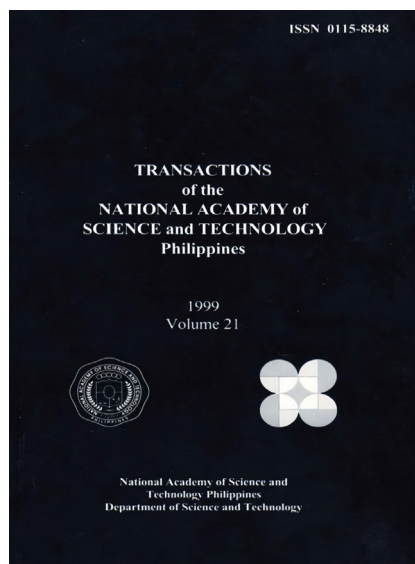


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Detection, Sequencing and Genotyping of Human Papilloma Virus Associated with Cervical Cancer among Filipino Women

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DETECTION AND GENOTYPING OF HUMAN PAPILLOMA VIRUS ASSOCIATED WITH CERVICAL CANCER AMONG FILIPINO WOMEN

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ABSTRACT

Infection of cervical epithelial cells with human papillomaviruses (HPV) is a major factor in cervical cancer development. Out of more than 70 HPV types identified, about 30 types have been found to infect the genital and cervical mucosa. This study aimed to detect HPV in cervical cancer patients and identify which HPV genotypes are prevalent among Filipino women. Cervical tissue biopsies were collected from patients with cervical malignancies from various hospitals. A broad spectrum of mucosal HPV types have detected by consensus primer-mediated polymerase chain reaction (PCR) using the degenerate form of the MY09/MY11 primer pair which amplifies a ~450 base pair segment of the HPV major capsid gene. Of the 162 patients in the study, 137 (84.6%) tested positive for HPV. PCR products generated are cloned into pUC18 and sequenced in ABI PRISM 310 Genetic Analyzer. Homology search and phylogenetic analysis of the resulting sequences from 11 samples show close sequence relation to HPV type 6.

Keywords: cervical cancer, human papillomavirus, oncogenic agent, polymerase chain reaction, degenerate consensus primer, major capsid gene, genotypes, cloning, DNA sequencing, phylogenetic analysis

INTRODUCTION

Epidemiological and molecular studies on cervical cancer, the second most common form of cancer in Filipino women, stimulated research on the role of sexually transmitted agents on the carcinogenesis of this neoplasia.

Human papillomaviruses (HPV) are the cause of common warts, plantar and genital warts. They are also associated with the majority of benign and malignant lesions of the anogenital tract. Out of more than 70 pathogenic human strains of HPVs identified so far, the HPV 6 and HPV 11 (low-risk types) have been predominantly associated with benign genital warts and low grades of cervical dysplasia, whereas HPVs 16, 18, 31, 33, 35, & 39 (high-risk types) have been found mainly in severe dysplasia and malignant cervical carcinoma (Choi, 1990; Garuti, *et al.*, 1989). A number of them have been implicated as an infectious oncogenic agent, first because the HPV DNA has been detected in more than 90% of all cervical cancers (Campion, 1987; Crum, *et al.*, 1984; Reid, *et al.*, 1988), and second since they encode proteins (E5-E7) which modify responses and form complexes with products of tumour suppressor genes (p53 and Rb) (Dyson, *et al.*, 1989; Scheffner, *et al.*, 1990).

HPV infections have been diagnosed through established cytologic and histologic criteria as well by electron microscope search for viral particles (Syrjanen, *et al.*, 1985) and immunologic detection of HPV group-specific capsid antigen (Woodruff, *et al.*, 1980). Due to the lack of appropriate tissue culture systems for *in vitro* propagation of HPV, diagnosis is predominantly based on the detection of its DNA. Originally, DNA hybridization techniques such as Southern blotting and *in situ* hybridization have been used for HPV detection. Early detection of cervical cancer in the premalignant stages is the goal of routine cervical cancer prevention efforts. The advent of polymerase chain reaction (PCR) significantly improved the sensitivity of HPV DNA detection.

The aim of this study is to detect HPV associated with cervical cancer by PCR and subsequently determine the viral genotype by nucleic acid sequencing and phylogenetic analysis. This study will provide epidemiological data on the presence of HPV in cervical cancer in the Philippines.

METHODOLOGY

Sample Collection

Freshly excised cervical biopsies from female patients referred to the OB-GYNE clinic for suspected cervical cancer were collected in sterile saline solution and transported frozen to the lab for processing. Collaborating hospitals include Dr. Jose Fabella Memorial Hospital, Philippine General Hospital, Dr. Jose Delgado Memorial Hospital, East Avenue Medical Center and St. Luke's Medical Center.

