13

Human Papillomavirus Genotyping by DNA Sequencing- The Gold Standard HPV Test for Patient Care

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Objective: Because the Digene HC2 test is known to generate numerous false negative results in human papillomavirus (HPV) testing and cannot perform HPV genotyping, we sought to find a practical approach to use a polymerase chain reaction (PCR)-based method to detect HPV followed by genotyping with direct DNA sequencing for routine patient care.

Methods: The cells in the alcohol-fixed liquid-based cervicovaginal samples were digested by proteinase K. An aliquot of the extract was used for MY09/MY11 primer PCR followed by nested PCR with the consensus GP5+/GP6+ primers to amplify a hypervariable segment of the HPV L1 gene. The positive nested PCR products were subjected to automated DNA sequencing, using the GP6+ primer as the sequencing primer. A 34 bp DNA sequence downstream to the GP5+ primer was compared with the standard sequences stored in the GenBank for final genotyping. The PCR/DNA sequencing results were compared with those obtained by Digene HC2 test.

Results: The established protocol of PCR/direct DNA sequencing was successfully modified for HPV detection and genotyping in a community hospital laboratory. Using this research tool as the gold standard method for comparison, the Digene HC2 test detected only 62.6% of the 107 HPV isolates derived from 513 clinical samples. The Digene HC2 test returned 11 out of 29 proven HPV 16 cases as "positive for high-risk HPV", a sensitivity of 37.9%. Although HPV 16 was the most prevalent HPV genotype in the suburban New Haven, the combined HPV 16 and HPV 18 positive cases constituted only 32.8% of the total isolates. The second most prevalent high-risk HPV, after HPV16, was HPV 56.

Conclusion: The Digene HC2 HPV kit shows serious deficiencies in the detection of high-risk HPV. It fails to identify 62% of the cases infected by HPV 16, the leading oncogenic HPV genotype. The future HPV assay should be PCR-based and the detected HPV be genotyped for clinical follow-up. Genotyping is urgently needed in the communities where the HPV vaccine is made available for protection against HPV 16 and HPV 18 diseases as the HPV vaccine may increase the risk of developing precancerous lesions in the patients already infected with the vaccine-related HPV genotypes.

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Introduction

Human papillomavirus (HPV) testing was introduced to compensate for the poor sensitivity and specificity of the Pap smear cytology often used as diagnostic tool for borderline precancerous lesions.(1) Most clinical laboratories rely on the Digene Hybrid Capture 2 (HC2) kit to detect the presence of the prevalent high-risk oncogenic HPV types as well as the low-risk HPV genotypes.(2) The HC2 test depends on signal amplification generated by the reaction product of an enzyme attached to antibodies against RNA-DNA hybrids to increase its sensitivity. The presence or absence of the target HPV DNA molecules is determined by a cut-off reading of chemiluminescent intensity (the signal) in the reaction system. Since its amplified signal is generated by the last of the multiple layers of reporter molecules over the target HPV DNA, the hybrid capture test is inherently associated with a significant percentage of false positive and false negative results (3-5). HC2 HPV test is reported to be negative in 25% of cases with biopsy-proven high-grade squamous intraepithelial lesion (SIL) although all these biopsies have been proven to contain high-risk HPV DNA by polymerase chain reaction (PCR).(5) The nucleic acid probes used for the HC2 RNA/DNA hybridization are designed to bind a mixture of predetermined high-risk or low-risk target HPV DNA molecules. Specific genotyping is not possible with this technique.

Recently many investigators have recognized the importance of specific genotyping in HPV testing since it has become more widely known that consistent detection of the same high-risk genotype of HPV in a patient on multiple occasions which may be indicative of a persistent infection is more clinically significant (6-8) than finding different high-risk HPV genotypes over a period of time. Sequential and multiple infections, even caused by high-risk HPV genotypes, are characteristically not associated with high risk of cancer development. The need for specific genotyping has become more urgent since the announcement by Merck & Co., Inc. that a vaccine against infection of oncogenic HPV types 16 and 18 (Gardasil™) has been approved for human use by the Food and Drug Administration (FDA). According to a Merck document filed with the FDA, Gardasil™, although highly effective against new HPV type 16 and 18 infections, may enhance development of high-grade cervical intraepithelial neoplasia (CIN grade 2/3 or more severe) in subjects who have evidence of persistent infection with vaccine-relevant HPV types prior to vaccination, with an observed efficacy of -44.6% compared to placebo. Furthermore, persistent infection due to other HPV types may counter the efficacy results of Gardasil™ for the HPV types contained in the vaccine (9). Therefore, the future standard HPV testing must provide information on genotyping of the viral DNA detected in the cervicovaginal specimens to assist the gynecologists who will face questions raised by these new issues.

