DETECTION OF HUMAN PAPILLOMAVIRUS TYPE 16 IN INVASIVE CERVICAL CANCER BY POLYMERASE CHAIN REACTION

DETECTION OF HUMAN PAPILLOMAVIRUS TYPE 16 IN INVASIVE CERVICAL CANCER BY POLYMERASE CHAIN REACTION

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Abstract

Human papillomaviruses (HPV) have been implicated as etiologic agents in the genesis of cervical carcinoma and certain other benign lesions of the cervix. Clinical and epidemiological data, and the demonstration of HPV 16 viral DNA sequences in cervical cancer biopsies lend support to the etiologic association of HPV type 16 and cervical carcinoma. Interpretation of the association between HPV 16 and cervical cancer is limited by methods of detection. Different methods of detection of viral DNA sequences have been used based on DNA-DNA hybridization. Recently, a method based upon the in vitro enzymatic amplification of specific viral DNA sequences or polymerase chain reaction (PCR) has been used. The purpose of this study was to compare PCR with DNA-DNA hybridization methods in clinical specimens obtained from invasive cervical cancer. The in vitro enzymatic amplification or PCR was carried out on three specific regions of HPV 16. E6, E7 and L1 regions of HPV 16 were chosen as the target sequences of amplification and primers were synthesized specific to these regions. PCR was performed on 163 cervical cancer specimens using primers specific for E6 and E7 regions of HPV 16. 112 of

these specimens were also analyzed using L1 primers of HPV 16. Estimates of sensitivity and specificity of the different methods to see if PCR is a better, more sensitive method compared to the other methods were computed. The results suggest that although percent positivity by PCR method increases significantly, thereby improving sensitivity of detection, the specificity suffers compared to the other methods. However the advantages of using PCR as a diagnostic tool are attractive, as it requires only picogram quantities of DNA, is rapid and easy to perform, and is amenable to automation.

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List of Abbreviations

PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
HPV	Human papillomavirus
CIN	Cervical intraepithelial neoplasia
PAGE	Polyacrylamide gel electrophoresis
ORF	Open Reading Frame
E6,E7	Early region 6 & 7
L1	Late region 1
EtBr	Ethidium bromide
UV	Ultra violet
O/N	Overnight
dNTP's	deoxy nucleotide triphosphates
SSC	Standard saline citrate
EDTA	Ethylene diamine tetra acetate
TBE	Tris borate-EDTA
SDS	Sodium dodecyl sulphate
mRNA	messenger ribonucleic acid
BSA	Bovine serum albumin
SV40	Simian vacuolating virus 40
RB	Retinoblastoma
TAE	Tris acetate-EDTA
TE	Tris-EDTA
Taq	Thermus aquaticus
BPV-1	Bovine papillomavirus-1

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I. INTRODUCTION

I.1. Papovaviridae.

The papovaviridae are a family of DNA viruses that have naked icosahedral nucleocapsids of 44-55nm diameter, which are composed of one major and one minor protein component. Papovaviruses fall into two distinct groups based on size a) papillomaviruses and b) polyomaviruses. Papillomaviruses possess both larger capsids and larger genomes than polyomaviruses. Papillomaviruses infect surface epithelium and cause benign tumors that may become malignant whereas polyomaviruses infect internal organs causing malignancy and also set up persistent lytic infections.

I.2. Papillomaviruses (HPV), structure and function:

Papillomaviruses are widespread in nature and are small non-enveloped DNA viruses containing circular genomes of approximately 8kb that replicates in the nucleus of the infected cell. Papillomaviruses are a difficult group of viruses to study because of the lack of a suitable tissue culture system capable of supporting viral replication.

Papillomaviruses have been classified into over 50 different types on the basis of their sequence homology. Over 40 different types have been molecularly cloned, sequenced, characterized and linked to specific infectious diseases involving benign or malignant hyperproliferation of either mucosal or cutaneous epithelia at different body sites.

Papillomaviruses induce squamous epithelial or fibroepithelial tumors in their natural hosts. They infect humans and animals with strict host and tissue specificity. Both animal and human papillomaviruses have been associated with squamous cell carcinomas. They cause benign epithelial tumors or warts in a variety of animal hosts. Some of these viruses are also associated with lesions which can progress to carcinoma of the cervix. This suggests a causative role of these human papillomaviruses (HPV) in the etiology of these neoplasias. All this knowledge is extrapolated from genetic studies of Bovine papillomavirus-1 (BPV-1), which is often used as a model system since it transforms certain mouse cell lines in vitro (Dvoretzky et al., 1980) and replicates extrachromosomally as plasmids (Law et al., 1981), just as viruses do in vivo. BPV-1 has therefore served as a prototype papillomavirus for studies involving in vitro transformation assays and has provided the basis for most of the knowledge of papillomavirus molecular biology (Lambert et al., 1988).

The HPV viral genome is a circular, double stranded DNA and has 8 major open reading frames (ORF) which are concentrated in approximately 90% of the genome and the other 735 bp segment located between open reading frame L1 and E6 constitutes the upstream regulatory region (URR) or non coding region (NCR). The genome is divided into two main regions: Early and Late regions. All of the detectable mRNA species are transcribed from a single strand and all the ORF's are located on the same strand suggesting that all transcription would be derived from one strand.

The upstream regulatory region (URR) contains an origin of DNA replication, promoters, enhancers and short 5' exons for certain mRNAs (Baker et al., 1987a: Chow et al., 1987a; Hirochika et al., 1987; Broker et al., 1986). It also has E2-independant enhancer elements that are cell type specific and glucocorticoid responsive (Cripe et al., 1987; Gloss et al., 1987).

The early (E) region is required for cellular transformation, episomal replication in the transformed cells and transcriptional control. The E1 ORF encodes transactivating factors necessary for stable plasmid replication (Lusky et al., 1983). The E2 ORF codes for a protein that has a transcriptional transactivating function that can activate enhancer elements in the URR or LCR of the virus (Phelps et al., 1987). E5 codes for a 44 amino acid membrane associated, hydrophobic polypeptide (Schlegel

et al., 1986) that has recently been shown to be capable of stimulating cellular DNA synthesis in growth arrested cells (Green & Lowenstein. 1987) and has transforming function. E6 is a 15.5 kilodalton nucleoprotein and has 4 Cys-X-X-Cys sequence within the E6 protein, which has led to the suggestion that E6 protein may be a nucleic acid binding protein (Androphy et al., 1985). This E6 protein is believed to code for a transforming protein (Bedell et al., 1987; Matlashewski et al., 1987). E7 codes for a predominantly cytoplasmic phosphoprotein that has transforming and transactivation function, distinct from E2 transactivation. Recent evidence has indicated that HPV16 E7 ORF encodes transcriptional transactivation and cellular transformation functions analogous to those of Adenovirus E1a proteins. E7 has two conserved domains 1 and 2 like E1a of Adenovirus and hence the similarity in function has been attributed to similarity in conserved domains (Phelps et al., 1988). A mutagenic analysis of HPV 16 E7 has indicated that transformation is not dependant on high level trans-activation activity of E7 protein. A single amino acid substitution analogous to a mutation in E1a which destroys the transforming ability of this protein abolished both transformation and trans-activation by E7 (Edmonds & Vousden. 1989). Recent evidence has suggested that HPV 16 E7, adenovirus E1a, and SV40 large T (LT) immortalize cells by a common mechanism, and these form a distinct class of

immortalizing genes, distinct from the myc gene (Vousden et al., 1988, 1989). The E7 of HPV 16, E1a of adenovirus and the SV40 LT all share a region of homology which appears to be important for retinoblastoma (RB) gene binding (Decaprio et al., 1988; Whyte et al., 1988). This suggests that RB binding is essential for the transforming or transactivating activity of these proteins (Edmonds et al., 1989). HPV16 is actively transcribed in cervical cancer, the most abundant transcripts map to the E6 and E7 ORF's.

The late (L) region encodes the virion structural proteins. L1 codes for a major capsid protein and L2 codes for a minor capsid protein. The expression of the late mRNAs seems to be intimately tied to the state of differentiation of the epithelial cell in that it is restricted to terminally differentiated keratinocytes of the fibropapilloma and are believed to be suppressed in non-virus producing lesions (Baker et al., 1987a; Chow et al., 1987a; Engel et al., 1983). Detectable late messages have not been found in undifferentiated basal cells. This suggests the possible involvement of differentiation specific cellular or viral factors in the viral replication cycle.

I.3. HPV infections:

Different papillomaviruses are linked to different types of diseases. They infect their specific hosts with

great host and tissue specificity. Cervical carcinoma is one of the common malignancies in women worldwide. Clinical and epidemiological data have implicated the human papillomaviruses as an etiologic factor in the initiation and progression of the disease. Papillomaviruses are believed to be venereally transmitted and probably act as oncogenic factors, thereby causing oncogenic transformation. In vitro transformation of cells with HPV 16 DNA, detection of HPV DNA sequences especially of HPV 16 in DNA extracted from tumor biopsies, and the epidemiologic features of cervical carcinoma strongly implicate HPV 16 in the etiology of the disease. It has recently been demonstrated that DNA cloned from a cervical carcinoma containing the putative transforming genes E6 and E7 from the HPV 16 prototype is sufficient for the immortalization of primary human keratinocytes (Kaur et al., 1989). Recent evidence also suggests that the E7 gene alone of HPV 16 is capable of immortalizing NIH3T3 cells in conjunction with activated ras in primary baby rat kidney cells (Phelps et al., 1988; Storey et al., 1988; Kanda et al., 1988; Matlashewski et al., 1987). Matlashewski et al (1988) have shown that HPV 16 early gene products can cooperate with EJ-ras to transform human fibroblasts and that these transformed cells were aneuploid. This supports the hypothesis that at the molecular level HPV 16 may only be one factor in the multi-step process of HPV related human cell neoplasia.

