

Detection and Partial Sequence Resolvability of Some Undefined Types of Human Papillomavirus

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ABSTRACT

Purpose: Infection with the high-risk human papillomaviruses (HPVs) has been shown to play an important role in the development of cervical cancer from precancerous lesions. Screening of clinical specimens for HPV by PCR and genotyping may be challenged by some unidentified types. This work was designated to detect and type HPV in 30 samples of cervicovaginal cells derived from women with cervical intraepithelial neoplasia grade 1 and further examination of the untypable samples.

Materials and Methods: The consensus primers MY09/MY11 have been used to amplify 450bp fragment of the viral L1 gene. Infected samples were typed using dot blot hybridization using a probe mix, which detects more than 40 different genotypes including the major high-risk members. Genotype-undefined samples were reamplified using GP5+/GP6+ primers, subcloned and sequence analyzed.

Results: The data indicates the prevalence of HPV in 25 (83.3%) of the 30 samples investigated. High-risk HPV genotypes were detected in 12 (40%) samples and multi-infections, with two or more genotypes, were observed in 17 (56.7%) samples. Two samples (No. 04 and 19) showed a clear hybridization signal with Pst I cleavage followed by Southern hybridization. However, they were negative for both MY09/11 PCR and dot hybridization. Re-amplification using GP5+/GP6+ primers, subcloning and sequence analysis, of these samples revealed that they are harboring HPV genotypes closely related to HPV-54 and to the newly identified HPV-91 with sequence homology 86% and 96%, respectively.

Conclusion: The results confirm the importance of HPV genotype determination to monitor the potential development of cervical cancer in patients infected with the high-risk members. Also, the results indicate the importance of careful examination of type-undefined samples as a potential source of variants, new types of HPV and for probe enrichment.

Key Words: Human papillomavirus - Cervical cancer - Genotyping - L1 gene - GP5+/GP6+.

INTRODUCTION

Human papillomaviruses (HPVs) are sexually transmitted pathogens. Over 100 putative HPV types have been identified and GenBank-documented, including 85 cloned and officially designated and others identified from the sequence of PCR amplification of the viral L1 gene. Persistent infection with high-risk HPVs induces dysplastic lesions of the lower genital tract. Some of these lesions eventually progress to invasive cancers, particularly of the uterine cervix [1]. Structurally, the viral genome is 8 kb, closed circular, double-stranded DNA. This genetic material encodes 8 proteins. Two proteins (L1 and L2) are expressed late in the viral life cycle and construct the viral coat proteins. The remaining proteins are expressed earlier and concerned with hi-jacking the host-cell systems to maintain and produce virus [2].

Up till now HPV detection is based on a molecular biological, more than serological, approaches. Antibodies against HPV detection using standard enzyme-linked immunosorbent assay, is restricted to the major oncogenic genotypes such as HPV-16, HPV-18 and HPV-33 [3]. This limitation values the predominant application of polymerase chain reaction and/or hybridization approaches for both viral detection and typing. Of the many detectable homologous segments present along HPV genome, a 450bp segment located in the L1 gene is notable because it is flanked by the MY09 and MY11 consensus primers and comprises a segment of highly conserved amino acid residues. This explains the predominant usage of L1 fragment in PCR-based detection of HPV [4,5]. Also L1

gene is usually targeted for nucleotide sequence alignment of different isolates for HPV-type identification. A cloned HPV genome whose L1 open reading frame (ORF) displays less than 90% nucleotide homology with all the previously designated types is defined as a novel type [6].

HPV typing based on hybridization approach, takes different formats including dot hybridization (DH), Southern hybridization (SB) or reverse line blotting (RLB) [7,8]. Mass screening of clinical samples, however, for PCR-based detection and/or type-identification of HPV is usually challenged by unidentified cases due to failure of either PCR amplification or hybridization. Discrepancies between the results obtained during well-optimized PCR and hybridization for the same specimens usually trigger the concern about fishing variants and new types of HPV. Ong and his coworkers [9], for example, identified 3 HPV sequences (clones LVX82, LVX, LVX100) as new HPV types. These sequences were isolated from cervical smears positive for HPV-DNA by PCR but negative by S.B hybridization of the viral sequence with probes for 19 different genital HPV types.