The gold standard method for accurate HPV genotyping is DNA sequencing.(10) PCR amplification of HPV DNA followed by direct cycle sequencing is a straightforward technique and has been widely used in microbiological and epidemiological HPV studies worldwide (3,4,11-15). The purpose of the current report is to introduce this research tool for routine patient care. It represents the first effort to use DNA sequencing to gather information on HPV genotypes in a community setting.

Materials and Methods

The clinical materials used for this study were alcohol-preserved cervicovaginal cells in liquid-based specimens (Cytyc or Surepath) from different women. They included 316 samples collected during regular gynecologic office visits by a solo private practitioner in the town of Milford, CT, a community with a relatively stable population, and 197 partially selected samples from a commercial cytology laboratory in New Haven, CT. Digene HC2 HPV test on samples of these two sources was performed by Quest Diagnostics Laboratory (Wallingford, CT) and Pathology & Laboratory Services, LLC (Woodbridge, CT), respectively. An aliquot of each specimen was blind-coded with an assigned number and was transferred to the laboratory at Milford Hospital for HPV PCR/DNA sequencing. The results were compared after all the tests were completed and the case numbers were decoded.

The general methodology of initial primary PCR amplification of a 450 bp segment of the HPV L1 gene with a pair of MY09/MY11 degenerate primers followed by nested PCR with GP5+/GP6+ consensus general primers was used for HPV detection; the 150 bp nested PCR products in the positive specimens were genotyped by direct DNA sequencing (4, 11) with minor modifications briefly summarized as follows.

The washed cell pellet was digested in 100 μL proteinase K solution according to a standard National Cancer Institute (NCI) protocol to prepare the DNA extracts for PCR (16).

For primary PCR amplification, 1 μL of the DNA extract and the MY09/MY11 primers were added to a 20 μL of LoTemp™ HiFi® DNA polymerase ready-to-use mix (HiFi DNA Tech, LLC, Trumbull, CT) which contains all the components needed for PCR, to reach a final 25 μL reaction volume. For thermocycling, the temperature steps of a TC-412 Thermal Cycler (Techne Incorporated, Burlington, NJ) were programmed for an initial heating at 85°C for 2 min, followed by 30 cycles at 85°C for 30 sec, 40°C for 30 sec, and 65°C for 1 min. The final extension was 65°C for 10 min. The full-length genomic DNA of human papillomavirus types 16, 18, 6B and 11 purchased from American Type Culture Collection (ATCC) was used as positive control. Deionized water instead of DNA extract was used as negative control.

Another 1μL of each DNA extract was placed in a separate PCR tube with a β-globin primer pair (17) for human genomic DNA amplification as a control of specimen adequacy.

After completion of the primary PCR amplification, a small amount of the MY09/MY11 PCR products was transferred by a glass rod of about 1.5 mm in diameter to a PCR tube containing 25 μL of final reaction mixture with 20 μL ready-to-use LoTemp™ HiFi® DNA polymerase mix and the GP5+/DP6+ primers for nested PCR, using the same thermocycling protocol as described above. Three PCR tubes per sample were used routinely for the β-globin gene, the MY09/MY11 primer and the GP5+/GP6+ nested amplification, respectively.

Aliquots of all three PCR products of each specimen were subjected to electrophoresis in a 2% agarose gel containing ethidium bromide, examined under UV light and photographed for permanent records. A clear 450 bp PCR product band in the MY09/MY11 lane or a 150 bp band

in the nested PCR lane of the agarose gel indicated the presence of HPV DNA in the specimen. One μL of the150 bp nested PCR product, if positive, was pipetted out from the nested PCR tube for direct DNA sequencing, using GP6+ as the sequencing primer, the BigDye® Terminator v 1.1/Sequencing Standard Kit for enzymatic termination reaction and the automated ABI 3130 four-capillary Genetic Analyzer for sequence analysis, according to the protocol supplied by the manufacturer (Applied Biosystems). Sequence alignments were performed against various HPV genotypes in the GenBank database by BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) arriving at the specific genotyping.