It is generally believed that the virus primarily infects basal cells of the epidermis, infection presumably depends on local abrasions of the skin. At the cervix uteri, proliferating cells are exposed at the squamous-columnar border, and interestingly, 90% of cervical HPV infections occur at this site. The persisting viral genome increases the rate of cell proliferation or prolongs the normal life span of the keratinocytes, both events leading to hyperplasia. Epidermal cells are not permissive for papillomavirus replication in the beginning of their differentiation process, but as differentiation proceeds, they become more and more permissive (Pfister. 1987). Viral DNA replication can be demonstrated by in situ hybridization in suprabasal layers, and structural proteins and mature virus particles appear in the upper epidermal layers. HPV infection of the genital mucosa frequently induces koilocytotic atypia. The koilocyte is characterized by a large clear perinuclear zone, and binucleation is frequently observed (Pfister. 1987). There is increased mitotic activity in the basal layer and varying degrees of dysplasia (Koss. 1987). Cytoplasmic changes appear first in cells of intermediate layers and extend to the surface of the epithelium. The lesions produced by papillomaviruses share common histological features including lack of differentiation, koilocytosis and in most severe cases, abnormal mitotic figures. On infection, the basal

epithelial cells which are the target cells for HPV infection, are inhibited from differentiating in a normal manner. Three methods are routinely used to evaluate neoplastic lesions of the cervix. These include cytology, colposcopy, and histology.

In very severe cervical intraepithelial neoplasia (CIN), little or no differentiation takes place and cells throughout the full depth of the epithelium have the appearance of abnormal basal cells. Therefore, genital epithelial cancers may be viewed as lesions in which both immortalization and inhibition of terminal differentiation have occured. Histological grading of the disease is therefore based on the proportion of the epithelial thickness occupied by undifferentiated basal like cells. Involvement of the entire epithelium by atypical cells has been called carcinoma <u>in situ</u>, whereas cellular abnormality confined to the more basal layers of the epithelium has been called dysplasia. McCance, et al(1988) have used the collagen raft system and shown that HPV 16 gene products are capable of altering the differentiation pathway of epithelial cells <u>in vitro</u> in a manner similar to that seen in genital intraepithelial neoplasia. Cervical intraepithelial neoplasias (CIN) are the precursors of cervical malignancies (cervical carcinoma). Carcinoma of the cervix arises at the junction between the squamous and columnar epithelium. Invasive cancer consists of abnormal

cells in the cervical epithelium as well as in the uterine stroma underlying the basement membrane.

I.4. Implications in cervical cancer:

Several HPV types have been associated with the lesions of the uterine cervix, and only some types of HPV are found in the female genital tract. Of particular interest are the mucosal tropic HPV's that are associated with venereal diseases and cervical carcinoma (Beaudenon et al., 1986; Boshart et al., 1984; Broker et al., 1986; de Villiers et al., 1981; Durst et al., 1983; Gissmann et al., 1982; Lorincz et al., 1986). HPV 6 and HPV 11 have been associated with condyloma acuminata, a benign lesion of the cervix in which the HPV DNA's exist as monomeric plasmids. HPV 16,18,31,33 and 35 are closely associated with cervical carcinoma and its precursors, known as cervical dysplasia or cervical intraepithelial neoplasia (CIN) (Gissmann et al., 1983; Schneider-Gadicke et al., 1986; Shirasawa et al., 1986; zur Hausen et al., 1987), in which HPV sequences integrate or exist as multimeric plasmids (Durst et al., 1985; Smotkin & Wettstein. 1986; Tsunokawa et al., 1986). Direct evidence for integration was obtained by cloning virus-cell DNA junction fragments (Durst et al., 1985). The integration event seems to occur sometime during tumor progression and could serve as an indicator of an early malignancy. The effect of integration may be to alter both

viral and cellular gene expression. Viral gene expression in cells containing integrated HPV DNA is limited predominantly to the E6 and E7 ORF's (Baker et al., 1987b). There is little evidence of E2 gene expression in majority of cases studied, either from invasive tumors or cell lines (Shirasawa et al., 1988). It has been found that integrated HPV-16 DNA is preferentially opened within the E1-E2 ORF's while maintaining the URR-E6-E7 ORF's intact (Choo et al., 1987; Tidy et al., 1989). The loss of the E2 protein as a result of interruption of the E1-E2 ORF due to integration, may allow aberrant expression of the E6 and E7 proteins, which have been shown to have the ability to transform and immortalize cells in culture. There has been evidence which suggests that when both episomal HPV DNA and integrated HPV DNA coexist in the same cell, transcription of the integrated HPV predominates over the expression of the episomal HPV DNA (Smotkin and Wettstein. 1986) and the low level of E2 expression from the episomal HPV may not be sufficient to regulate the E6-E7 expression from the integrated HPV DNA, thereby allowing aberrant E6-E7 expression in these cells. Tidy, et al.(1989) have shown that deletion and rearrangement of HPV DNA may occur as a result of events other than integration. They have also described a novel deletion in the URR and a point mutation in episomal HPV 16 from two tumors, indicating the probable existence of a defective virus, which is believed to be a

transmissable variant. The change in the physical state of HPV DNA from episomal DNA to integration within the host genome has been proposed as an important event in the subsequent development of neoplasia.(Tidy et al., 1989).

The presence of HPV 16 and HPV 18 viral sequences therefore appears to be a major risk factor for progression of dysplasia to carcinoma. Due to the lack of a productive tissue culture system, very little is known about the life cycle of these viruses in spite of their medical importance (Chow et al., 1987b). The worldwide prevalence of HPV 16 is noteworthy and may possibly indicate an increased cancerogenic potential of this virus, although it is apparent that only a small percentage of women with HPV16 infection eventually develop cervical carcinoma after a long latent period. This suggests that secondary events additional to HPV infection are important for the progression of the premalignant lesion to invasive carcinoma (zur Hausen. 1986; zur Hausen & Schneider. 1987).

Since E6 and E7 ORF's of HPV 16 are actively transcribed in cervical cancer, it seems likely that these functions are of central importance to the role of HPV 16 in initiation and/or progression of the associated human malignancies, and also suggests that these gene products (E6 and E7) may be necessary for maintenance of the transformed phenotype (Schneider-Gadicke & Schwarz. 1986;

Smotkin & Wettstein. 1986; Baker et al., 1987). HPV 16,18,31,33 and 35 have been found in over 80% of all cervical tumors, suggesting that these viruses play a causative role in the development of these malignancies (Boshart et al., 1984; Durst et al., 1983).

I.5. METHODS OF DETECTION OF HPV DNA IN CLINICAL SAMPLES:

Epidemiologic features of cervical cancer strongly implicate a venereally transmitted agent in the etiology of the disease. Herpes simplex virus type 2 and human papillomaviruses (HPV 16 and HPV 18) are venereally transmitted and have been suggested as causal agents. Evidence of viral DNA sequences in the tumor tissue is one point favouring an etiologic relation between a virus and a cancer. HPV DNA sequences have been found in a majority, but not in all cancer cases, while HSV-2 DNA sequences have been detected in only a few (~15%). For HPV, the cancer cells appear to contain most of the genome while for HSV, only segments of viral sequences are found. HPV infection of the cervix has been associated with neoplastic change (Howley. 1987).

The current hypothesis based on epidemiological evidence suggests that the human papilloma viruses are neither necessary nor sufficient by themselves in causing cancer of the cervix. However, because of its prevalence in cancer of the cervix, HPV could be oncogenic and could thus

act either independantly or in association with other possible risk factors. The prevalence of HPV infection in the normal female population suggests that HPV may be a relatively ubiquitous agent (de Villiers et al., 1987), which implies that secondary events following HPV infection, probably combined with cellular factors, are of importance in the development of cervical neoplasia. One of the secondary events that may be relevant to this process is the integration of HPV DNA into the cellular genome, the consequence of which could be alteration of both HPV and cellular gene expression (Tidy et al., 1989).

Although a clear association between papilloma viruses and human squamous cell carcinomas has recently been established (Pfister. 1987; zur Hausen et al., 1987), the significance of the association needs to be determined (Munoz. 1988; Melnick. 1989). An important development in cancer research has been the definition of some of the genetic changes that occur in human cancer, such as evidence of mutations and rearrangements in oncogenes. Yet some other studies indicate that viral genomes are involved in the process of carcinogenesis. In order to understand the association between cellular or viral genomes and carcinogenesis, studies on DNA from tumors is critical. Therefore efficient and sensitive methods of diagnosing or detecting viral sequences are needed. To date, a number of

diagnostic techniques of virus detection have been used for detecting papillomaviruses.

The presence or absence of virus in the clinical specimens can be detected either by looking for antigens specific for the viral capsid protein or by looking for viral DNA. Because of the fact that the viral capsid protein (L1) is type common in that it is shared by all the papillomaviruses and the expression of this late protein is rather intimately tied with the state of differentiation of the infected cell, L1 is not expressed in undifferentiated epithelial cells (Gupta et al., 1986). Hence, detection of viral capsid protein is not a very sensitive assay. Demonstration of the presence or absence of viral DNA therefore seems to be more sensitive and hence superior in general.