In the present work, 30 cervicovaginal samples obtained from women with cervical intraepithelial neoplasia grade 1 (CIN1) were used. Molecular investigations included PCR-based detection of HPV using MY09/MY11 consensus primers followed by HPV typing. Out of 30 samples we have investigated, two samples were found to be HPV DNA-positive by Pst I cleavage and Southern hybridization, negative MY09/11 PCR and did not show signal with DB. For further characterization of such cases, HPV-DNAs were amplified using GP5+/GP6+ primers rather than MY09/MY11 set and their nucleotide sequence was analyzed.

MATERIAL AND METHODS

Sample processing: Cervicovaginal lavage was self collected from 30 women suffering from CIN1 during a mass screening program for HPV conducted in Albert Einstein College of Medicine, NY, USA. Samples were received from different sources either frozen or at 4°C for detection and type identification of HPV. About 40 µl of each sample were processed

with 100 µl digestion buffer containing 100 mM Tris-HCl, 2mM EDTA and 0.4 µg/µl proteinase K. After incubation at 55°C for 2 hours, proteinase K was heat-inactivated at 95°C for 10 minutes and DNA was extracted by phenol/chloroform using standard technique [10].

PCR Amplification: HPV L1 MY09/MY11 fragments were amplified from 10 µl processed (DNA extracted) samples in a 100 µl PCR mix containing 10X PCR buffer (100 mM Tris-HCl pH 8.5, 500 mM KCl, 40 mM MgCl₂), dNTP mix (10 mM each), 5 pmole of each MY09 and MY11 primers (the sequence is shown in Table 1) and 2.5 U Taq polymerase. HPV positive and negative control reactions were carried out in parallel with the investigated samples. Reactions were overlaid with 50 µl mineral oil and then subjected to a thermal cycling program consisting of initial denaturation at 95°C for 10 minutes, 35 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 30 seconds, primer extension at 72°C for 45 seconds. This was followed by a single extension step at 72°C for 5 minutes. Amplification products were resolved in a 2% agarose gel, visualized and photographed under UV transilluminator.

For L1-GP5+/GP6+ fragment amplification, processed samples were amplified in 100 µl reactions containing 10X PCR buffer (100 mM Tris-HCl pH 8.5, 500 mM KCl, 40 mM MgCl₂), 1X dNTP mix (10 mM each), 5 pmole of each GP5+, GP6+ (the sequence is shown in Table 1) and 2.5 U Taq polymerase. Reactions were overlaid with 50 µl mineral oil and subjected to a thermal cycling program consisting of initial denaturation at 95°C for 5 minutes and 30 cycles of denaturation at 95°C for one minute, annealing at 55°C for 30 second, primer extension at 72°C for 30 seconds followed by a single extension step at 72°C for 5 minutes.

Hybridization: For dot hybridization (DH), an aliquot (65 µl) of the PCR products, (or processed DNAs) and a set of HPV positive and negative internal controls were denatured with denaturing solution (0.0.25M EDTA, pH8.0 and 0.4N NaOH) and blotted onto a set of positively charged nylon membranes (Millipore, Billerica, Mass., USA). After washing with 20X SSC buffer, the membranes were baked at 80 °C for 2 hours then hybridized with 10 pmole of HPV-specific probe at 65°C for 12 hours.

After washing, they were treated with streptavidine conjugated horseradish peroxidase (Calbiochem, CA, USA) in washing solution, for 15 minutes followed by enhanced chemiluminescence (Amersham Biosciences) treatment for one minute at room temperature and then exposed to an X ray film. The results were recorded according to the signal intensity.

For Southern blot hybridization, different HPV-specific probes were labelled with ^{32}P αdCTP (Amersham Biosciences) and used to hybridize Pst I-cleaved extracted DNAs which were transferred to a positively charged membrane. The prehybridization and hybridization steps were performed following the standard hybridization protocol [10].