DNA Isolation

Tissue specimens were incubated at 55°C overnight in a digestion buffer containing 10mM Tris-HCl, pH 8, 100mM NaCl, 25mM EDTA, 0.5% (w/v) SDS and 100ug/ml Proteinase K. Samples were deproteinized twice with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated with 7.5M

ammonium acetate and absolute ethanol, and resuspended in 100 μ L double distilled, deionized water.

Polymerase Chain Reaction

Four microliters (4 μ L) of the DNA solution was supplemented with 5 μ L 10X PCR buffer (500 mM KCl, MgCl₂, 100 mM Tris-HCl, pH 9), 50 pmoles of each primer (5'CGTCCMARRGGAWACTGATC3'/5'GCMCAGGGWCA-TAAYAATGG3'), 0.25 μ L 20 mM dNTP mix, 1U of Taq DNA polymerase (Pharmacia), and autoclaved distilled water to a final volume of 50 μ L. Samples were overlaid with mineral oil and subjected to 40 cycles in an Eppendorf Mastercycler. The temperature profile was as follows: 10 min initial denaturation at 94°C, 1 min denaturation at 94°C, 1.5 min primer annealing at 45°C, 1 min extension at 72°C, and a 10 min final extension at 72°C.

Cloning of the PCR Product

One hundred microliters (100 μ L) of the PCR product mix was loaded onto a 1% low-melting temperature gel with large wells. The PCR product was recovered using the GFX^ä PCR DNA and Gel Band Purification Kit (Pharmacia) and resuspended in 50 μ L sterile distilled water. Using the SureClone^ä Kit (Pharmacia), the purified PCR product was blunted, phosphorylated and ligated unto blunt-ended pUC18 overnight at 16°C. Transformation was done by mixing 4 μ L of the ligation mix with 50 μ L competent *E.coli* XL-1 Blue cells. The transformation mix was incubated on ice for 30 minutes, heat-shocked at 42°C for 30 seconds, and was placed back on ice for 2 minutes. Four hundred fifty microliter (450 μ L) SOC medium was then added and the mix was incubated at 37°C with gentle shaking in a Thermomixer^ä (Eppendorf, Germany) for an hour. Forty microliters (40 μ L) of X-Gal solution (40 mg/mL) was spread on previously prepared LB agar plates containing 100 ug/mL ampicillin, and was allowed to soak for at least 30 minutes. Fifty μ L and 200 μ L of the transformation mix were spread on separate LB plates and grown overnight at 37°C. The plates were then shifted to 4°C for 2-3 hours for proper color development.

DNA Sequencing

The clones were sent to the Department of Virology at the Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan for DNA sequencing. Capillary gel sequencing using the ABI PRISM 310 Genetic Analyzer was utilized for this purpose.

RESULTS

One hundred sixty three (163) cervical specimens were collected from the collaborating hospitals. Genomic DNA was extracted from the tissue samples and

subjected to PCR using the consensus L1 MY09/MY11 primer pair. One hundred thirty seven (137) samples yielded the expected 450-bp PCR product (Figure 1).

PCR products of eleven samples were cloned. After screening the clones with the β -Gal colorimetric (blue/white colony) selection, plasmid preparations were checked further for inserts. The plasmid DNA from five clones from each sample were analyzed on a 0.9% agarose gel along with circular pUC18 as negative control. The size of the plasmid DNA from the clones should be predictively greater than the vector without the inserted PCR product. Plasmid DNA that exhibits a band which migrated slower than pUC18 was considered positive for the insert. Furthermore, these selected plasmids were double digested with EcoRI and BamHI to identify the ligated 450-bp PCR product fragment.

All DNA sequences were derived from at least five independent clones of each of the 11 samples and were done at the Institute for Tropical Medicine in Nagasaki University, Japan. Alignment of the DNA sequences with the corresponding region for all other HPV in data bases and computer-assisted homology search from the BLASTN programs showed a close sequence relation with HPV type 6. An aligned sequence (Figure 2) shows base differences with the prototype (type 6) and differences among samples.

