

METHODOLOGY

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A Simple and Robust Method for Semi-Quantitative Detection of Human Papillomavirus Nucleic Acids (HPV) Helps Oncological Clinicians to Assess the Severeness of HPV Cellular Changing

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Abstract: A simple and robust method for the detection of nucleic acids of human papilloma virus (HPV) has been developed. The assay exploits the excellent sensitivity and specificity of “nested” polymerase chain reaction (nPCR) that is designed in the original single tube configuration to effectively prevent the carry-over contamination. This approach theoretically covers the amplification of all cancer developing genotypes currently known. The nPCR, paired with very simple nucleic acids isolation steps, is a real alternative to the standard method. This manuscript shows its capacity for routine use under clinical conditions. It is shown that the strategy is at least as sensitive as the standard two tube nPCR and the data are acceptably reproducible.

Keywords: HPV-DNA, nPCR, contamination, semi-quantitative, userfriendly

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Introduction

The human papilloma virus (HPV) is considered to be the agent that triggers the malignant process in the cervix tissue.¹ The molecular biology approach for detection of HPV nucleic acids can help to assess the cytological findings especially the so-called “high risk” genotype determination and/or quantification of the HPV DNA. A differentiation between high risk and low risk HPV subtypes is not necessary since only high risk HPV causes precancerous lesions. However, the practical utility of this detection is questionable since a high percentage (about 10%) of women under 25 years of age are positive for HPV DNA although the risk of developing the malignant disease is negligible. The simple finding of HPV DNA positivity does not change the physician’s decision to prevent cervix carcinoma development without other signs of pathogenicity.

On the other hand, very high prevalence of the HPV DNA in the cancerous and precancerous cervical tissue is alarming. The affinity of E6/E7 proteins (coded and expressed from HPV genome) to the anti-oncogene p53 protein supports the role of HPV in the malignant process.

We have decided to address the question whether quantitative changes of HPV DNA are useful parameters for making a prognosis for HPV positive women or not. The project preceeds the methodological work where we tested the quantification method that is both acceptably reproducible and user friendly to be established in clinical laboratory conditions.

The nPCR is known to be the most sensitive and specific amplification method (with good chances for detecting even single template nucleic acid sequences), however, the recruitment of this method for a routine detection has not been successful mostly due to the high risk of “carry over” contamination leading to false positive results. We have developed a new strategy for the single tube PCR amplification where the modified and simplified nPCR exploits the fluorescent primers enabling simultaneous (semi) quantitative data obtaining.^{2,3}

The amplification is run as a duplex reaction, enabling reduction of the contamination risk to the minimum and simultaneously evaluation of false negatives results. Moreover, the quantification can be performed from the same amplified sample and the

same stands for genotype determination, either by the PCR-RFLP technique⁵ or any other techniques,⁶ that positively influence the cost effectiveness of the diagnosis.

Material and Methods

DNA isolation

Cervicovaginal swabs from 8 patients were rinsed with 1 ml of 150 mM NaCl and 3 × 150 µl aliquots were prepared. The first aliquot was used for isolation of DNA by the standard phenol-chloroform extraction followed by ethanol precipitation method when final dilution was in 10 µl of double distilled water. The second aliquot was centrifuged 2 minutes at 10000 g and supernatant discarded. The pellet was resuspended with 10 µl 5% Chelex-100 resin (Bio-Rad, USA) in double distilled water, overlaid by 20 µl of mineral oil and heated at 102.5 °C for 5 min in the heating box. One microliter of the solution (carefully aspirated not to take the Chelex resin) was used for PCR. The third aliquot was centrifuged 2 minutes at 10000 g and supernatant discarded. The pellet was resuspended in 10 µl of double distilled water, overlaid with 20 µl mineral oil and heated at 102.5 °C for 5 min in the heating box.