Subcloning and DNA Sequencing: The PCR amplified products were treated with phosphokinase (PK) in a reaction containing 1X PK buffer, 0.5 μl 100 mM DTT, 0.05 pmol PCR product or 9 ng DNA positive control. Tubes were incubated at 22°C for 40 minutes. After heat-inactivation of phosphokinase at 75°C for 10 minutes, the PCR product was ligated with EcoRV-predigested pT7Blue-3 vector (Novagen) using 5 units (0.5 μl) T4 DNA ligase (Invitrogen). The recombinant plasmid (ligation product) was used to transform E. coli competent cells. For colony screening, in addition to β galactosidase insertional inactivation-based screening, an inoculum of each colony was incorporated into PCR reaction mixture containing PG5+/PG6+ primers. The reaction was performed following the same cycling protocol described above. Miniprepations from the plasmids harboring the GP fragment were prepared by alkaline lysis and used for subsequent sequence analysis.

DNA sequencing was performed by the chain termination method described by Sanger et al. [12] using T7 Sequenase Version 2.0 DNA sequencing kit (Amersham, Life Science Inc.) and T7 & SP6 sequencing primers (Table 1). The obtained nucleotide sequences were processed using MacVector™ align (Scientific Imaging System-Kodak), assembly LIGN™ (Eastern Kodak Company) and DNA Strider 1.2 softwares. The verified nucleotide and amino acid sequences of each clone were matched with HPV sequences submitted to the GenBank (accession numbers AY601350 and AY601351

for isolates 04 and 19, respectively) by a Blast Search through the NCBI site on the World Wide Web.

RESULTS

HPV detection and typing:

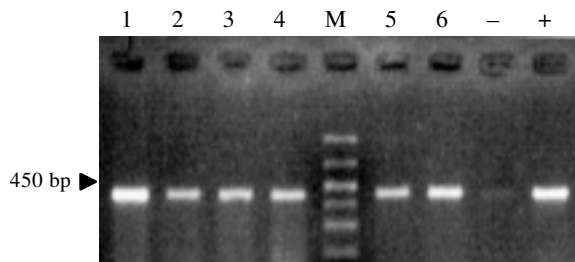
In this study, 30 human cervicovaginal cell samples obtained from American women enrolled in an epidemiologic investigation were screened for the presence of HPV-DNA by MY09/11 PCR and subsequently type-identified using a set of 40 different HPV-specific probes.

Table (2) summarizes the results of HPV amplification, hybridization and genotyping. Out of 30 (100%) samples investigated, 5 (16.7%) were conclusively HPV-free as indicated by negative MY09/11 PCR and absence of hybridization signal. MY09/MY11 primer set was successful in detecting HPV in 23 (76.7%) (Fig. 1A). Also a clear type-indicating hybridization signals were observed in these samples using DH. The distribution of HPV genotypes detected in these samples (shown in Table 2) ranged from a single genotype up to 4 different types per sample. Of the positive cases, 6 samples were infected with a single type (HPV-53, HPV81, HPV-88, HPV-66 (2 cases) and HPV-16. Infection with more than one genotype was detected in 17 samples including infection with 2 genotypes (13 cases); 3 genotypes (one case) and 4 genotypes (3 cases). Collectively the high-risk (HR) types HPV, 6,16,18,33,45,51 and 56 were detected in 12 samples. These genotypes were observed in one sample as a single infection (sample No. 16) and concomitant with low risk types in the remaining samples. The genotype was inconclusively determined in two samples (cases 04 and 19). These samples were negative for both MY09/11-mediated PCR and dot blot hybridization. However, they showed a hybridization signal by Pst I cleavage and Southern blotting.

