPV.

INTRODUCTION

Human papilomavirus (HPV) genital is the most common infection among sexual transmitted diseases (Koutsky 1997). Most of these infections are transitory and the virus becomes undetectable within two years (Schiffman & Kjaer 2003). Under pathological criteria, HPV types are classified in low and high risk to genital cancer (Muñoz et al. 2003). Because of their inability to grow in cell cultures, papilomavirus presence in infected tissue is detected throughout molecular methods. Polymerase chain reaction (PCR) is the common assay for HPV detection. The primer sets My09/11 (Manos et al. 1989), GP5/GP6 (Snijders et al. 1990), or modified as GP5+/GP6+ (Roda Husman et al. 1995) and SPF10 (Gravitt et al. 2000) are usually employed to target the conserved L1 region of the virus genome thus allowing the amplification a large range of HPV types DNA. However, the nested-PCR system has shown better sensibility than the PCR only. It is characterized by amplification inside a DNA fragment still amplified (Johnson et al. 2003). The inner primers can be hybridized with smaller DNA sequences increasing the sensitivity and specificity of the method. So, GP primers may be used in a nested-PCR after amplification with MY primers (Husnjak et al. 2000). Nested-PCR with the primers GP5+/6+ after MY 09/11 amplification detect above 500 copies/ml.

Young girls begin their sexual life each time early. If they do not practice safe sex they have high probability of acquiring HPV infection. Such infections are, in the most of the cases, transient and asymptomatic. However, about 25% of adolescents and youth develop low grade squamous intra-epithelial lesions (LSIL) (Moscicki et al. 2001) with a spontaneous regression in 90% of these people (Moscicki 1999). In spite of this, LSIL cases associated to high risk HPV types can progress to high grade intra-epithelial lesions (HSIL). However, viral load is not always a predictor for high grade lesions (Johnson et al. 2003) and small viral load can bias the virus presence. Sensible molecular techniques as the nested-PCR are reliable method to monitor this infection, improving studies about virus
distribution among young people. In this sense, we evaluated the nested PCR GP5+/GP6+ performance in young female asymptomatic to cervical lesions that were negative to HPV infection when tested with PCR assay using My09/11 primers.

We analyzed seventy and one samples from women screened for cytological examination with previous negative result to HPV infection. They proceeded from public high school of Niterói City, Rio de Janeiro. Cervical smears were collected between 2004 and 2005. In this time, participants gave written consent according to the project approved by Ethical Committee of Faculdade de Medicina, UFF. Briefly, to perform HPV detection, the samples were incubated for 4 hours at 50°C in digestion buffer [10 mM trishydrochloric acid pH 8.3, 1 mM EDTA, pH 8.0, 0.5% Tween 20, proteinase K (Invitrogen, São Paulo, SP, Brazil); final concentration of 400 µg/ml]. Later, they were extracted with phenol-chloroform-isooamyl alcohol (25:24:1) (USB Corporation, Cleveland USA). DNA was precipitated with one-tenth volume of 0.3 M sodium acetate and three volumes of 100% ice-cold ethanol, washed with 70% ethanol, air-dried and suspended in 50 µl of sterile water. MY09/11 consensus primers (Invitrogen, São Paulo, Brazil), which amplify 450-bp (base pair) DNA sequences within the L1 region of HPV, were used to detect generic HPV DNA (Manos et al. 1989). Amplification was carried out in 50 µl of reaction mixture (1 X polymerase chain reaction [PCR] buffer, 200 mM dNTPs, 1.5 mM MgCl2, 50 pmol of each primer, 0.25 U unit of Taq polymerase and 5 µl of sample, Invitrogen, São Paulo, SP, Brasil) with 35 cycles of amplification. Each cycle included a denaturation step at 94°C for 1 minute, an annealing step at 55°C for 2 minutes, and a chain elongation step at 72°C for 2 minutes using DNA Thermal Cycler (Perkin Elmer, CETUS, USA). The beta-actin primers Ac1 and Ac2 (0.1 pmol each, Invitrogen, São Paulo, Brazil), which amplify a 330-bp region of the human DNA, were used as a sample internal control (Gal et al. 1993). Negative controls for background contamination did not add to the DNA template.