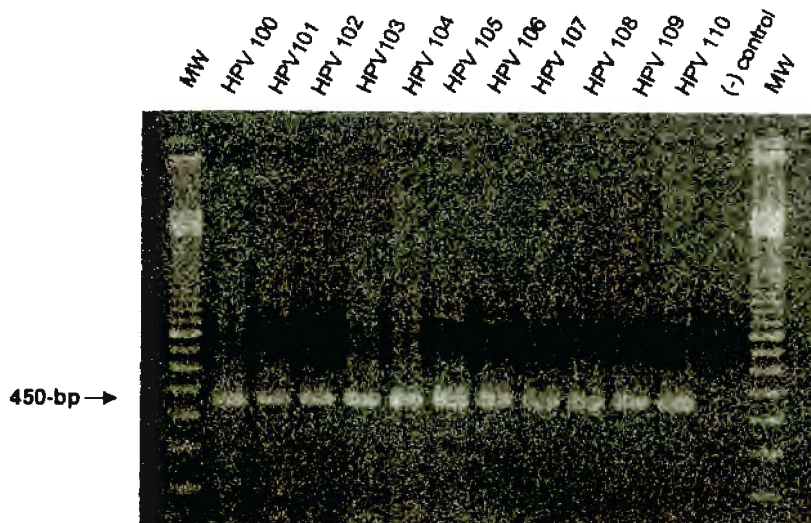


Figure 1. PCR products from genomic DNA isolated from cervical biopsies using MY09/MY11 primer pair after electrophoresis in a 2% agarose gel. Numbers above the lanes represent the isolate code. Negative control contains no DNA as template. Molecular weight (MW) marker used is a 100-base pair ladder (Pharmacia: 800-bp mark is the most intense band).

A phylogenetic tree (Figure 3) was constructed by distant matrix algorithms (DNADIST) and by UPGMA method of clustering using the PHYLIP 3.5 package.

DISCUSSION

Unlike other viruses whose members are classified into serotypes based on antigenic relatedness, papillomaviruses are designated as types based on the relatedness of their genomes. An HPV isolate is considered a new type if it meets certain criteria. For an isolate to be recognized as a new HPV type, the nucleotide sequences of the E6, E7 and L1 open reading frames (ORF) should demonstrate <90% identity with established types; isolates that differ from an established type by 2-10% are considered subtypes, and those that have <2% divergence are termed variants (Bernard *et al.*, 1994).

Since comparing three ORFs is a laborious undertaking, an alternative method is to analyze only a segment of the HPV genome. The HPV L1 ORF contains both conserved and divergent regions. The regions that are flanked by the MY09 and the MY11 primers appear to be highly conserved between, if not all, genital HPV types (Peyton & Wheeler, 1994). It is therefore, reasonable to believe that isolated genital HPVs could be identified by comparing sequences of this region.

Sequence comparison of the 11 isolates to those of reference type HPVs identified these isolates as variants of the HPV type 6 for they all exhibited greater than or equal to 98% sequence similarity. Since the homologies are quite high, there seems to be no significant differences in the genetic distances. Although HPV 16 is the type highly related to a number of cervical neoplasia cases and prevalence of the type 6, a low-risk HPV, in these cases is unpopular, some papers indicated otherwise. Shen *et al* reported a prevalence rate of 0% type 16 in all their specimens for routine hysterectomy and cervical neoplasia; while Melkert *et al* reported a low but consistent 1-2% rate of type 16 in women 35-55 years of age. Both papers noted a significantly lower rate of type 16 infection in older women. The generalization about the specific types being associated with certain forms of precancerous and cancerous lesions of the cervix is not absolute. HPV 16 (Syrjanen *et al.* 1986; Shirasawa *et al.*, 1986) has been detected in mild dysplasia and in clinically normal women (Gissman, 1986] while HPV 6-like sequences have been reported in CIN III and in invasive carcinoma (Hoepner & Loning, 1986).

HPV infection is a multicentric disease. The spectrum of HPV infection ranges from subclinical infection to exophytic condyloma to CIN and invasive cancer (Schiffman, 1992). Although an individual may exhibit one clinical symptom over another, multiple HPV types and clinical presentations can coexist. If one has to rule out the possibility of contamination with lower-risk HPVs in the lower genital tract during specimen collection, then one must consider the possibility of multiple-type infection (especially in immuno-compromised women) in one subject. LR-HPV DNA is usually found in episomal form and in high-copy number within

HPV 6 CAGGGA-CATAAC ATGGTATTTGTTGGGGTAATCAACTGTTTGTACTGTGGTAGATACCACACGCAGTACCAACATGACATTATGT89
 HPV 003 --- T --- T ---
 HPV 046 --- T --- T ---
 HPV 047 --- T --- T ---
 HPV 050 --- T --- T ---
 HPV 053 --- A --- T ---
 HPV 054 --- T --- T ---
 HPV 056 --- T --- T --- G ---
 HPV 093 --- T --- T ---
 HPV 094 --- T --- T ---
 HPV 095 --- T --- T ---
 HPV 096 --- T --- T ---

HPV 6 GCATCCGTAAC TACATCTTCCATACACCAATTCTGATTATAAAGAGTACATGCGTCATGTGGAAGAGTATGATTTACAATTTATTTTT180
 HPV 003 --- --- --- --- C ---
 HPV 046 --- --- --- --- C ---
 HPV 047 --- --- T C --- C ---
 HPV 050 --- --- --- --- C ---
 HPV 053 --- --- --- --- C ---
 HPV 054 --- --- --- --- C ---
 HPV 056 --- --- --- --- C ---
 HPV 093 --- --- --- --- C ---
 HPV 094 --- --- --- --- C ---
 HPV 095 --- --- --- --- C ---
 HPV 096 --- --- --- --- C ---