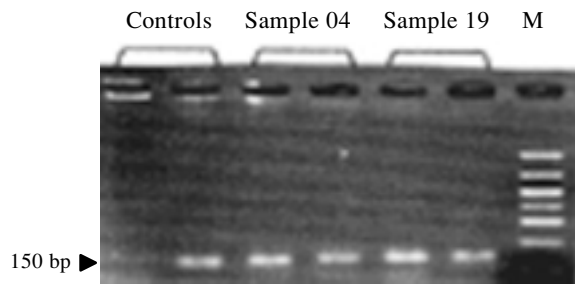
To investigate the type of these two type-undefined samples, 10 μl of the proteinase K digests of these undefined samples were used in a PCR reaction using the general consensus primers GP5+ and GP6+. The amplification products (Fig. 1B) of these two samples (150 bp) were purified and then subcloned. Fig. (2) illustrates the sequencing gel of the isolate derived from sample 04. To ensure sequencing

fidelity, 2-3 different PCR reactions were carried out and 2-4 clones were sequenced for each isolate (the open reading frame) and the corresponding amino acid sequence of HPV isolates was determined. For each isolate, both the forward and reverse sequencing reactions were aligned to determine the consensus nucleotide sequence for each clone (Fig. 2C). The blast sequence homology analysis showed that the nucleotide sequence of sample 04 was most closely related to HPV-54 with 86% sequence homology. Isolate number 19, on the other hand, was most closely related to the most recently identified HPV-91 with sequence homology 96%.

Based on the nucleotide sequence of each sequence probe, MH04 and MH19 (corresponding to isolates 4 and 19, respectively) were designated and incorporated into the probe mix used for screening (data not shown).

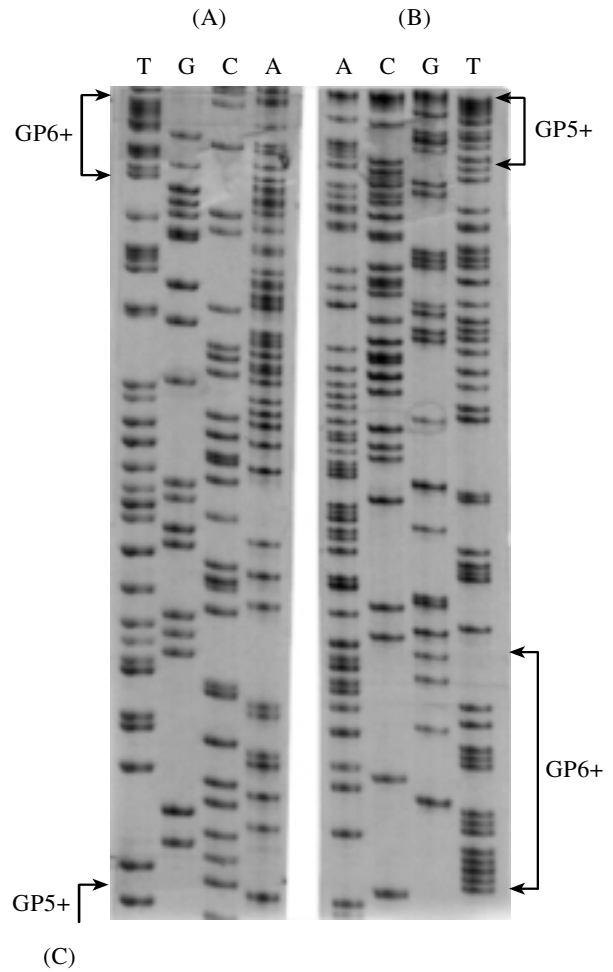


(A)



(B)

Fig. (1): Detection of HPV. Samples were treated with proteinase K and 10 ml were incorporated in the PCR reaction. Amplification products of MY09/11-mediated PCR, 450 bp bands, are resolved in 2% agarose gel (A). Lanes 1-5 correspond to samples No. 9,10,11,12 and 13, respectively. Two type-undefined samples are amplified (in duplicate) using GP5+ and GP6+ primers where 150 bp bands are resolved in 3% agarose gel (B). Lanes 1, 2 are HPV negative and positive controls, respectively. Lanes 3,4 and 5,6 are loaded with different products of samples 4 and 19, respectively. (M) is molecular weight marker (Biomarker).



Sample No. 04 CCGCAGTACTAACCTAACTTTATG
TGCTACAGCAACCTCGCAGGATA
CGTTTAATAATGCTAACTTTAAG
GAGTATATAAGACATGTGGAG

Sample No. 19 TCGCAGCACTAACTTAACCTTGT
GTGCATCCACTGAGTCTGTGCTA
CCTACTACATATGACAACACAAA
GTTCAAAGAATATTTAAGGCATG
CAGAA

N.B.: Sequences are cleaned from primers.