Primers

The published general primers MY09, MY116, GP5+ and GP6+7, originated from the L1 gene of HPV were selected. The primers TET1 to TET4 (TET1 = 5'ACTTT ACCCA GATCA ACTC 3', TET2 = 5'GTAGA TGGTC TGTTA TGG 3', TET3 = 5'GTTCT CCAGA GACAG ACT 3', TET4 = 5'GTGAG TCAAT TCCCC AAG 3') were used for D21S11 locus amplification.³ Four primers anti-GP6+, anti-TET3, anti-MY09 and anti-TET1 have the complementary sequence to the corresponding primers without a prefix “anti”. Primers anti-GP6+ and anti-TET3 were labelled at the 5' end with the 6-FAM (6-carboxyfluorescein) stain to enable the fluorescent detection in ABI-PRISM automatic sequencer.

Standard nested PCR

The 20 µl PCR mixtures contained 1x GeneAmp II buffer (Perkin-Elmer, USA) supplemented with MgCl₂ (2.5 mM of final concentration), 200 µM deoxyribonucleotide triphosphates (dUTP instead



dTTP), 50 ng of each primer (MY11, MY09, TET1 and TET2), 1U AmpliTaq Gold DNA polymerase (Perkin-Elmer, USA) and 1 µl of template from the isolation step. The cycling was: (94° C for 15 minutes) 1x followed by (94° C for 30 seconds, 47° C for 30 seconds and 72° C for 30 seconds) 20x. The second PCR was run in the new tube and under identical conditions, except that oligonucleotide primers GP5+, GP6+, TET3 and TET4 respectively were used at 50 ng amount each per reaction and oligonucleotide primers anti-MY09 and anti-TET1 at 2 ng amount each per reaction. About 0.2 µl of the solution from the first PCR was introduced into the new tube and the second PCR was started there. The cycling was also identical as for the first PCR but 40 cycles were programmed. The 5 µl of the second PCR were tested in 2% agarose electrophoresis.

Modified nested PCR

Both mixtures at the volumes 20 µl were prepared simultaneously as described at the standard PCR with the following differences: 1) for the first PCR mix only 1 ng each of the MY11, MY09, TET1 and TET2 primers were used, 2) for the second PCR the mix was supplemented with glycerol to a final concentration of 2.5%. The mix of the first PCR was pipetted to the bottom of the Eppendorf tube and overlaid by 20 µl of the low melting wax (melting point 5° C, Genesis Consortium, Prague). The whole volume of the second PCR mix was introduced to the cap of a 0.5 ml PCR tube and sealed with a high melting wax (Genesis Consortium, Prague) with melting temperature about 40° C. These premixtures in tubes can be stored in a freezer for up to six months without significant reduction of PCR signals.

The actual PCR was started by introducing 1 µl of the solution from the DNA isolation step. The cycling conditions were identical to the standard PCR (TC300, ConBrio, Czech Republic). After the first PCR finished the second mixtures was briefly spun down (30 seconds at 5 000 g) to the first mix without opening the tube and the cycling left for 40 cycles. The detection of the amplified product was made by the agarose electrophoresis.

Semi-quantitative measurements with fluorescence detection. After the second PCR of the nPCR half a microliter of the final product was used as an inoculum

for semi-quantitation with the primers anti-GP6+ and MY09, TET1 and antiTET3 respectively. The standard PCR mix contained 20 µl. The cycling was identical to standard nPCR but only 20 cycles were run. 1 µl of the final PCR product was mixed with 12 µl formamide and 0.5 µl of TAMRA (6-carboxy-tetramethyl rhodamine) labelled DNA weight marker and tested in ABI Prism 310 sequencer for FAM and TAMRA signals. The peak height and peak area were recorded and used for calculation.

Results

Cervical swabs of eight patients with high grade cervical intraepithelial neoplasia (CIN III) and of 4 patients with no known CIN history were tested for the presence of HPV by general primers mediated nPCR using both standard and modified amplification protocols in conjunction with different methods of the DNA isolation (the standard phenol-chloroform method, the Chelex-100 method and the simple boiling method).

All eight CIN III samples were positive for the HPV derived signal and the D21S11 derived signal as well as with the standard PCR and were also 100% positive by the modified nested PCR when the standard nucleic acid isolation step was incorporated. The same results were obtained also for the Chelex-100 nucleic acid isolation procedure. Surprisingly six samples were positive even when the simple boiling method was used for the isolation of nucleic acids (data not shown). On the other hand the control samples were all negative for HPV amplification but positive for D21S11 amplification. Since the simple boiling method of the nucleic acid isolation did not provide acceptable results this protocol was abandoned and for the quantification only the standard and Chelex-100 methods were used.

