**Methods for HPV Detection: Polymerase Chain Reaction Assays**

*Suzanne M. Garland*<sup>a,b</sup>, *Sepehr Tabrizi*<sup>a,b</sup>

<sup>a</sup>Department of Microbiology and Infectious Diseases, The Royal Women’s Hospital, and <sup>b</sup>Department of Obstetrics and Gynaecology, University of Melbourne, Melbourne, Australia

**HPV and Cervical Cancer**

Worldwide, carcinoma of the uterine cervix is a common cancer of women, being second only to breast cancer. Since the initial reports by Harald Zur Hausen in the 1970s, suggesting a role for HPV in the development of cervical cancer, there have been a number of molecular, epidemiological and clinical observational studies clearly implicating HPV as an etiological agent in various anogenital cancers, including the cervix [1]. The lack of ability to utilize conventional viral culture methods initially made detection and diagnosis for HPV difficult until the advent of molecular methods, particularly amplification technology (such as polymerase chain reaction, PCR), which has allowed detection of low-level virus copy numbers in clinical samples. Utilizing these more sophisticated and sensitive diagnostic assays for HPV DNA detection, the consensus conference of the International Agency of Research on Cancer (IARC) in Lyon, France, concluded that certain high-risk (HR)-HPV genotypes (i.e. HPV16 and HPV18), which collectively contribute to around 70% of cervical cancers worldwide, be formally named as human carcinogens [2]. Moreover, these same IARC scientists in a multinational case-control study showed that the strength of the association with less-prevalent HR-HPV genotypes, including 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, and cervical cancer was also very strong with odds ratios of 72–347 being the strongest ever observed for human cancer [3]. Analyses of cervical cancers from different regions of the world using PCR methods have shown oncogenic HPV DNA in 95–99.7% and with consistent findings in a large number of investigations in different countries and populations [4].
Despite this role as a necessary cause for cervical cancer, HPV is a common viral infection of squamous epithelial tissues, with genital HPV infections being the commonest viral sexually transmitted infection (STI). This apparent enigma that HPV infection alone is not sufficient as a cause for cancer reflects the fact that most genital HPV infections are transient and asymptomatic (i.e. natural immune surveillance usually clears infectious HPV). It is only in a small number of women, with chronic carriage of oncogenic or HR genotypes that severe dysplasia (CIN2/3) eventuates, over several decades, to cancer. Carcinogenesis requires additional genetic changes such as HPV integration and possibly other cofactors in complex pathways not totally understood [5]. In contradistinction, CIN1/HPV is a transient manifestation of productive cervical HPV infection (median duration 6 months), with natural immune surveillance usually clearing the virus, leading to resolution of the lesion.

Genotyping, through comparison of viral sequences and comparison of genetic homology of viral genomes, has shown HPV to be very heterogeneous, with the presence of over 100 HPV genotypes fully sequenced and identified to date, with many more likely in the near future [6]. Biologically, HPV's are divided into two groups: cutaneous and mucosal. A subset of about 30 appears to regularly infect the genital/mucosal epithelium, with the remaining infecting cutaneous areas (skin warts and other lesions). Genital mucosal HPV types such as 6, 11, 42, 43, 44, 53, 54, 55, 62 and 66 are mainly found in low-grade cervical lesions with type 6 and 11 being the primary cause of genital warts. They almost never occur in cervical cancer (apart from the rare malignant change in giant condylomata acuminata of Buschke-Lowenstein tumor) and are designated low-risk (LR) HPV types. HR genotypes are found regularly in high-grade dysplasias or high-grade squamous intraepithelial lesions (HSIL), as well as in cervical cancers. Whilst there is consistency in findings for types 16 and 18 in cervical cancers worldwide, there is some geographical variation in prevalence of types following these two HPV types [7]. Not only do epidemiological studies require reliable and reproducible identification of genital HPV genotypes, but with the imminent licensure of prophylactic HPV vaccines, there will be a need to evaluate the efficacy of the vaccine and potential changes in prevalence of HPV genotypes in the years to come.