Fig. (2): DNA sequencing of HPV GP5+/GP6+ fragment. HPV-DNA fragment amplified from samples 04 and 19 were subcloned into the EcoRV site of pT7-Blue-3 vector and the recombinant vector was sequenced using T7 and SP6 primers. Denatured sequencing reactions were resolved in 8% polyacrylamide gel. Both the forward and reverse sequencing reactions of sample 19 are shown (A and B, respectively). The obtained sequences for both isolates were aligned to obtain the consensus sequence (C).

Table (1): Sequences of the general primer sets MY09, MY11, GP5+ and GP6+ used in detection of HPV-DNA and T7 and SP6 used in DNA sequencing.

Primer	Target size	Nucleotide sequence
GP5+		5'TTT GTT ACT GTG GTA GAT ATC AC 3'
GP6+	150 bp	5'.GAA AAA TAA ACT GTA AAT CAT ATTC 3'
MY09		5'CGT CC(C/A) A(G/A)(G/A) GGA (T/A)AC TGA TC 3'
MY11	450 bp	5'GC(C/A) CAG GG(T/A) CAT AA (C/T) AAT .3'
SP6		5' GATTTAGGTGACACTATAG 3'
T7		5'GTAATACGACTCACTATAGGG 3'

Table (2): Molecular biological investigations used in HPV detection and genotype identification.

Sample No.	PCR	D.B	S.B	HPV genotype
1	+	+	+	53
2	+	+	ND	51,56
3	+	+	+	81
4*	-	-	+	?
5	+	+	-	88
6	+	+	-	33,87
7	+	+	ND	66
8	-	ND	-	Negative
9	+	+	+	59,88
10	+	+	ND	39,51,53
11	+	+	+	16,45
12	+	+	+	16,53
13	+	+	ND	16,45,51,58
14	+	+	ND	61,81
15	+	+	+	33,45
16	+	+	+	16
17	+	+	ND	40,88
18	-	ND	-	Negative
19*	-	-	+	?
20	+	+	+	70,87
21	+	+	ND	6,88
22	+	+	ND	66
23	-	-	-	Negative
24	+	+	+	61,81
25	+	+	ND	33,45
26	-	ND	-	Negative
27	+	+	ND	40,88
28	+	+	+	6,31,61,88
29	+	+	+	16,70,87,89
30	-	ND	-	Negative

Abbreviations: DB and SB refer to Dot blot and Southern blotting hybridization, respectively. ND: Not done, (+): Positive, (-): Negative, (*) Untypable samples.

DISCUSSION

Worldwide, cervical carcinoma is the second leading cause of death in women [13-16]. HPV infections are associated with benign and malignant lesions of cutaneous and mucosal epithelia [17,18,19]. It is estimated that HPV-DNA is present in over 99% of these cancers [20].

The initial goal of this work was to revise the prevalence of HPV in 30 clinical samples using MY09/MY11 general primers [21]. This primer set is located in the late gene (L1) that encodes a major capsid protein and considered the most polymorphic part of the viral genome [22,23]. L1 specific primer is a valuable tool, takes the advantage of general amplification where it is common to the vast majority of HPV types. The intra M09/M11 fragment sequence variability extends from variants of the same genotype up to completely different genotypes. This gives the primer the ability to amplify many genotypes particularly in clinical specimens harboring more than one genotype. Also, its detection fidelity precedes both direct probes and signal amplification methodologies used for viral detection. Out of 23 specimens positive for HPV this primer set was able to amplify a 450 bp segment for more than one type in 17 samples (2 different genotypes in 13 samples, 3 genotypes in 1 sample, 4 genotypes in 3 samples).