The tests for sensitivity were performed by taking the isolated nucleic acids from CIN III positive patient. One was diluted with water down to the dilution 1 + 100,000 i.e. to the concentration 10⁻⁵. In the dilution 1 + 100,000 no signal was obtained for any isolation method and amplification protocol. However, in the dilution 1 + 10,000 they were positive for standard and Chelex-100 isolation methods and both amplification protocols. These data indicate that the combination of the Chelex-100 method and modified



nPCR amplification protocol gives at least the same sensitivity for HPV detection and sensitivity can be compared to the references where general primers were used.^{7,8}

Discussion

We have addressed the possible strategy for sensitive detection of viral particles, a strategy that would be easy to handle and cost effective for clinical laboratories. For this purpose we have linked the original nPCR protocol with extremely simple isolation of nucleic acids in order to detect the HPV in the cervical swab material. The use of nPCR is fully legitimate since this method is the most sensitive amplification technique known, with an astronomical theoretical amplification factor about 1018. The use of this technique brings a rather high risk for false positive results. A possible solution is the multiplex PCR which enables the determination simultaneously of both the false negative and false positive cases. The highly polymorphic human genomic tetranucleotide repeat from the D21S11 locus⁴ region has been selected as a true internal control with the highest fidelity to monitor the whole diagnostic process. The fragment to be amplified is designed from the region without transcription activity, i.e. it is effectively amplified only from genomic DNA (not from mRNA). Indeed, the genomic DNA is an excellent control template because of the amount of human cells (with possible HPV particles) included to the individual test may vary individually either by the actual patient status or by the subjective swabbing technique performed. However, the ratio of HPV DNA to the human genomic DNA should be independent of the swab performance and the semi-quantitative data can be extracted.

Another critical moment in nPCR occurs when a small volume of the first PCR is transferred to the new tube for subsequent PCR (the “carry over” template inoculation). We solved this problem by adaptation of the original single tube amplification technique. The total number of cycles is sixty, the consumption of the heat sensitive material is divided in two parts that diminishes the inverse amplification effect of accumulating dNMPs. The use of AmpliTaq Gold DNA polymerase, where the enzyme must be activated by high temperature treatment prevents any misamplification in the upper buffer and both reactions

undergo the “hot” start that prevents the mispriming and reduces the irritating background signals.

To test if the single tube amplification system reduces the amplification efficiency we have found that the sensitivity of the system reaches the sensitivity of the standard PCR. Although the direct comparison between the new strategy system and the Digene hybrid capture technique⁹ was not tested (the transport medium in the Digene kit is not compatible with Chelex-100 isolation) it can be anticipated that the new system of nPCR will maintain the advantage of PCR over the Digene system, i.e. substantially higher sensitivity and specificity,^{10–16} here also supported with an economical effect.

Although we have not noticed the total inhibition effect in our amplification, the presence of inhibitors is described,¹⁷ and reliable tests should solve this problem. The genomic template control follows all isolation and amplification failures and reveals also all human factor (i.e. bad pipetting) mediated failures. Very tempting is the use of D21S11 hypervariable locus to test possible false positive results. Indeed, the highest probability for most frequent allele combinations is only 5% which means that there should be different allelic signals for most samples detected. The cumulation of the same allelic variants signalizes the presence of contamination and hence the false positive signal.

The biggest advantage in our strategy is the possibility to handle the large number of samples. The “hand in” time is remarkably low due to the extremely fast nucleic acids isolation step (about 10 minutes) and two joined PCRs (1 minute for transfer of solutions between two PCRs and 1 minute for centrifugation and start of second PCR).

In this article we have present the single tube nPCR amplification joined with a simple isolation step for nucleic acids. We believe that this system is robust enough to provide important parameters even in clinical conditions, and to be the method of choice for very sensitive determinations.

Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest.



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