**HPV Detection**

Initial methods of HPV detection used were direct probe hybridization such as dot blot and Southern blot. Besides being labor-intensive and time-consuming they had low sensitivity, required large amounts of DNA in clinical
samples and have largely been superseded by amplification technology, which has allowed detection of low-level virus copy numbers in clinical samples.

Two such methods, currently used diagnostically include PCR and Hybrid Capture 2 (HC2) (Digene Corporation, Gaithersburg, Md., USA). In their infancy are newer assays detecting HPV RNA, which await large-scale clinical trials to assess their clinical diagnostic value. This review specifically describes PCR assays in detecting HPV DNA.

**Polymerase Chain Reaction**

PCR is a selective target amplification assay capable of exponential and reproducible increase in the HPV sequences present in biological specimens. The amplification process can theoretically produce one billion copies from a single double stranded DNA molecule after 30 cycles of amplification. When performing PCR, care must be taken to avoid false-positive results, which may be derived from cross-contaminating specimens or reagents with PCR products of previous rounds. Although this was a serious problem in laboratories when PCR was first utilized, most laboratories now implement procedures to overcome this.

The sensitivity and specificity of PCR-based methods can vary, depending on the DNA extraction procedures, site and type of clinical sample, sample transport and storage, primer sets, the size of the PCR product, reaction conditions and performance of the DNA polymerase used in the reaction, the spectrum of HPV DNA amplified and ability to detect multiple types. Generally a sensitivity of 1–10 copies per PCR reaction is achieved by most methods utilized.

Most laboratories use PCR assays, which utilize consensus primers, directed to a conserved L1 gene, and hence able to detect all mucosal HPV types. Consensus primers described include GP5/6 and modified GP5+/6+, MY09/11 and modified PGMY09/11, and SPF primer set. Amplification with each of these primers will result in different size amplification products (fig. 1) and this can result in varying sensitivity for detection of certain HPV genotypes [8]. This is particularly an issue when samples contain multiple HPV types. In a recent study, 120 of 11 (9.2%) specimens had multiple infections. The PGMY09/11 method detected most of them (9/11, 81.8%), MY09/11 detected 2/11 (18.2%), whereas the GP5+/6+ method detected none. The inability of the GP5+/6+ method to detect multiple infections was at least partly due to subsequent typing by sequencing that had difficulties in revealing multiple types [9].

Subsequent to PCR, analysis of the amplified products and distinction of HPV types can be achieved by sequencing [10] or hybridization with type-specific oligonucleotide probes, using various methods. The latter can be achieved using
various hybridization formats, dot blot (fig. 2a), Southern blot (fig. 2b), microtiter ELISA plate (fig. 2c), reverse line blot strip assays (fig. 2d), and microchip format assays (fig. 2e). The sensitivity and reproducibility of results are different depending on the methods used by the laboratory.

PCR assays can also be greatly affected by various extraneous substances in a clinical sample and which can inhibit the amplification reaction. Most laboratories have incorporated the amplification of an internal control, such as beta-globin gene (present at one copy per human cell) in each PCR reaction as a measure to detect potential inhibition and/or sample integrity. This is especially important in paraffin-embedded archival tissue where there may be degradation of DNA; then it is important to assess sample integrity.

Comparing results of PCR assays from various studies is difficult as there have not been standardized methodologies described and utilized. However, a number of commercial HPV detection and typing assays have recently been released in order to address this need. The commercial assay by Roche Diagnostics (AmpliCior, Indianapolis, Ind., USA) has recently been released, although not yet FDA approved although this is anticipated in 2007. This assay, similar to HC2 is able to detect 13 HR-HPV types and will allow diagnostic laboratories to detect HPV-DNA by PCR, although not to discriminate genotypes specifically.