The high prevalence of HPV in this work (83.3%) may reflect the high sensitivity of the MY09/11-mediated PCR. This high sensitivity permits detection of HPV DNA in samples with a low HPV viral load, which would probably be recorded negative with alternative methods. Enzyme immunoassay (EIA)-based methods reported [3,24] for both detection and type-identification of HPP are hampered with the limited number of HPV type which could be resolved. Also there is evidence from a study comparing results from several different laboratories that PCR-EIA is prone to give occasional false positive results [25]. MY09/II has the ability to amplify a wide range of different HPV genotypes including low-risk and high-risk types. The failure of MY09/MY11 PCR to detect 2 samples of screened positive cases by SB may be due to the mismatching of these primers with the targeted sequences (7047-7066 and 6615-6634) on the viral genome.

PCR detection of HPV is not conclusive unless accompanied with, or followed by, a strategy for type identification which determines the susceptibility of cancer development. Although the method we used is long, costly, tedious and has a potential of handling of radioactive material, it is, however, considered the most confidential way for both detection and type identification of HPV in clinical samples. Rather than amplification, MY09/MY11 could be used in genotype identification by several methods such as RFLP, reverse hybridization [26] or direct sequencing followed by matching the sequence with the previously reported HPV sequences [27,28] accessible through Blast or Blitz searches of the Genbank.

The validity of type identification using the hybridization approach depends mainly upon the enrichment of probe set used in hybridization. In this work, samples were hybridized with an improved cocktail of type-specific oligonucleotide probes. This probe mix can detect more than 40 different types. The majority of subjects were infected with multiple genotypes (range from one type per patient up to 4 genotypes). This may reflect the fidelity of the probing conditions we applied. It also indicates that the frequent exposure of these patients to multiple HPV genotypes may be due to unprotected sexual contacts (samples were collected from American patients). Such multiple infections were reported frequently with an increased incidence in HIV patients compared to HIV-negative individuals [29]. Failure of the immune system to clear the infection may increase the persistence rate of HPV [30]. It may hypothesize that the rate of HPV clearance in the tested group is lower than the rate of acquisition of new types. Also, the viral replication may be more efficient in some hosts, which could result in an increased detection rate as well as a higher chance of developing persistent HPV infection with possible viral mutations [31]. If exposure to novel infection continues, this may result in accumulation of different genotypes and a higher prevalence of women infected with multiple HPV genotypes.

Among the samples we investigated, MY09/MY11 primer set failed to detect 2 samples, meanwhile they had clear hybridization signals only by S.B. Untypable HPV was reported in many studies. Many reasons step behind the

failure of typing. This discrepancy triggers our interest for further molecular characterization of such samples. HPV-DNAs from these samples were successfully amplified with the GP5+ and GP6+ general primers generating about 150bp fragments. The sequence analysis revealed the average nucleotide homology of isolate 4 to HPV-54. Isolate 19, on the other hand, matches the previously reported isolate JC9813 of HPV late major capsid protein (L1) gene with 96% sequence homology. Isolate JC9813 was isolated and identified as a new type by Feoli-Fonseca et al. [32] and more recently by Terai and Burk [33]. The GP5+/GP6+ fragment (about 100bp excluding the primer sequence) does not contain sufficient sequence information for conclusive type identification or phylogenetic study. However, it provides a base for further amplification, possibly by using walking PCR, inverse PCR or long PCR, which ultimately provide a sufficient fragment for genotyping. Identification of promising new types is usually followed by a well confirmed detection and typing approaches, investigation of the prevalence and correlation between the infection with this type and the possible clinical outcomes. This, in turn determines if it will be categorized as a high-risk or a low risk type. Although the nucleotide sequences of the GP fragments we obtained do not confirm if they are new types, this sequence, however, could be used to design isolate-specific probes, which could be used to enrich the fidelity of the probe mix for other HPV screening programs.

Conclusion:

Although this work is derived from a screening program for HPV detection and typing, it shows how the discrepancies in molecular data may lead to fishing of variants, subtypes and potentially new types of HPV. We identified two unique clones and potentially new types of HPV. The data presented for these clones permits a good base for further investigation including cloning of the entire genome (8 kb fragment) starting with the GP fragment we obtained. Detailed characterization of HPV genomes of such cases will provide insight into their prevalence, biological and clinical behaviour.

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