The hybridization methods of DNA detection are Southern blotting, dot or slot blot hybridization, filter in situ and tissue in situ hybridizations.

Identification and characterization of nucleic acid fragments is an important part of molecular biology. The development of a standardized, highly sensitive and specific detection system in which specific gene sequences can be detected would facilitate rapid diagnosis and screening. The detection process itself needs to be quite specific and sensitive.

The HPV DNA sequences have been detected in clinical specimens by DNA-DNA hybridization techniques. The classic method of detection of viral DNA is the Southern blot method in which DNA is extracted from biopsies of lesions, fragmented with restriction enzymes, and transferred to nitrocellulose paper by blotting (Southern. 1975). On hybridization of the blot with the specific radiolabelled viral DNA probe, the presence of the correct sized restriction fragments can be seen by autoradiography. Dot and slot blot hybridization methods in which DNA on being extracted from biopsies, is directly added onto the nitrocellulose filters without restriction, and probed with viral DNA probes and subsequently visualized by autoradiography is another method of detection. Filter <u>in situ</u> hybrization involves exfoliated cells which are directly trapped onto nitrocellulose paper wherein the DNA is denatured and the proteins are removed enzymatically. The hybridization reaction of the DNA is carried out after fixing the DNA to the paper with heat (Schneider et al., 1985). Hybridization can also be carried out on tissue sections on glass slides (Gupta et al., 1985). Although Southern blot hybridization method has been considered the gold standard, it is cumbersome and none of the above methods are sensitive enough to detect low copy numbers of the virus. The most recent method of detecting specific viral DNA sequences is by <u>in vitro</u> enzymatic amplification

of viral DNA sequences using Taq polymerase. This amplification of specific HPV sequences by polymerase chain reaction (PCR) is the main technology utilized in this study and in theory is the most sensitive method of viral DNA detection. Picogram quantities of DNA are required, thus making PCR a feasible diagnostic tool.

I.6. Polymerase chain reaction:

The polymerase chain reaction (PCR) technique developed by Mullis and coworkers (1987) and described by Saiki et al., (1985, 1988) is a very sensitive and improved method of all DNA detection methods, by virtue of the fact that the concentration of specific nucleic acid sequences are amplified exponentially. At the end of the PCR technique, the most abundant species of DNA is the sequence of interest. Polymerase chain reaction (PCR) involves specific amplification of target viral DNA sequences and is capable of detecting sub picogram quantities of DNA sequences. This method requires considerably less material and is based on <u>in vitro</u> enzymatic amplification of DNA sequences by extension of specific primers which flank the DNA sequence of interest, thereby considerably enhancing the sensitivity of DNA-DNA hybridization.

The PCR technique is conceptually a very simple method for amplifying nucleic acids and mimics the natural DNA replication process in that, the number of DNA

molecules generated by the end of a typical cycle using PCR doubles after each cycle in a way similar to in vivo DNA replication, thereby producing a selective enrichment of a specific DNA sequence by a factor of 10^6. This method is based on the repetition of a set of three steps, all conducted in succession under somewhat different and controlled temperature conditions (Christian Oste. 1988). PCR involves two synthetic oligonucleotide primers that are complementary to sequences that flank the DNA segment to be amplified, which on incubation with the deoxynucleotide triphosphates (dNTP's), target DNA sequences and the enzyme [Klenow frament of E.coli DNA polymerase I or Taq polymerase purified from thermophilic bacteria, Thermus aquaticus (Perkin Elmer, Cetus)], and subjected to repeated cycles of heat denaturation (to denature the target sequence) of the DNA, annealing of the primers to their complementary sequences and extension of the annealed primers with Taq polymerase results in exponential increase in target sequences thereby increasing the sensitivity of DNA-DNA hybridization. Since each primer is complementary to opposite strands of the target DNA sequence and since they are oriented such that DNA synthesis occurs across the target sequences between the primers, it results in doubling of the target sequences at the end of each cycle. Taq polymerase has several advantages over Klenow fragment of DNA Pol I in that, there is increased specificity,

sensitivity and since this enzyme can survive extended incubation at 95°C, simplifies the procedure considerably (Saiki et al., 1988). The efficiency of the PCR amplification can be calculated according to the formula: $(1 + X)^n = Y$, where X is the mean efficiency per cycle, n is the number of PCR cycles, and Y is the extent of amplification (yield) after n cycles (Saiki et al., 1988). The sensitivity of this method of DNA detection is, in a way, a disadvantage, in that contamination is a problem which might lead to false positives. So, extreme care in handling all materials and reagents used in the PCR assay needs to be exercised.

I.7. Hypothesis:

Since HPV is etiologically related to cervical carcinoma, it is expected that HPV is present in all cases of cervical cancer. This is with the assumption that HPV 16 DNA is present in low copy numbers in all cases of cervical carcinoma. The limits of detection by the other methods of DNA detection based on hybridization is about a picogram of HPV DNA which represents approximately 70,000 genome equivalents. This might mean that any clinical specimen with lower copy numbers of HPV viral DNA could yield a false negative result. PCR being in theory a very sensitive method should in fact increase the percent positivity of

HPV DNA in clinical specimens, thereby increasing sensitivity of detection of viral DNA.

I.8. Purpose of study:

It is proposed to verify the above hypothesis by using PCR technology to detect HPV 16 viral DNA sequences in invasive squamous cell carcinoma of the cervix. Since evidence suggests that E6 and E7 ORF's are actively transcribed in cervical cancer tissue and in cervical cancer cell lines, these sequences may have a role in oncogenesis. If so, then the prevalence of these virus specific sequences in cancer cases should be fairly high. But, the virus may be present in low copy numbers and could go undetected by methods which are not sensitive enough. PCR will be used to detect presence or absence of virus. The PCR results will be compared to hybridization methods of assay, such as Southern and Slot blot methods in terms of sensitivity and specificity. Interpretation of the statistical analyses performed will be discussed.

II. MATERIALS AND METHODS

II.1. Clinical specimens.

II.1.1. Study population.

All newly diagnosed invasive cervical cancer patients attending the National Oncology Institute, Panama City, Panama between January 1986 and June 1987 were enrolled in a study detailed elsewhere (Acs et al., 1989). After histological confirmation of the diagnosis, women between 18-69 years of age who had received no previous treatment and who had resided in Panama for at least 6 months were invited to participate in the study. All 200 eligible cases volunteered to participate. The cases had a standard clinical evaluation, which included staging of the cancer, by the Oncology Institute staff. All participants were administered a standardized interview (requiring approximately 60 minutes) by a trained interviewer. Biopsy samples of the lesion yielding sufficient DNA for HPV testing were obtained from 94% of the cases enrolled.

II.1.2. Processing of biopsies:

Biopsy samples were frozen in liquid nitrogen and transported to Gorgas Memorial Laboratory where they were

stored at -70°C until shipment on dry ice to McMaster University. DNA was purified from the biopsies as previously described (Prakash et al., 1985). All DNA specimens were stored frozen at -20°C.

The DNA extracted was initially tested for HPV DNA using DNA-DNA hybridization assays. These included Southern blot analysis of specimens, using plasmid DNA containing HPV 16 sequences to probe for viral DNA, under moderately stringent conditions of hybridization. The specimens were then analyzed by slot blot hybridization under less stringent conditions of hybridization using a 637 base pair fragment of the L1 open reading frame of HPV 16 and HPV 33 plasmid DNA probes. To assess the potential utility of the Polymerase chain reaction (PCR) in detecting HPV DNA in cervical cancers, DNA of the specimens were then tested by this assay. Sufficient DNA was available from 163 samples to test by polymerase chain reaction (PCR) using oligonucleotide primers specific for the E6 and E7 regions of HPV 16. 112 of these samples were also tested by PCR using oligonucleotide primers specific for L1 region of HPV 16. Products of amplification of the E6 region were detected by probing with an endlabelled oligonucleotide probe (20mer) specific for E6 region. The E7 and the L1 amplification products were detected by electrophoretic separation of ten μl of the amplified mixture on 8% polyacrylamide gels. DNA products were visualized by staining with ethidium bromide.

II.2. DNA-DNA hybridization assays:

II.2.1. Probes for HPV DNA:

DNA probes used in the DNA-DNA hybridization assays were derived from recombinant plasmids containing DNA from HPV 16 (Durst et al., 1983), and HPV 33 (Beaudenon et al., 1986). DNA preparations were radiolabelled by the random primer method (Feinberg and Vogelstein. 1983) using α^{32}P-dCTP. In addition, a purified fragment of HPV 16 DNA was used as a probe. This was a 637 base pair fragment of the L1 open reading frame generated by digestion of the DNA with Bam H1 and Pst 1 restriction endonucleases and purified by polyacrylamide gel electrophoresis (Maniatis et al., 1982).