HPV 6 CAATTATGTAGCATTACATTGTCTGCTGAAGTAAATGGCCTATATTCACACAATGAATCCCTCTGTTTGGGAAGACTGGAAC TTTGGGTTA270
 HPV 003 ---
 HPV 046 ---
 HPV 047 ---
 HPV 050 ---
 HPV 053 ---
 HPV 054 ---
 HPV 056 ---
 HPV 093 ---
 HPV 094 ---
 HPV 095 ---
 HPV 096 ---

```

HPV 6   TCGCCTCCCCAAATGGTACATTAGAA-ATACCTATAGGTATGTGCAGTCACAGGCCATTACCTGTCAAAAGCCCACTCCTGAAAAGGA360
HPV 003 -----
HPV 046 -----
HPV 047 -----
HPV 050 -----
HPV 053 -----
HPV 054 -----
HPV 056 -----
HPV 093 -----
HPV 094 -----
HPV 095 -----
HPV 096 -----

HPV 6   AAAGCCAGATCCCTATAAGAACCTTAGTTTTGGGAGGTTAATTTAAAAGAAAAGTTTTCTAGTGAATTGGATCAGTAT-CC-T-TTG-G450
HPV 003 -----
HPV 046 -----
HPV 047 -----
HPV 050 -----
HPV 053 -----
HPV 054 -----
HPV 056 -----
HPV 09  -----
HPV 094 -----
HPV 095 -----
HPV 096 -----

HPV 6   GACG 454
HPV 0   -----
HPV 046 -----
HPV 047 -----
HPV 050 -----
HPV 053 -----
HPV 054 -----
HPV 056 -----
HPV 093 -----
HPV 094 -----
HPV 095 -----
HPV 096 -----

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Figure 2. DNA sequence alignment of the 11 isolates with HPV type 6 generated using DNAsis; HPV 6 sequence was obtained from Genbank. For the 11 isolates, only the bases different from those of HPV type 6 are shown.

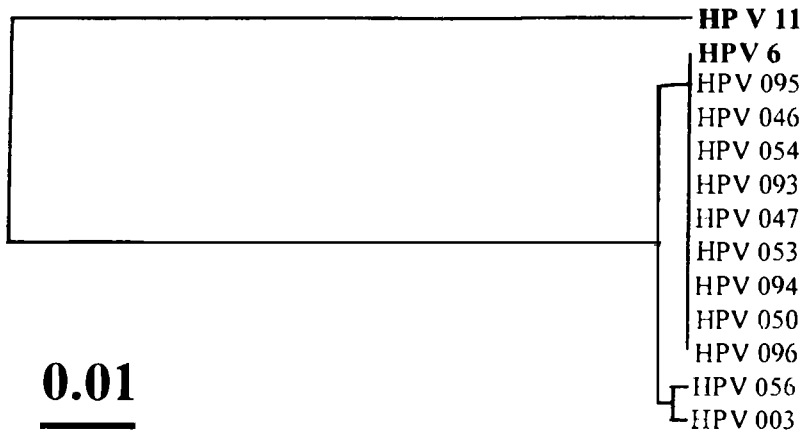


Figure 3. Dendrogram showing relationship among the 11 isolates and two established HPV types (HPV 6 & 11). The dendrogram was constructed by distant matrix algorithm (DNADIST) and by UPGMA method of clustering in the PHYLIP 3.5 package.

the cells. In contrast, a marked reduction in HPV DNA replication occurs in high grade precursor lesions and cancers because of the integration of the viral DNA into the chromosome (Shen *et al.*, 1995).

It has also been speculated that the currently used L1 consensus MY09/MY11 primers has a lower stringency with the low-risk HPV (6/11) DNA which are distantly related to other mucosal HPVs [Galloway, 1994]. More recent reports on the modification of these primers into a degenerate form [Ong *et al.*, 1994; Meyer *et al.*, 1995] allowed a broader range of HPVs to be detected and amplified. HPV typing in genital specimens is a diagnostic challenge because of the numerous types that must be detected and distinguished. Our PCR-based procedures can still be modified to an extent to provide sensitivity and specificity in the identification of a still growing variety of HPV. Beyond this, RFLP analysis can be utilized (refer to Figure 6) to assess the established types [Meyer *et al.*, 1995] although DNA sequencing is still the one recommended to identify novel HPV types and for designing probes for these new types.

With a growing number of pathogenic agents that are yet to be documented and analyzed, rapid and sensitive methods are needed. The advent of PCR and DNA sequence analysis allows easy and accurate detection. Without an established excellent clinical and histopathological data, one can rely on these molecular techniques.

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