QUANTITATIVE ANALYSIS OF HIGH-RISK HUMAN PAPILLOMAVIRUS TYPES IN ABNORMAL CERVICAL SMEARS

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ABSTRACT
Although high-risk human papillomavirus (HPV) is known to be a major risk factor for the development of cervical cancer from cervical intraepithelial neoplasias (CIN), the amount of viral DNA could represent an important aspect for course and strength of disease. Here we analysed samples of cervical cells from twenty women with known abnormal cytological cervixes with aim to measure HPV viral load using SYBR® Green - based real-time PCR. Quantitation over a range of 10^2 to 10^6 initial HPV copies was possible to detect in patients infected with HPV types. The assay provides a fast and reliable way for estimating the viral load of high-risk HPV types and might be of prognostic-diagnostic significance.

Introduction
Human papillomavirus (HPV) infection is etiologically related to the development of precancerous lesions of the cervix and cervical cancer (11). Until now more than 180 types of HPV have been identified (2). The types associated with diseases of the anogenital tract can be classified on the basis of phylogenetic relationship and of association frequencies with benign or malignant cervical lesions as high-risk types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -54, -56, -58, -59, and -66) and low-risk types (HPV-6, -11, -34, -40, -42, -43, -44) (2). Currently, the viral diagnosis of the infection is based on detection of the HPV genome (5, 11). Because of the high specificity and sensitivity provided by target DNA amplification, the most widely used method for HPV detection in cervical cancers is based on polymerase chain reaction (PCR) using either general or type-specific primers for the amplification. The introduction of real-time reporting of PCR amplification provides quick and convenient tool for sequence variation detection (genotyping) and has the potential to measure viral load (5).

Several studies have shown that the patients with high viral load of the HPV types most commonly found in cervical tumours may be at increased risk for developing cervical cancer (10). Almost all of the investigations involve real-time quantitative PCR (4, 9, 11, 12). Some researchers have applied the 5′-exonuclease assays either for endpoint determination of the amount of HPV PCR product (10) or for real-time detection of HPV (12). Additional methods for the quantification or semiquantification of HPV have also been described, based on PCR and seven-color fluorescence hybridization (9), or with scorpion probes in real-time PCR (4).

In this study we describe the application of a conventional/real-time PCR technique
for detection and typing of HPV DNA and viral load measurement of high-risk HPVs. We believe that the routine application of such technique will enhance the quality of HPV diagnostics and will aid both physicians and their patients.

**Materials and Methods**

**Patients and samples preparation.** Twenty women with known abnormal cytological cervices (Pap smear grade 3-d) were included in the study. Samples of cervical cells taken by cytobrush were obtained from each woman and were resuspended in transport buffer (7). Cells were washed twice and resuspended in 200 µl TE buffer (pH 7.2). The extraction of viral DNA was performed using Quigen DNA mini kit, according to the manufacturer's protocol.

**Conventional PCRs.** HPV detection and identification were made by conventional PCR with universal HPV primer pairs (termed "consensus primer") and primer pairs specific for the HPV-6, -11, -16, -18, -31, -33 types. The frequently used MY09/MY11 primer pairs contain several degenerate nucleotides and are capable of amplifying a 450-base pair fragment within the conserved L1 region common to numerous HPV types and therefore recognizes a wide range of HPV types. This primer pair is routinely used in our lab. To control internally the quality of the isolated DNA, the 110 bp sequence of β-globin gene was co-amplified using PC03 and PC04 primers in the multiplex PCR with the MY primers (7). After the consensus primer-based PCR, the PCR using primers specific for HPV types 6, 11, 16, 18, 31, 33 was performed using respective amplification protocol and conditions described previously (7).

**Real-time PCR.** Viral load of the samples positive for high-risk HPV types as determined by the preceding conventional PCR was determined by SYBR Green-based real-time PCR. The Opticon-2 (MJ Research) and the BIOPAP-QT kit for HPV DNA detection and quantification in human clinical samples (Biotools B&M, S.A.) were used. The oncogenic HPV mixture, included in the kit was used to amplify a conserved sequence of high-risk HPV E6-E7 region. The amplification conditions were 10 min at 96°C, 97°C at 10 sec, 50°C at 8 sec, 72°C at 30 sec for a total of 45 cycles. Melting curve analysis was performed starting from 65°C; the temperature in the thermal chamber was raised to 95°C at a transition rate of 0.1°C with 1 sec hold. The standard curves were prepared using the oncogenic positive control from the kit. Serial dilutions were made and analyzed to generate the standard curves.

**Results and Discussion**

All tested samples were positive for high-risk HPV DNA. Thirteen of them were typed as HPV-16 and 7 - as HPV-18. A standard curve was generated by plotting fluorescence signal versus cycle number indicating that the concentration of dsDNA in each sample rose above background fluorescence levels (Fig. 1). The c(t) values decrease proportionally with the increase in initial DNA concentration (8). This trend is expected since higher amounts of initial template more quickly generate the amount of product necessary to be detected with SYBR® Green. The linear range extends from 10⁶ to 10² copies of the oncogenic positive control initially present in the reaction.

A plot of log quantity versus c(t) cycle for the 250-base-pair amplicon is shown in Fig. 2. The regression coefficient is 0.970, indicating a strong linear correlation.

Melting curve analysis (Fig. 3) shows the oncogenic positive control dilutions containing 10⁶-10² copies per reaction. The curve shows the typical smooth decline in fluorescence with an increase in temperature as the strands of dsDNA dissociate and SYBR® Green is released. The
Fig. 1. Quantitation curves obtained from a dilution series of the oncogenic positive control ranging from $10^6$ to $10^2$ initial template copies per reaction.

Fig. 2. Standard curve plot of log copy number versus $c(t)$ value. The regression coefficient is $r^2=0.970$.

Fig. 3. Melting curves depicting the fluorescence intensity for oncogenic positive control dilutions containing $10^6$-$10^2$ copies/5µl reaction. Melting temperature (Tm) is seen as a single, common peak at approximately 83°C. This peak corresponds to the predicted melting temperature of the amplicon, indicating amplification of the amplicon of interest. Anomalies due to contamination, primer-dimer, false priming, etc., are not evident, as indicated by the lack of additional peaks or of abnormal broadening of the single peak.

Viral load was measured in the samples containing high-risk HPVs by interpolation on the standard curve (Fig. 4). The detected amount of viral copies per reaction (5µl) varied from 41,353.5 to 185,158 suggesting various degree of expression of viral oncogenes E6/E7.

Real-time PCR allows detection and quantification of products generated during the process of specific gene amplification.
Fig. 4. Standard curve with a selection of three patients plotted according to c(t) value.

(E6/E7) of HPV. We used SYBR® Green technology because it is easier to handle in routine applications and to optimize. The protocol listed here can be used to provide accurate quantitation data for high-risk HPV types over a broad concentration range as the lower limit of the standard was 100 copies of the oncogenic positive control per reaction and the higher – 10^6 copies/reaction. This makes the assay particularly valuable when analyzing samples with unknown or widely varying DNA concentration.

Viral load determination of lesions, caused by high-risk HPVs might be of prognostic-diagnostic significance (1, 3, 6). With increasing amounts of the viral copies, the risk for the development of invasive cervical carcinoma is likely to increase (1). Although high-risk HPV is known to be a major risk factor for the development of cervical cancer from CIN, the amount of viral DNA present in the tumor could represent an important aspect for course and strength of disease (4). The described conventional/real-time PCR technique has a number of advantages for HPV detection, typing, and in particular with respect to the quantitation ability.

REFERENCES