Linear array assay and microarray systems are, however, two methods offering rapid detection and simultaneous typing of multiple HPV types. HPV oligonucleotide microarray has been developed by Biomedlab Company (Seoul, Korea), which allows for detection of 22 HPV types using an aldehyde-derivatized slide glass. PCR products are generated in the presence of fluorescein-tagged nucleotides and hybridized onto the chip and scanned by laser fluorescence, thus able to detect multiple infections with one hybridization step. Ideally, a larger number of HPV type-specific oligonucleotides could be spotted on the chip, although this method requires expensive equipment and may not be suitable for many (fig. 2e).

**Fig. 1.** L1 consensus PCR assays.
The Linear array® assay also developed by Roche allows detection of 37 different HPV genotypes. It detects amplified type specific HPV DNA with oligonucleotides immobilized onto a nylon membrane, which is an easy to perform detection method (fig. 3). The advantages of this method are speed, with
a turnaround time of 1 day, plus ease in interpreting individual and/or multiple genotypes, yet without requiring expensive, specialized instrumentation.

Utility of real-time and quantitative PCR in detection of HPV has also been investigated in a number of studies and can provide tools for quantitation of various HPV genotypes in specimens [11].

**Standardization of Assays**

Laboratories using molecular assays for detection of infectious organisms should use standardized tools when performing such assays. Although such standards are not available for HPV DNA assays yet, the World Health Organization (WHO) has initiated an International Collaborative Study enrolling several laboratories worldwide [12]. The aim of developing HPV International standard reagents as well as proficiency panels, and which should become available in the very near future for HPV detection and typing [Sonia Pagliusi, Geneva, WHO, pers. commun., 2005], means that clinical diagnostic laboratories will be able to validate their own assays and determine their analytical sensitivity. Moreover, for epidemiological prevalence studies and surveillance studies, this will allow comparisons of HPV DNA detection and typing results over time.
between different geographic locations, populations and anatomical sites. This is, in particular, important as the licensure of prophylactic HPV vaccine studies is imminent, and these standards will allow for accurate documentation and comparison of various methods in determination of the prevalence of HPV in trial pre- and postvaccine populations responses across various studies and geographic areas.

Clinical Indications

There are three areas in clinical care where HPV DNA testing has been endorsed or considered, and these will be briefly discussed here.

Primary Screening in Conjunction with the Papanicolaou (Pap) Test or as a Stand-Alone Test for Women over 30 Years of Age

Well-organized, high-quality Pap cytology screening programmes, which adequately reach a high proportion of those at high risk have markedly reduced cervical cancer incidence and mortality rates. However, there are limitations to cytology: in studies with the least biased estimates of sensitivity, it ranged from 30 to 87% with a mean of 51% [13]. As persistent infection with oncogenic HPVs precedes virtually all HSIL or neoplasias, HPV DNA detection can be used as a marker for current or subsequent development of precursor lesions.

Longitudinal studies show HPV DNA testing has a higher sensitivity for predicting prevalent high-grade dysplasias than cytology. Further, the negative predictive value approaches 100%; superior to Pap smears alone. Ultimately, combined HPV DNA and Pap cytology could result in increasing the screening interval for those with a normal Pap and negative HPV DNA, making the combination cost-effective. Most of the published studies to date have been conducted utilizing HC2, with studies based on PCR detection of HPV DNA only just coming to light. In the recently published ALTS study where HPV genotyping was evaluated, authors concluded that ASCUS and LSIL patients who have a positive HPV16 diagnosis by genotyping are at significantly greater risk for detection of high-grade abnormalities during a 2-year follow-up period when compared to those who test positive for another oncogenic type of HPV or who are HPV-negative [14].