II.2.2. Southern blot analysis:

The biopsy DNA and 1 μg of HPV 16 plasmid DNA (positive control) were initially analyzed by the Southern blot method using 20 ug of DNA digested with 200 units of Bam H1 according to the instructions of the manufacturer (Bethesda Research Laboratories,Gaithersburg,MD). The digested DNA was electrophoresed on 1% (w/v) agarose gels and transferred to nitrocellulose filters by the Southern blot method (Southern. 1975) as modified by Wahl and co-workers (1979). The nitrocellulose filters were prehybridized in a solution containing 50 mM Tris HCl, pH 7.5, 5 X Denhardt's solution [1 X Denhardt's solution =

0.1% (w/v) ficoll, 0.1% (w/v) polyvinyl pyrrolidone, 0.1% (w/v) bovine serum albumin], 5 X SSC [1 X SSC = 8.765% (w/v) NaCl, 4.41% (w/v) trisodium citrate], 20% (v/v) formamide and 200 ug/ml sonicated and heat denatured salmon sperm DNA]. Hybridization was carried out at 42°C in the same solution modified to contain 20 mM Tris HCl (pH 7.5), 10% (w/v) dextran sulphate and ^{32}P labelled DNA probe ($2x10^5$ cpm/cm^2 nitrocellulose filter). After 16 hours of incubation at 42°C the nitrocellulose filters were washed extensively in 2 X SSC containing 0.1% SDS at 65°C and then exposed to X-ray film for 7 days. The autoradiographs were independently examined by 3 observers. Specimens were considered HPV DNA positive only when all observers agreed.

II.2.3. Slot blot analysis.

The DNA specimens of the cancer biopsies were also analyzed for HPV DNA sequences using a slot blot apparatus (Minifold II, Schleicher & Schuell, Inc. Keene, NH). Two micrograms of specimen DNA were diluted in 40 ul of 0.5M NaOH, 6 X SSC and heated at 80°C for 5 minutes. The specimens were placed on ice for 15 minutes and neutralized with 10 ul of 5M ammonium acetate and then spotted on nitrocellulose filter using the slot blot apparatus. Two micrograms of human placental DNA (Sigma, St.Louis, MO) was added to every eighth well for control purposes. The nitrocellulose filters were baked at 80°C for 2 hours

followed by prehybridization and hybridization as described above. After hybridization at 42°C the filters were washed 5 times in 5 X SSC at 50°C. They were then dried and exposed to X-ray film (Kodak, XAR 5) for 2 days. The nitrocellulose filters were initially probed with the 637 base pair fragment of the L1 region of HPV 16.

The filters were dehybridized in 5mM Tris HCl, pH 8.0, 0.5 mM EDTA and 0.05% sodium pyrophosphate. Boiling dehybridization solution was poured over the filters which were then maintained at 70°C for 2 hours, washed briefly in 0.2 X SSC containing 0.1% SDS and baked at 80°C for 10 minutes and the filters were subsequently probed with HPV 33 DNA. The autoradiographs were scanned on a Biorad model 620 densitometer with a model 3392 integrator. The density values were recorded to 10^6. Values obtained for the control placental DNA were subtracted from those obtained for the specimen DNA to yield values thought to be attributable to HPV DNA sequences. The mean and standard deviation of 14 slots containing control placental DNA and probed with the L1 fragment were 2.69 and 1.0, respectively. Values of samples exceeding 3 standard deviations (i.e. 3.0 or greater) were considered positive. Density values of 2.0 or greater were considered positive for samples probed with HPV 33 DNA. Reconstruction experiments indicated that 12 to 16 picograms of HPV 16 DNA could be detected in 2 micrograms of placental DNA using the L1 probe.

II.3. Analysis by Polymerase chain reaction:

DNA extracted from the cancer biopsies were also analyzed for HPV DNA sequences using polymerase chain reactions (PCR). DNA was used at concentrations of 10ng/μl and 1μg/μl. 100ng or 1μg was used for the PCR assay. Sufficient DNA from 163 specimens were available for analysis by PCR using E6 and E7 primers, and DNA from 112 specimens were also analyzed by PCR using L1 primers. The DNA from clinical specimens were tested by PCR assay in batches and each batch included positive and negative controls in each assay. HPV16 plasmid DNA (cloned into pBR322) was used as a positive control in all the experiments to show that the assay worked well each time to give the right product, either detected by hybridization with the specific probe or by the presence of the correct sized product visualized on 8% polyacrylamide gels.

Human Placental DNA (Sigma, St.Louis, MO) and DNA extracted from monocytes (Carl's DNA) were used as negative controls in PCR assays using E6 primers. DNA extracted from an EBV transformed human cell line homozygous for the HLA gene (SA.DR1), and water with all reagents except DNA (No DNA), were used as negative controls in experiments involving E7 region and L1 region of HPV16.

II.3.1. Primers and probes used in PCR assay:

Oligonucleotide primers and probes specific for E6, E7 and L1 regions of HPV16, were synthesized <u>in vitro</u> by

the Central facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Lyophilized primers were reconstituted in sterile distilled water to a 10μM concentration of each of the oligonucleotides. The choice of the primers and probes were based on published sequences of HPV16 DNA (Seedorf et al. 1985). The probes for E6 and E7 regions were within the amplified sequences of E6 and E7 regions, respectively. The probes were end labelled using τ-[^{32}P]ATP (3000 Ci/mmole) and T_4 polynucleotide kinase.

II.3.2. PCR Assay:

The PCR amplification assay with Thermus aquaticus (Taq) polymerase was carried out in 100 μl reaction mixtures containing genomic DNA in 10 mM Tris HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 1 μM each of (+) and (-) primers and 200 μM of each of the four deoxynucleotide triphosphates (dATP, dTTP, dGTP and dCTP), 200 μg/ml of gelatin and 2 units of Taq polymerase. This was mixed in a 0.5 ml eppendorf tube by combining 53 μl of sterile double distilled water, 9 μl of the reagent buffer containing 5 μl of 1M KCl, 1 μl of 1M Tris (pH 8.4), 1 μl of 250 mM $MgCl_2$ and 2 μl of 10 mg/ml gelatin, 4 μl of dNTP's mix, 10 μl of each of the two primers (AB282 & AB283, or AB565 & AB566, or AB380 & AB381, depending on whether E6, E7 or L1 region is amplified) and 10 μl of DNA (100 ng or 1 μg) to a total volume of 96 μl. The reaction mix was then heated for 5-10

minutes at 96°C to denature the DNA and also to inactivate any exonucleases. Two units of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) was added to give a total reaction volume of 100 μl. The contents were then mixed and spun briefly in a microcentrifuge. This reaction mix was then overlaid with 100 μl of light mineral oil, spun again and then amplified for either 20 or 40 cycles using an automated thermal cycler (Ericomp, Ca). Positive and negative controls included in each assay were subjected to the same conditions as were the tubes receiving specimen DNA. All reagents were made in the hood and autoclaved, and care was taken to avoid contamination.

Each cycle in the thermal cycler has three steps conducted in succession under carefully controlled conditions of temperature and time as follows:

1. Denaturation for 1 minute at 94°C.
2. Annealing for 2 minutes at 42°C or at 53°C.
3. Activation of Taq polymerase and extension of primers for 2 minutes at 55°C or at 72°C.

A typical cycle of PCR using E6 primers consisted of 1 minute at 94°C, 2 minutes at 42°C, and 2 minutes at 55°C. A typical cycle for PCR using E7 and L1 primers consisted of 1 minute at 94°C, 2 minutes at 53°C, and 2 minutes at 72°C.

The step of extension in the last cycle was programmed to proceed for 10 minutes in order to ensure

complete extension. Once the reaction was complete, the reaction mix was spun briefly and the reaction mix was separated and transferred into a clean eppendorf using a pasteur pipette. This was stored at -20°C until further analysis.

II.3.3. Detection of amplified product:

The method of detection of E6 amplified product was by dot or slot blotting the amplified sample onto nitrocellulose filters with subsequent hybridization with a radiolabelled E6 specific oligonucleotide probe. After hybridization, the filters were exposed to XAR-5 Kodak film for 1 day and specific signals on the autoradiograph were quantitated by densitometry. The method of detection of E7 & L1 amplified product consisted of electrophoresing the amplified product on an 8% polyacrylamide gel, staining with ethidium bromide and looking for the correct sized band, using pBR322 cut with HaeIII as a size marker (Sigma # D9655,St.Louis,MO). This was then photographed, and values assigned on a scale of 0 - 5 by visual inspection, based on band intensity.

II.4. PCR for E6 open reading frame of HPV 16:

DNA specimens extracted from 163 cancer biopsies were amplified by PCR for 20 cycles, using primers specific for E6 region of HPV16. Two oligonucleotide primers (AB282

and AB283), one of which is complementary to one strand of HPV16 and the other complementary to another strand, were used to amplify part of the E6 region of HPV16. A probe (AB285) was synthesized to detect the amplified product. The sequences and base positions of the primers and probes are as follows:

AB282 [b106 - 126]

5' GTT TCA GGA CCC ACA GGA GCG 3'(21mer)

AB283 [b209 - 227]

3' GTC AAT GAC GCT GCA CTC C 5'(19mer)

AB285 [b141 -160]

5' CAC AGT TAT GCA CAG AGC TG 3'(20mer)

Ten μl of the amplified DNA mixture was placed in an eppendorf tube and the volume made up to 80 μl with 6 fold SSC, 0.5M NaOH and boiled for 10 minutes at 80°C. The tube was chilled on ice immediately for 15-20 minutes, spun briefly and neutralized with 20 μl of 5M ammonium acetate. The mixture was dot or slot blotted onto nitrocellulose filters using the dot blot or slot blot apparatus (Minifold II, Schleicher and Schuell, Inc. Keene, NH). The nitrocellulose filter was air dried and baked for 30 minutes at 80°C.