Specimens that did not show MY09/MY11 and GP5+/GP6+ bands, but showed evidence of positive β-globin gene amplification were interpreted as HPV-negative. Specimens that did not show any PCR products in all three lanes were considered unsatisfactory for evaluation due to low DNA extraction or presence of a PCR inhibitor. There were two such unsatisfactory cases. They were excluded from the total number of 513 for final analysis.

Specimens infected with more than one genotypes of HPV were indicated by occurrence of numerous ambiguous or overlapping peaks in the DNA sequencing tracings. For each of these mixed infections, the nested PCR products were subjected to additional four individual type-specific primer sequencing reactions (10) to rule out infection by the vaccine-related HPV types 16, 18, 6 or 11. DNA sequencing with multiple primers in one single reaction (10) was found to be unreliable.

Results

The image of a typical agarose gel slab after electrophoresis showed a bright fluorescent 450 bp PCR product in the MY09/MY11 primer amplification and/or a 150 bp band in the nested PCR products if the specimen was positive for HPV (Fig. 1).

After automated sequencing, a DNA sequence in the hypervariable segment of the L1 gene downstream to the GP5+ binding site was excised for sequence alignment. A BLAST analysis of 34 bp in this region based on the GenBank database determined the genotype of the HPV detected (Fig. 2).

Of the 513 liquid-based cervicovaginal samples, the PCR method detected at least one HPV strain in 107 with a general positive rate of 20.9% (Table 1). The positive rate for the samples collected in a private practitioner's office was 11.7% and that for the samples from a commercial cytology laboratory which received specimens from several health care clinics was 35.5%. A total of 23 single HPV genotypes were identified by DNA sequencing. There appeared to be no major difference in the HPV type prevalence between the women under the care of a private gynecologist and the women visiting health care clinics. In both groups, the most prevalent genotype in the New Haven area was found to be HPV 16, followed by HPV 56. The combined rate of prevalence of HPV 16 and HPV 18 constituted 32.8% of the total isolates.

The HC2 high-risk HPV test identified only 11 (37.9%) of the 29 HPV 16 cases as positive. However, it successfully identified all the cases containing HPV 56 and HPV 18 as positive for high-risk HPV.

Although HPV 54, 66, 83, 53 and 62 are not included in the hybridization cocktail probe, at least some samples containing these genotypes were classified as positive for high-risk HPV by HC2 test.

In the real-life practice, HC2 low-risk HPV test was not uniformly performed on all clinical samples for economic reasons. Whether there is a "high-risk" HPV in the sample is the only question that most of the practicing gynecologists were concerned about. Therefore, the "Negatives" under Digene HC2 test in Table 1 actually referred to negative results obtained by the high-risk oncogenic HPV cocktail probe. Some of the cases listed as "Negative" by HC2 test in Table 1 might have been classified as "positive" for low-risk HPV genotypes if a low-risk cocktail probe had been applied on all samples. The samples found to be positive for both high-risk and low-risk HPV by HC2 test were listed under "High-risk +".

Among the 107 positive nested PCR samples, DNA sequencing with GP6+ consensus general primer yielded multiple overlapping sequence traces in 5 cases. Using the individual type-specific primer sequencing for HPV 6, 11, 16 and 18 proved that one of them contained HPV 16, but not the other three genotypes, and that one contained a mixture of HPV 16 and HPV 18, but not the other two genotypes. For the remaining 3 mixed infection samples, repeated individual DNA sequencing failed to produce a readable primer extension/termination reaction with any of the four type-specific primers. Therefore, these latter 3 cases were considered to be mixed infections by multiple HPV types other than the four tested.

In all 513 cases, HC2 test listed 8 PCR-negative samples as positive for high-risk HPV.

Discussion

A sensitive and type-specific HPV testing is needed for the current practice of gynecology. There are two reasons for this need.