The Nested-PCR was performed by inner amplification of 2µl of the primary PCR products using GP5+/GP6+ primers set (150 pmol each, Imprint do Brasil Ltda., Campinas, SP) to amplify 150 bp products. It was performed with 20 cycles consisted of steps at 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 2 minutes. The protocol was made with minor modifications according to Johnson et al. (2003). Positive and negative controls were also performed. To avoid false positive results, besides the standard controls, a tube
containing extracted DNA negative to HPV was amplified with GP5+/GP6+ primers. Nested-PCR products were analyzed on 1.3% agarose gel with ethidium bromide staining for visualization of DNA under ultraviolet light and checked by comparison with a 100-bp DNA ladder. Statistical significance of results has been analyzed by using the X2 test. The significance level of tests (p) was set at 0.05.

Study population was constituted by 71 young female aged from 14 to 25 years old, with average of of 20.2 years. The results show high sensitivity of nested-PCR assay. Among 71 samples that were considered HPV negative by single PCR with My09/11 primers, 19 (26.7%) of them presented the 150 bp band of L1 fragment from HPV DNA on agarose gel, after nested-PCR. No difference was found between HPV infection and age. Virus frequency in people aged less than 18 years was similar to people in the same age range without infection (Table 1). Under cytological examination, most of sample presented normal result (94.4%). Both ASCUS and LSIL were each 2.8% of the cases, being 18 with normal cytology. Again, cervical cytological alterations were not a risk factor to HPV infection in these women (Table 2). Considering that PCR assay using My09/11 primers detect above 5000 copies/ml and GP5+/GP6+ primers above 500 copies, we detected women infected with low viral load.

<table>
<thead>
<tr>
<th>Age</th>
<th>HPV+ (%)</th>
<th>HPV - (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-18</td>
<td>7 (36.8)</td>
<td>17 (32.6)</td>
<td>24</td>
</tr>
<tr>
<td>19-25</td>
<td>12 (63.1)</td>
<td>35 (67.3)</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
<td>19 (100)</td>
<td>52 (100)</td>
<td>71</td>
</tr>
</tbody>
</table>

Improvement of PCR assay is advantageous for detection of low amounts of HPV and likewise, can be used to become the cervical cancer diagnosis more sensible. The use of two pairs of primers in a nested PCR has showed an increasing sensitivity if compared to single primers pair set. For that, this method has profit to epidemiological studies (Evander et al. 1992 Husnjak et al. 2000, Remmerbach et al. 2004).
In this work, women HPV infected presented low number of viral DNA only detected by nested-PCR, justifying the need of the use of this method. If this particular infection does not present damage at short term, its persistence can represent a source of viral transmission as well a risk to cervical cancer. Since this method allow detecting between 500-5000 copies of DNA HPV, it was possible to show virus infection in samples supposedly negative to HPV.

Table 2– HPV infection versus cytological results

<table>
<thead>
<tr>
<th>Cytology</th>
<th>HPV+ (%)</th>
<th>HPV - (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/Inflamatory</td>
<td>18 (94.7)</td>
<td>49 (94.2)</td>
<td>67</td>
</tr>
<tr>
<td>ASCUS/LSIL</td>
<td>1 (5.3)</td>
<td>3 (5.8)</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>19 (100)</td>
<td>52 (100)</td>
<td>71</td>
</tr>
</tbody>
</table>

According to Schiffman and Castle (2003), soon after the beginning of sexual life, most of women acquire the virus. In few years, the infection is not detectable, because it was cleared or it became latent. This status could conduct to a small number of HPV DNA copies, misunderstanding the diagnostic. Otherwise, this reducing number of virus, in particular high risk HPV types could be maintained as persistent infection, becoming a risk to precursor cervical cancer lesions (Morrison 1994).

Early studies verified that the most of cervical cytological abnormalities are not clinically visible and/or microscopically evident (Moscicki 1999). This fact was confirmed in our experiment. Of the total, 67 samples (94.3%) presented normal cytological results. Among them, 18 (26.3%) were HPV infected. In view of low range age, it is possible that most of the youths had acquired the virus recently, with few length time for developing lesions.

Diagnostic accuracy is important in order to detect HPV in women aged from 14 to 18 years, who are beginning their sexual activity. This status is an additional risk to cervical cancer, since the columnar epithelium in this stage is actively changed into squamous epithelium becoming vulnerable to sexual infections, as HPV (Sulak 2002).
In conclusion, we confirmed the sensibility of nested-PCR for low viral load samples in young female. This method is useful tool to increase the performance of the diagnostic, become more efficient the screening of infection. Besides, its importance in epidemiological surveillance is evident.

ACKNOWLEDGEMENTS

This work was supported by FAPERJ, PROPP-UFF and CNPq.

REFERENCES