Hybridization solution containing 20% formamide, 5X Denhardts solution, 5 X SSC, 50 mM Tris HCl buffer (pH 7.5), 200 μg/ml of salmon sperm DNA was made. For example, 10 ml of hybridization solution was made by mixing 2 ml of

100% formamide, 1 ml of 50 X Denhardts solution, 2.5 ml of 20 X SSC, 0.5 ml of 1M Tris (pH 7.5), 0.2 ml of salmon sperm DNA (10 mg/ml) and 3.8 ml of distilled water. The oligonucleotide probe was end labelled with τ-[^{32}P]ATP using T_4 Polynucleotide kinase. To an eppendorf tube, 2 μl of oligonucleotide probe (200 ng/2 μl) was added. Also added were 2.5 μl of 10 X Kinase buffer I, 9.5 μl of sterile distilled water, 1 μl of T_4 polynucleotide kinase (kept on ice) and 10 μl (10 μCi/μl) of τ-[^{32}P]ATP (sp. activity = 3000 Ci/m mole), to a total volume of 25 μl. After mixing, the tube was spun briefly and incubated O/N at 37°C. The labelled DNA was separated from the unincorporated τ-[^{32}P]ATP by spun column chromatography through Sephadex G-50 (fine) suspended in TE buffer (pH8.0). The labelled DNA probe (25 μl) was applied to the column in a total volume of 100 μl, made up by adding 75 μl of TE buffer (pH 8.0). The effluent was collected in an eppendorf tube and the radioactivity of 1 μl of the purified radiolabelled DNA probe was counted. Between 5 X 10^5 or 5 X 10^6 cpm of purified radiolabelled probe was used per ml of hybridization solution.

The nitrocellulose filters were prehybridized with 10 ml of the hybridization solution in sealed plastic bags at 37°C for a minimum of 4 hrs. This was followed by hybridization O/N at room temperature with 5 ml of hybridization solution containing 5X10^5 or 5X10^6 cpm of

purified radiolabelled probe (AB285) per ml of hybridization solution.

The filters were then washed for 5 minutes at room temperature with 5 X SSC containing 0.1% SDS to remove excess probe, followed by a 1 hr wash with the same solution at 37°C, air dried, wrapped in saran wrap and exposed to Kodak XAR-5 film for 1 day at -70°C. The autoradiograph was then analyzed by densitometry. The amplification product for E6 region of HPV 16 was 122 bp long. Since the negative control DNA yielded a positive signal with AB285, and increased stringency of wash conditions did not decrease signal intensity of negative control DNA significantly, it suggested that the signal detected was HPV related sequences. This was confirmed by washing at incresed temperatures and the signal washed off close to the T_m (melting temp) of the probe, which is 60°C (Suggs et al. 1981).

II.4.1. Calculation of T_m of E6 probe (AB285):

$T_H = T_m - 5°C = 2°C$ (A-T bp) + 4°C (G-C bp) - 5°C

$T_H = T_m - 5°C = 2$ (10) + 4 (10) - 5°C

$T_H = 20 + 40 - 5 = 55°C$

$T_m = T_H + 5°C = 55 + 5 = 60°C$

$T_m = 60°C$

II.5. PCR for E7 open reading frame of HPV 16:

DNA samples from 163 specimens were also analyzed for E7 region by PCR using two oligonucleotide primers (AB565 and AB566) specific for E7 region and a probe(AB596) which represents sequences in the amplified segment of HPV16 DNA sequences. The sequences and base positions of these primers and probe are as follows:

AB565 [b556 - 581]

```
5' GAA TTC ATG CAT GGA GAT ACA CCT AC  3'(26mer)
    *  *
```

AB566 [b836 - 863]

```
3' GGT AGA CAA GAG TCT TTG GTC CTA GGA C  5'(28mer)
                              *  *    *
```

AB596 [b701 - 720]

```
5' CGG ACA GAG CCC ATT ACA AT  3'
```

These primers for E7 region of HPV 16 were designed based on the paper by Scharf et al (1986), for directly cloning enzymatically amplified segments of genomic DNA into a vector for sequence analysis. Therefore, one primer (AB565) had an Eco RI restriction site at it's 5' end and the other primer (AB566) had a Bam HI site at it's 5' end. The DNA sample was amplified for 40 cycles using the thermal cycler wherein a typical cycle was made up of denaturation at 94°C for 1 minute, annealing at 53°C for 2 minutes and activation of Taq polymerase and extension of the primers at 72°C for 2 minutes. The final segment of the last cycle was extended for 10 minutes to ensure complete synthesis of all products.

To visualize the amplification product, an 8% polyacrylamide gel was utilized (Maniatis et al., 1982). Ten μl of the amplified DNA was aliquoted into an eppendorf and 1.1 μl of 10 X loading buffer was added to it. [The 10 X loading buffer was prepared by mixing 25 mg of bromophenol blue (=0.25%), 25 mg of Xylene cyanole FF (0.25%), 2.5 gm Ficoll 400 (0.25%) and 10 ml of double distilled water. This buffer is used in 1 X concentration.] After mixing, the tube was spun briefly and it's contents loaded onto the gel. pBR322 cut with Hae III was used as the size marker. The gel was run O/N at 50 volts, stained in ethidium bromide solution (200 μl of 3 mg/ml solution of EtBr added to approximately 500 ml of distilled water) for about 45 minutes, and photographed under ultraviolet illumination. The presence of the correct sized band (308 bp) with respect to the marker indicated the presence of the amplified product. Values were assigned on visual inspection, on a scale of 1 - 5 as described in the definition of positivity below.

The samples which yielded bands which were greater than expected size were electrophoresed on 3% Nusieve-Agarose (FMC Bioproducts, Rockland, ME) gels. This was done by first warming 0.6 gms of Nusieve in 15 ml of 1 X TAE in a boiling water bath and simultaneously boiling 0.3 gms of agarose in 15 ml of 1 X TAE until it is dissolved. The two solutions were then mixed together with constant stirring,

cooled, and the contents poured into the gel apparatus and allowed to set. The samples were prepared for loading by mixing 8 μl of amplified DNA and 2 μl of 10 X loading buffer, briefly spun in a microcentrifuge and loaded onto the gel. pBR322 cut with Hae III was used as a size marker. The gel was run at 70 volts, stained with ethidium bromide for about 30 minutes and photographed under an ultraviolet transilluminator. The bands on the gel were transferred onto nitrocellulose filters by standard Southern blotting procedures and the filters were subsequently probed with an end labelled oligonucleotide probe (AB596) specific for the E7 region using the hybridization conditions described above. The filter was then washed with 5 X SSC containing 0.1% SDS for 5 minutes at room temperature, followed by a one hour wash in the same solution at 37°C, air dried and exposed to a Kodak XAR-5 film for 2 days at -70°C. The presence of a signal on the autoradiograph, indicated that the specimen was positive for HPV 16 related sequences.

II.6. PCR for L1 open reading frame of HPV 16:

DNA samples from 112 biopsies were available for analysis by PCR using two oligonucleotide primers (AB380 and AB381) which were specific for the L1 region of HPV16 DNA. The sequences of these primers are as follows:

AB380

5' CAA CTA TTT GTT ACT GTT GT 3'

AB381

5'AAA TCA TAT TCC TCC CCA TG 3'

The PCR assay using L1 primers for the 112 specimens was performed as described above for the E7 region. The amplified product was also analyzed the same way as described for the E7 product. The amplification product for L1 region was 134 bp long, and was quantitated by visual inspection. Values on a scale of 1-4 were assigned to the samples based on band intensity. A set of 78 samples were also quantitated by densitometry in order to compare how the values obtained by visual inspection correlates with densitometric values.

II.6.1. Densitometry:

Densitometric measurements were done on 78 samples to see whether or not the values assigned on visual inspection were reasonably accurate. This was done by first making an enlarged negative of the photograph of the gels. The negatives were subsequently scanned by a densitometer. The values obtained by densitometry were compared with the values assigned on visual inspection. The correlation coefficient between density values and scores assigned by visual inspection was = 0.905, which suggests that the two values are highly correlated. Therefore, it was felt that the use of values assigned on visual inspection of the bands on the gel were sufficiently quantitative.

II.7. Definition of positivity:

The Southern blot hybridization yields qualitative data based on the presence or absence of the correct sized band on the autoradiographs of the nitrocellulose filters.

Slot blot hybridization assays were used to test DNA specimens and controls which were probed with a 637 bp L1 fragment of HPV16 and were subsequently exposed to a Kodak XAR-5 film O/N at -70°C. The resulting autoradiographs were scanned by a densitometer and values obtained. The mean and standard deviation of 14 slots containing placental DNA control was 2.69 and 1.0 respectively, on being probed with the L1 fragment. Values of samples exceeding 3 standard deviations (ie. 3.0 or greater) were considered positive. Since 99% of the controls lies within mean ± 3 Standard deviations, this was decided as the cutoff point and any assigned value ≥ 3.0 was considered positive. Density values of ≥2.0 were considered positive for specimens probed with HPV 33 DNA.

Since PCR using E6 primers yielded a signal with the negative control DNA on probing with a radiolabelled oligonucleotide probe specific for E6 region, a cutoff point had to be established. This necessitated the need for a ratio of reactivity to be established by dividing the density of the amplification product of the sample by the density of the amplification product of the control DNA. A large proportion of the samples yielded ratios of 1 or

less. It was reasoned that ratios of 1 represented reaction product that could have arisen from contamination of the reagents used in these assays and densities greater than 20% of control would be attributable to viral DNA in the samples. Thus, a ratio of 1.2 or greater was arbitrarily selected as the criterion of positivity.