First, several recent studies have confirmed the observation that persistent infection caused by a high-risk HPV is more significant in cervical carcinogenesis than multiple sequential infections. For the development and maintenance of high-grade SIL, the risk is greatest in women positive for the same genotype of HPV on repeated testing.(6-8) High-grade SIL is often associated with a viral DNA load lower than that observed in less severely affected cells.(18) The commonly used Digene HC2 kit test can neither provide specific HPV genotyping information nor is it sensitive enough to detect a low number of viral DNA copies.(19) The HC2 test requires 10^5 – 10^6 copies of HPV genome for a positive reading as compared to the PCR-based methods which can detect 10 –100 copies of the HPV genome.(20)

Secondly, a quadrivalent vaccine, Gardasil™, composed of virus-like particles of the L1 capsid polypeptides of HPV 6, 11, 16 and18 has been recently approved by the FDA for human use to

prevent persistent infection, cervical lesions and carcinogenesis related to these four types of virus, especially against the cancer-causing effects of HPV16 and 18. However, it is also documented, although not widely reported, that the vaccine may cause more harm than placebo when it is administered to subjects who have already contracted the infection of HPV 6,11,16 or18. In a subset of clinical trial data, among the 156 subjects who were seropositive and PCR-positive for these so-called vaccine relevant HPV types, 31 subjects developed grade 2/3 or worse CIN lesions after receiving the vaccine while only 19 of the 137 subjects in the same subgroup developed such precancerous lesions after receiving placebo.(9) In other words, the vaccine may increase the risk of developing high grade dysplasia by 44% in a patient if she has already been infected by one of the four HPV types contained in the vaccine. In addition, diseases due to other HPV types also have the potential to counter the efficacy results of Gardasil™ for the HPV types contained in the vaccine, according to this document. Therefore, a sensitive method for viral detection and accurate genotyping is needed in evaluating the HPV prevention strategy and its effectiveness for the individual patients.

The most sensitive and accurate HPV testing is PCR DNA amplification followed by direct DNA sequencing of the PCR products. Under ideal conditions, PCR can detect a single copy of target DNA and can make enough copies of the target DNA in the reaction sample for sequencing in a few hours. This technique has been used as a research tool for microbiological and epidemiological studies.(3, 4, 11-15) Among all the HPV PCR primers, the MY09/MY11 and GP5+/GP6+ are the best characterized and the most commonly used consensus primers for a site located in the L1 region of the HPV genome. A matched alignment of a 34 bp DNA sequence immediately downstream to the GP5+ primer site allows the correct typing of all the 125 clinically relevant HPV genotypes (11,12). Since the efficiency of DNA amplification by the MY09/MY11 primers varies greatly, as demonstrated in Fig.1, GP5+/GP6+ nested PCR is needed to maximize its detection rate.(4,11,12,14,15)

We have adapted and streamlined the HPV PCR/DNA sequencing procedure for routine HPV testing in a community hospital. A few technical modifications have been implemented for extraction of DNA from liquid-based fixed cytology specimens, and to eliminate the tedious steps usually required for purification of PCR products by choosing a more efficient DNA polymerase system in the protocol. However, any PCR polymerase available commercially can be used for this gold standard HPV testing. The technology has been widely published and is easily modified for in-house application in any hospital laboratory.

Although our series reported here is small, it represents the first attempt to provide a database on the prevalence rates of HPV genotypes detected by nested PCR and confirmed by DNA sequencing among the residents of a US community. In the past, MY09/MY11 consensus primer PCR amplification has been used to detect HPV in unfixed cervicovaginal specimens for research purpose by several investigators.(21-24) However, genotyping of the PCR products in those earlier studies was based on hybridization analyses. The result of the latter technology is generally limited by the number and specificity of the probes available.