Using a mathematical model to evaluate clinical and economic outcomes, Goldie et al. [15] concluded from evaluation of several different screening strategies that for those ≥30 years, using HPV DNA plus cytology were more effective in reducing cancer incidence, as well as being more cost effective than conventional cytology. Thus, less-frequent screening with more sensitive tests is likely to provide a reasonable balance between benefits and costs.
Furthermore, HPV DNA testing, in particular PCR, can be performed on self-collected samples; these have similar sensitivity to clinician-collected samples. Therefore this approach could provide a simple, less expensive and highly feasible tool for primary screening in low-resource settings many of whom have no Pap programs [16].

**Triage of Women with Minimally Abnormal (Borderline, ASCUS) Pap Smears**

In the USA, HPV DNA testing has an accepted role in the management of women with minor cytological abnormalities [17], the rationale being that a high proportion of the ASCUS group, on consensus cytological review are normal when HPV DNA is negative (around 50%), and hence at extremely low risk for HSIL. The role of HPV DNA testing is to focus on those women diagnosed with ASCUS that are HPV-positive where colposcopy assessment is justified. These recommendations largely came from the large ALTS (ASCUS /LSIL triage study), whereby various options for management of ASCUS and LSIL were evaluated. For ASCUS smears, HPV DNA testing predicted abnormalities sooner, with greater sensitivity and resulted in around 50% requiring referral to colposcopy, as compared to two thirds by repeat Pap [18]. In the recent follow-up of the ALTS study, in evaluating various test sensitivities for detection of cumulative CIN3+ (n = 306) over 24 months, a single HPV DNA identified all those found subsequently to have HSIL and more effectively than a single colposcopy or two Paps [19].

**To Predict Cure and Residual Disease after Ablation for Cervical Dysplasia**

Following treatment with cryosurgery, laser ablation or LEEP (loop electrosurgical excision procedure) for HSIL up to 10% may have residual disease. Standard of care has been close cytological and colposcopic follow-up at 6, 12 and 24 months post-procedure. However, as follow-up Pap has a low specificity in detecting residual HSIL, and HPV DNA is cleared from the cervix following adequate treatment, DNA testing has been evaluated to predict the presence of residual dysplasia [20].

In a recent meta-analysis of 11 studies evaluating HR-HPV DNA testing and monitoring of women after treatment of CIN 3, the NPV for residual disease was 98%, resection margins 91%, cytology 93% with combined HPV and Pap 99%. Therefore, in combining HR-HPV DNA testing with cytology, this allowed women double-negative for HR-HPV and Pap (70% of this population) less intensive follow-up. Adopting such a clinical algorithm would mean resources could be focused on those most at risk (i.e. women positive for Pap
and HPV DNA for colposcopy) [20, 21]. We await larger studies with cost-benefit analyses.

**Conclusions**

With the development of highly sensitive molecular assays such as PCR for HPV DNA and high-quality epidemiology, the natural history of HPV and various grades of dysplasia are being unraveled. Furthermore, evidence from clinical trials evaluating the use of assays of HPV in various clinical scenarios, is forming a basis for their use in the management of patient care. In the recent IARC consensus statements, the use of high-risk HPV types in screening and patient management is endorsed as a justified practice [22].

Concurrent with this, is a necessity to appropriately educate the health profession and the general public, particularly women and their partners, about HPV infection. Many women would not be aware that Pap cytological abnormalities relate to a viral infection, nor for that matter that the virus is transmitted sexually. There is a need to convey clear and consistent information about HPV to the general community; and to destigmatize and demystify the whole area of HPV. This will be particularly relevant to ensure appropriate use of HPV DNA assays in clinical care, as well as in the introduction of the HPV prophylactic vaccine in the very near future.

**References**


Prof. Suzanne M. Garland, MD
Department of Microbiological Research, Department of Microbiology and Infectious Diseases
The Royal Women’s Hospital, 132 Grattan Street
Carlton, Vic. 3053 (Australia)
Tel. +61 3 9344 2476, Fax +61 3 9344 3173, E-Mail suzanne.garland@rch.org.au