Since PCR using E7 primers with samples gave the expected sized band (308 bp) on an 8% polyacrylamide gel and did not show a band with the negative controls, values were assigned on visual inspection on a scale of 0 - 5 where 0 represented samples negative for HPV16. Values of 1 - 5 represented the degree of intensity of the correct sized band, 1 being the least intense 308 bp band and 5 representing the most intense 308 bp band. A few samples yielded bands that were greater than 308 bp, which were assigned values of 0.1 or 0.2 based on band intensity. Some samples had the correct sized band in addition to the off-sized band and these were assigned values of 1.1 or 2.1 based on band intensity. All samples assigned values of $\geq$ 1.0 were considered clearly positive and any sample assigned values of less than 1.0 were considered negative, till further analysis.

The amplification product which was greater than 308 bp was suspected as representing a variant of HPV16 and hence these samples were electrophoresed on a 3% Nusieve-Agarose gel (FMC Bioproducts, Rockland, ME), and

transferred onto nitrocellulose filters by standard Southern blotting procedures. The filters were probed with a radiolabelled oligonucleotide probe specific for the E7 region (AB596) and the filters were subsequently washed, air dried and exposed to a Kodak XAR-5 film O/N at -70°C. Those specimens that gave a positive signal on autoradiographs were also considered positive, the reasoning being that it was either a related sequence or a variant of HPV16.

PCR using L1 primers on clinical specimens also yielded bands on electrophoresing on an 8% polyacrylamide gel. The correct sized bands (134 bp) were assigned values on a scale of 0 - 4 based on band intensity by visual inspection. All specimens assigned values of ≥1.0 were considered positive.

II.8. Statistical methods:

The correlation coefficients were calculated to estimate the degree of relationship between distribution of PCR results with slot blot analysis using L1 area probe and PCR results with slot blot analysis using HPV 33 probe (Colton 1974). To see how the values assigned to band intensities from autoradiographs on visual inspection correlates to densitometric measurements, the correlation coefficient was calculated.

To compare distribution of L1 area results with HPV 33 results, analysis of variance was attempted. A chisquare test to compare distribution of L1 area results which are Southern positive with the distribution of HPV 33 results which are Southern positive was done. A chisquare test to compare the distribution of L1 area results with distribution of HPV 33 results in the proportion of samples negative by Southern was also done (Colton, 1974).

The PCR method using E6 and E7 primers, Southern blot method and the slot blot methods were compared. The reliability of the four methods used was assessed by the direct estimation of sensitivity and specificity (Maximum likelihood parameter estimates) of the four different methods in relation to each other using a program which made use of the E-M algorithm. The initial prevalence estimate here is based on the number of individuals which two or more methods rated positive (Walter. 1984).

III. RESULTS

III.1. Parameters for PCR using primers representing the E6 sequences of HPV 16.

The E6 region of HPV 16 was chosen as our target sequence for enzymatic amplification and two primers (AB282 and AB283) designed specifically for E6 region of HPV 16 were synthesized for the PCR assay as explained in the methods. 1μg of the DNA sample was amplified for 20 cycles using Taq polymerase. A typical cycle in the PCR assay for E6 region consisted of denaturation at 94°C for 1 min, annealing at 42°C for 2 mins, and activation of Taq and extension of primers at 55°C for 2 mins. The last segment of the last cycle was extended for 10 minutes in order to allow for completion of extension reactions. The reaction mixture was separated from the mineral oil and 10 μl of the reaction mixture was slot blotted onto nitrocellulose filters under suction. The filters were prehybridized and hybridized with an end labelled oligonucleotide probe (AB285) specific for the E6 amplified region of HPV 16. The filters were subsequently washed, air dried and exposed to autoradiography. The autoradiograph was then scanned by a densitometer and values assigned as has been outlined in the methods.

III.1.1. Limits of detection by E6 probe:

Initial experiments were performed to determine the limits of detection by E6 probe (AB285) by dot blotting varying amounts of HPV 16 plasmid DNA (1ng - 1μg) including 1μg of placental DNA as control, and probing with E6 oligonucleotide probe (AB285) radiolabelled at the 5' end using $\gamma[^{32}P]$-ATP and T_4 polynucleotide kinase as described in the methods section. The probe could detect 10ng of HPV16 as shown in figure 1. which suggests that the limits of detection of the E6 probe was 10ng of HPV16 DNA.

III.1.2. Specificity of E6 probe:

In order to determine whether or not the E6 probe is specific for HPV 16, 10 ng and 100 ng of each of the HPV types 6,16,18,31 and 33 plasmid DNA, pBR322 and placental DNA were dot blotted and probed with radiolabelled AB285 (E6 probe). Figure 2. shows that the probe hybridized specifically with HPV16, which suggests that AB285 could be used as a specific probe for E6 region of HPV 16.

III.1.3. Specificity of PCR assay:

To determine whether or not the primers specifically prime the HPV 16 E6 sequences, 100 ng of HPV 16 plasmid DNA was amplified for 20 cycles using PCR. The same experiment was repeated in which 100 ng of HPV 16 plasmid DNA was added to 2μg of placental DNA. Ten μl of

Figure 1: Autoradiograph obtained by dot blot hybridization method to test for limits of detection by E6 probe.

Different concentrations of HPV 16 plasmid DNA (1ng - 1μg) and placental DNA (1μg) were dot blotted and probed with endlabelled E6 specific oligonucleotide probe of HPV 16 (AB285) as described in the text. Rows A to D are HPV 16 plasmid DNA and row E is Placental DNA. Row A is 1 μg of HPV 16, Row B is 100 ng of HPV 16, row C is 10 ng of HPV 16, row D is 1 ng of HPV 16 and row E is 1 μg of placental DNA (Negative control DNA).

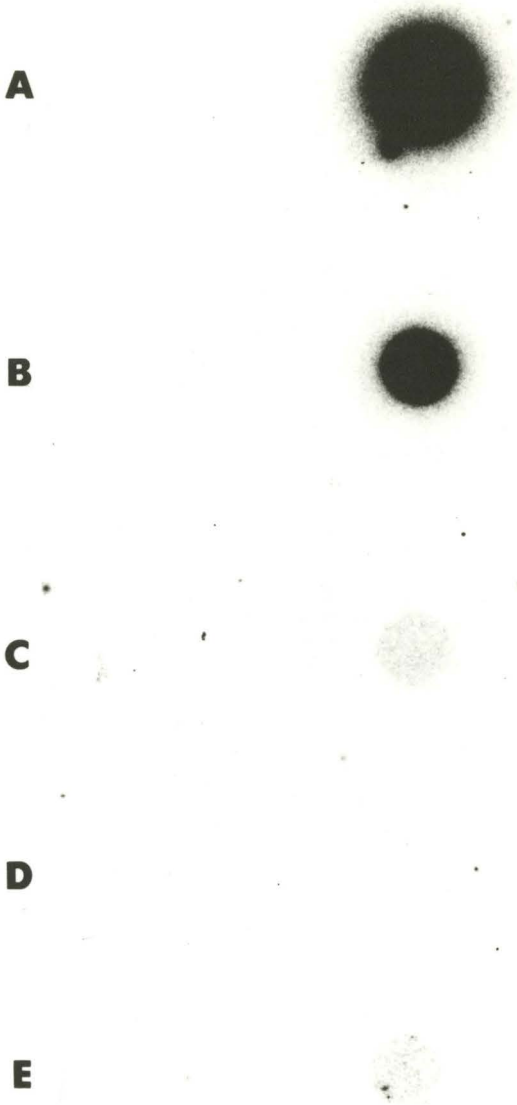

A
B
C
D
E

Figure 2: Autoradiograph obtained by dot blot hybridization method to test for specificity of E6 probe.

Different types of HPV, placental DNA and pBR322 were dot blotted and probed with E6 specific probe. Column A represents 100 ng of DNA and Column B represents 10 ng of DNA that was dot blotted. Row 1 is pBR322, row 2 is HPV type 6, row 3 is HPV type 16, row 4 is HPV type 18, row 5 is HPV type 31, row 6 is HPV type 33 and row 7 is placental DNA.