Different prevalence profiles of HPV genotypes have been observed in different geographic areas. Although both overall HPV prevalence and HPV16 prevalence are highest in sub-Saharan Africa, HPV-positive women in Europe are significantly more likely to be infected with HPV16

than were those in sub-Saharan Africa, and are significantly less likely to be infected with high-risk HPV types other than HPV16. Women from South America had HPV-type distribution in between those from sub-Saharan Africa and Europe.(25) We found that the combined numbers of HPV 16 and 18 cases constitute only 32.8% of the single HPV isolates in our series although HPV16 is the most prevalent high-risk genotype. This raises the question if Gardasil™ can be as effective as claimed in prevention of HPV-related cervical diseases among the women living in the county of New Haven, Connecticut. A similar question has been raised by a group of investigators in Quebec, after reviewing their DNA sequencing data which show that HPV18 seems to play a relatively minor role among the high-risk HPV infections in Canada.(12)

In our series, HPV 56 is the second most prevalent high-risk genotype detected (8.5%), followed by HPV 31, 18, 54, 58 and 66, sharing about the same degree of prevalence (5.6-6.5%).

The Digene HC2 high-risk test kit may identify HPV types 53, 54, 62, 66 and 83 and classify them as high-risk cases due to RNA/DNA cross reaction although these genotypes are not intentionally targeted in the high-risk cocktail probe.(26) Our findings confirm this cross reaction (Table 1).

HPV 66 has been reported to be associated with cancer or precancerous lesions and most prevalent in high risk populations, such as among commercial sex workers and imprisoned women (CSW/IPW).(26, 27) Its prevalence rate is 0.5% of the HPV isolates found in the general women population, but 2.1% of the HPV isolates among the CSW/IPW in Spain.(26) In the USA, its prevalence rate is 0.8% of the isolates in the general women population in Portland, Oregon (23), but is 5% of the isolates in the young women enrolled in the University Health Service at the University of California, Berkeley.(21) It is noteworthy that HPV 66 has a 5.6% prevalence rate in the HPV-positive New Haven suburban women population, the highest percentage among all published series, and is consistently identified as high-risk by HC2 test.

Fifty percent (50%) of the HPV 54 isolates are identified by HC2 test as high-risk HPV although it is not a target of the cocktail probe. This genotype was not known to be prevalent in high-grade dysplasia cases in the early studies carried out in many foreign countries. However, it is found to be associated with a 40-fold increase in risk among American Indian women with CIN 2/3. Only HPV type 16 has shown a higher risk than HPV 54 among this subpopulation. (28)

Our findings are consistent with those reported by others that the HC2 kit is associated with a significant number of false positive and false negative results.(3-5) The most serious defect of the Digene HC2 test is its failure to identify 18 of the 29 cases of HPV 16 positive samples which are confirmed by PCR/DNA sequencing. Since HPV 16 is the leading oncogenic high-risk genotype in all studies worldwide, the Digene HC2 high-risk kit has not fulfilled its role as a triage device for patient management. An alternative mechanism is needed in its place. Based on our experience and the recent publications cited above, we propose that the future HPV testing be based on PCR amplification and genotyping by direct DNA sequencing. A standard laboratory report should include a DNA sequence and the DNA sequence alignment (Fig. 2) to document the correct genotyping of the HPV detected for clinical follow-up.

When multiple genotypes are encountered in one specimen, we recommend DNA sequencing with single primers specific for HPV 6, 11, 16 and 18 (10) to determine if the mixed infections include one of these vaccine-relevant HPV types. With this approach, we have found that 2 of the 5 multiple infections contain at least one of these four HPV genotypes, with one sample infected by both HPV 16 and HPV 18. The other 3 samples contain more than one HPV types other than HPV 6, 11, 16 and 18; no sequence can be generated with the latter four type-specific primers. The inclusion of multiple HPV types in one sample, which is more common in young women, does not seem to be associated with a higher risk factor for the development of cervical neoplasia than single HPV infection.(8) The natural course of multiple infections needs to be further investigated when routine HPV genotyping becomes readily available.

Our study confirms that PCR/DNA sequencing, a research tool and the gold standard method for HPV typing (10), can be readily used for routine patient care.(3, 4,14,15) It is far more sensitive and specific than the Digene HC2 test kit. However, our findings do not suggest that any nucleic acid-based HPV testing be a gold standard for Pap smear cytology because there is no simple linear relationship between HPV positivity and cytological abnormalities. HPV infection is often transient in sexually active young women with normal cervicovaginal cytology; in 93% of initially infected women, the same viral type is usually not detected upon re-examination four menstrual cycles later.(29)

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