A

B

1

2

3

4

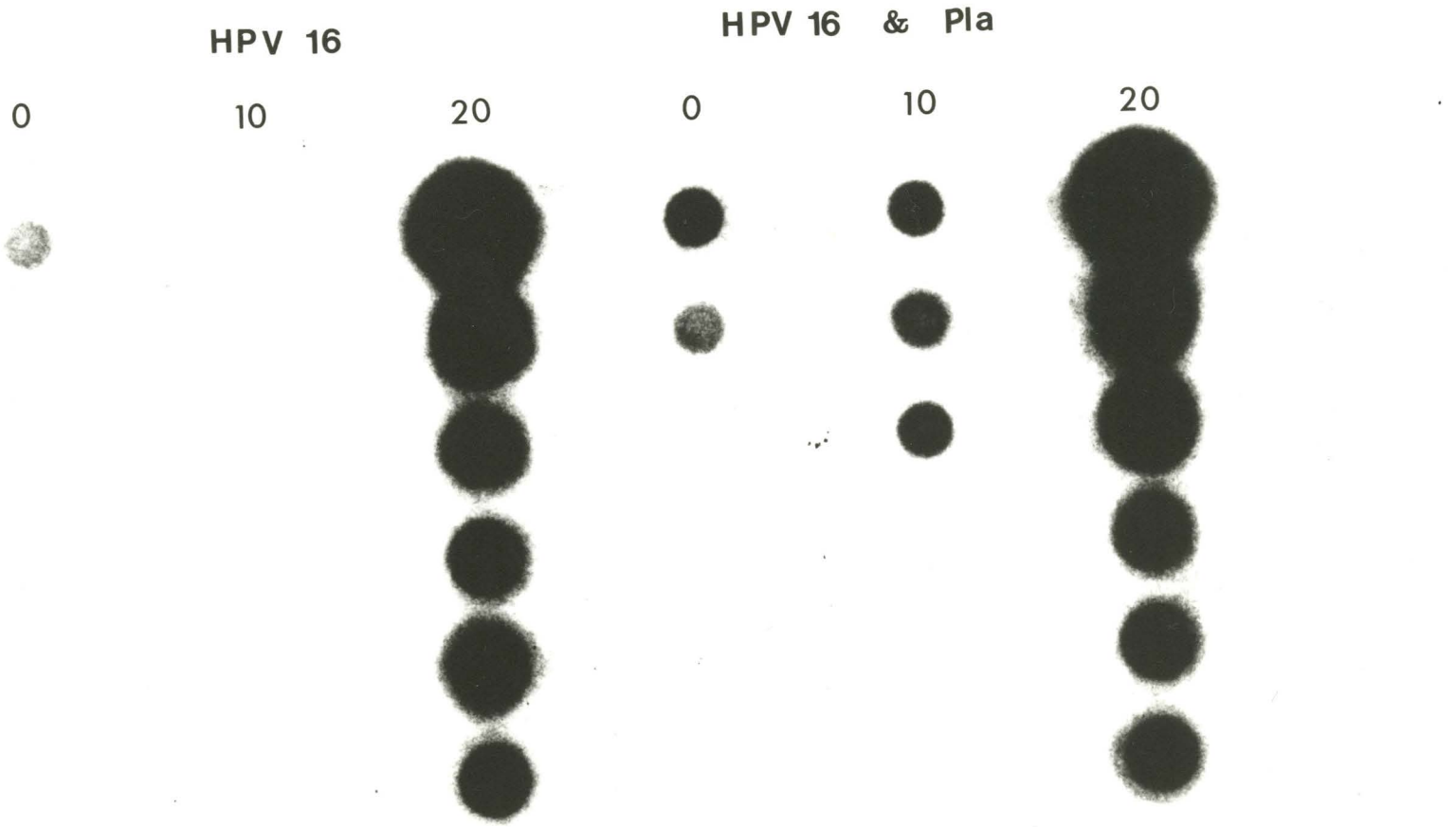
5

6

7

Figure 3: Autoradiograph obtained by dot blot hybridization method to test for specificity of amplification.

HPV 16 plasmid DNA (100 ng) and reconstructed form of HPV 16 (100 ng) in placental DNA (2μg) were amplified for 0, 10 and 20 cycles using PCR and dot blotted in 10 fold serial dilutions. This was probed with E6 specific probe. 0, 10 and 20 represent number of cycles of amplification. The first three columns represent HPV 16 plasmid DNA and the next three columns represent reconstructed form of HPV 16 (in placental DNA).


HPV 16
0
10
20
HPV 16 & Pla
0
10
20

the amplified product was dot blotted in 10 fold serial dilutions and probed with the radiolabelled E6 probe (AB285), and then washed, air dried and exposed to autoradiography. The results are shown in figure 3. Also, dehybridizing the blot and reprobing it with a non specific probe, AB284 (specific for L1 region of HPV 16), did not give a signal with HPV 16. The primers used, AB282 and AB283, therefore appeared to specifically amplify the E6 region of HPV 16. A separate experiment suggested that deoxynucleotide triphosphates and primers were limiting and needed to be replenished after 20 cycles of amplification.

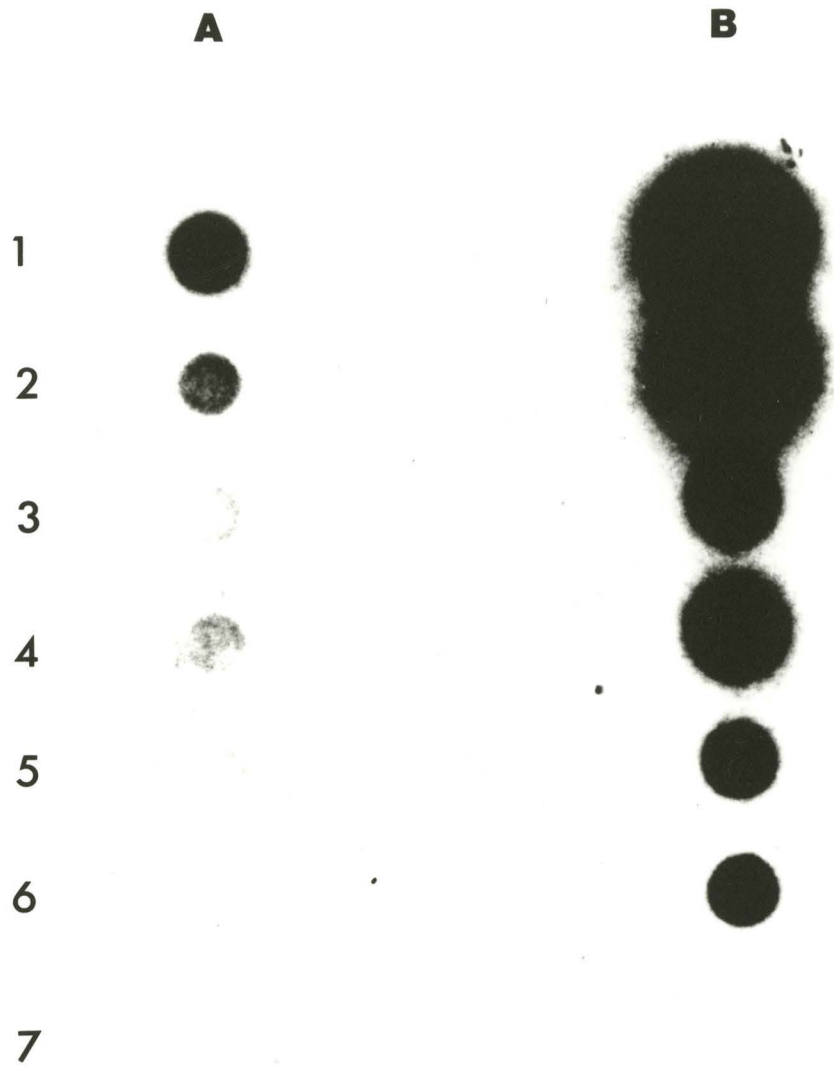
III.1.4. Limits of detection by PCR assay:

In a reconstruction experiment to determine the limits of detection by the PCR assay, varying amounts of HPV 16 plasmid DNA (0.1 pg - 100 ng) were added to 1 μg of Carl's DNA, amplified for 20 cycles, dot blotted and probed with the E6 probe. 1 pg of HPV 16 could be detected in reconstructed form on amplification as can be seen in figure 4. This suggests at least a 10,000 fold amplification of HPV 16 DNA sequences in the reconstructed form.

Interestingly, the negative control DNA also gave a positive signal in some experiments. To assess the specificity of the signal, the nitrocellulose filters were rewashed for 1 hr with 5 X SSC, 0.1% SDS at 42°C, 45°C and

Figure 4: Autoradiograph obtained by dot blot hybridization method to determine the limits of detection by the PCR assay.

Different concentrations of HPV 16 plasmid DNA (100 ng - 0.1 pg) was reconstructed in 1 μg of Carl's DNA (negative control) and amplified for 20 cycles, dot blotted and probed with E6 specific probe. Column A represents unamplified DNA and column B represents amplified DNA. All the rows have Carl's DNA in addition to which row 1 has 100 ng of HPV 16, row 2 has 10 ng of HPV 16, row 3 has 1 ng of HPV 16, row 4 has 100 pg of HPV 16, row 5 has 10 pg of HPV 16, row 6 has 1pg of HPV 16, and row 7 has 0.1 pg of HPV 16.


A
B
1
2
3
4
5
6
7

50°C. Increased stringency of wash conditions did not decrease signal intensity of negative control DNA significantly, which suggested that the signal represented amplification of HPV 16 related sequences in the control DNA. Two explanations for this observation were considered. First, that the placental DNA and the monocyte DNA (Carl's) constituting the negative control contained HPV 16 related sequences. Second, HPV 16 DNA could have inadvertantly contaminated either the negative DNA or the reagents used in the PCR assay. The former possibility was explored by amplifying another region of HPV 16, ie. E7 region which is described below. It was found that even the new set of primers gave similar results, which rules out the possibility of a homologous sequence being present endogenously. The latter possibility of molecular contamination was examined by sizing the amplified product of E6 region and by sequencing the amplified product of E6 region. The sizing experiment indicated that the size of the amplified product of HPV 16 and of the negative DNA control were both 122 bp long. The amplified products of both were then sequenced. Both HPV 16 and the negative control DNA were both found to have HPV 16 sequences (Dr. Martin Kraemar - personal communication).

These results suggest that molecular contamination accounted for the signal seen in the negative control DNA. This aspect of molecular contamination and means of

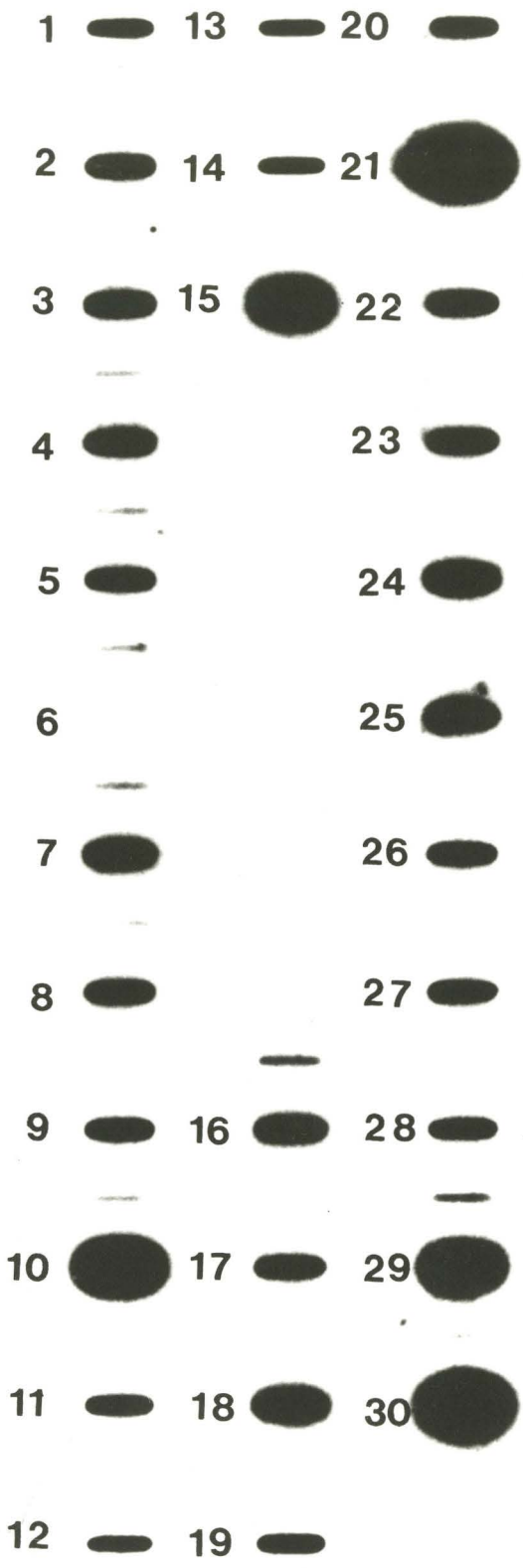
reducing it was looked into in greater depth using E7 primers, as described below. The presence of contamination does not negate the use of the assay since the level of amplification in the negative control DNA can be used to standardize for background amplification. Thus, DNA samples from 163 cervical cancer specimens were assayed for HPV 16 DNA sequences using PCR and E6 primers, including a positive (HPV 16 plasmid DNA) and a negative control (Carl's DNA) in each assay. 1 μg of the DNA extracted from cancer biopsy specimen was used for the PCR assay, using E6 primers and 20 cycles of amplification. Ten μl of the amplified DNA was slot blotted onto nitrocellulose and probed with the E6 specific probe. The results of a representative batch of clinical specimens is shown in figure 5.

III.2. Parameters for PCR using primers representing E7 sequences of HPV 16.

The primers for E7 (AB565 and AB566) were designed primarily as a check for contamination and to see if the above problems of contamination were unique to the E6 set of primers. 100 ng of the DNA samples were subjected to 40 cycles of amplification using E7 primers, and each cycle consisted of denaturation for 1 min at 94°C, annealing for 2 mins at 53°C and extension for 2 mins at 72°C. The assay for the E7 region was carried out on an automated thermal

Figure 5: Autoradiograph obtained by slot blot hybridization method of the clinical specimens after 20 cycles of amplification.

1 μg of DNA extracted from tumor biopsies was subjected to 20 cycles of amplification, slot blotted and probed with E6 specific oligonucleotide probe (AB285). 1 & 16 represent Carl's DNA (negative control) after amplification and all other slots represent amplified clinical specimens.


1
13
20
2
14
21
3
15
22
4
23
5
24
6
25
7
26
8
27
9
16
28
10
17
29
11
18
30
12
19

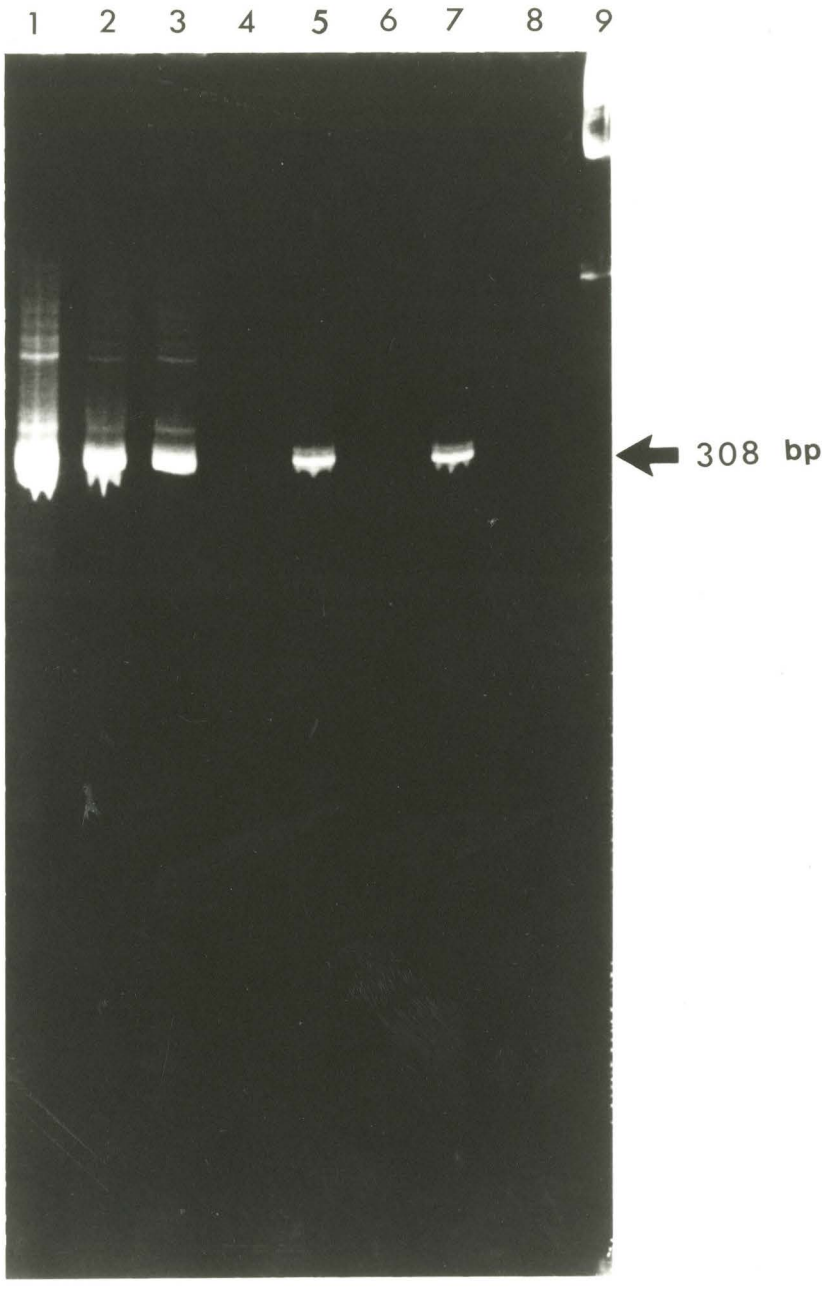
cycler. 10μl of the amplified sample was electrophoresed on an 8% polyacrylamide gel O/N at 50 volts, stained with ethidium bromide for 45 minutes and then photographed. Values were assigned based on visual inspection as described in the methods section.

III.2.1. Check for contamination:

As a check for contamination in the PCR assay, 100ng each of DNA extracted from monocytes (Carl's DNA), pBR322, water (No DNA) and HPV16 plasmid DNA were amplified for 20 cycles and 40 cycles using E7 primers. 10 μl of the amplified product was electrophoresed on an 8% polyacrylamide gel, stained with ethidium bromide and photographed under an ultraviolet illuminator. Lambda DNA cut with Hind III was used as a size marker. The E7 amplification product of HPV 16 was expected to be 308 bp long. At 20 cycles, only HPV 16 had a 308 bp band whereas at 40 cycles, all negative DNA's including water (No DNA) had a 308 bp band as can be seen in figure 6, which again suggested molecular contamination of reagents. This also suggested that this was not unique to one primer set, although the sensitivity in picking up contamination varied considerably between primer sets.

Figure 6: Gel after PCR assay as a check for contamination.

As a check for contamination, negative and positive control DNA were amplified for 20 cycles and 40 cycles using E7 primers and the amplified DNA was run on 8% polyacrylamide gels and visualized on ethidium bromide staining, under an ultraviolet illuminator. Lanes 1 & 2 are HPV 16 plasmid DNA, lanes 3 & 4 are pBR322, lanes 5 and 6 are Carl's DNA, lanes 7 & 8 are No DNA (water), and lane 9 is lambda DNA cut with Hind III which was used as a size marker. All odd numbered lanes except 9 are after 40 cycles of amplification and all even numbered lanes are after 20 cycles of amplification.


1 2 3 4 5 6 7 8 9
308 bp

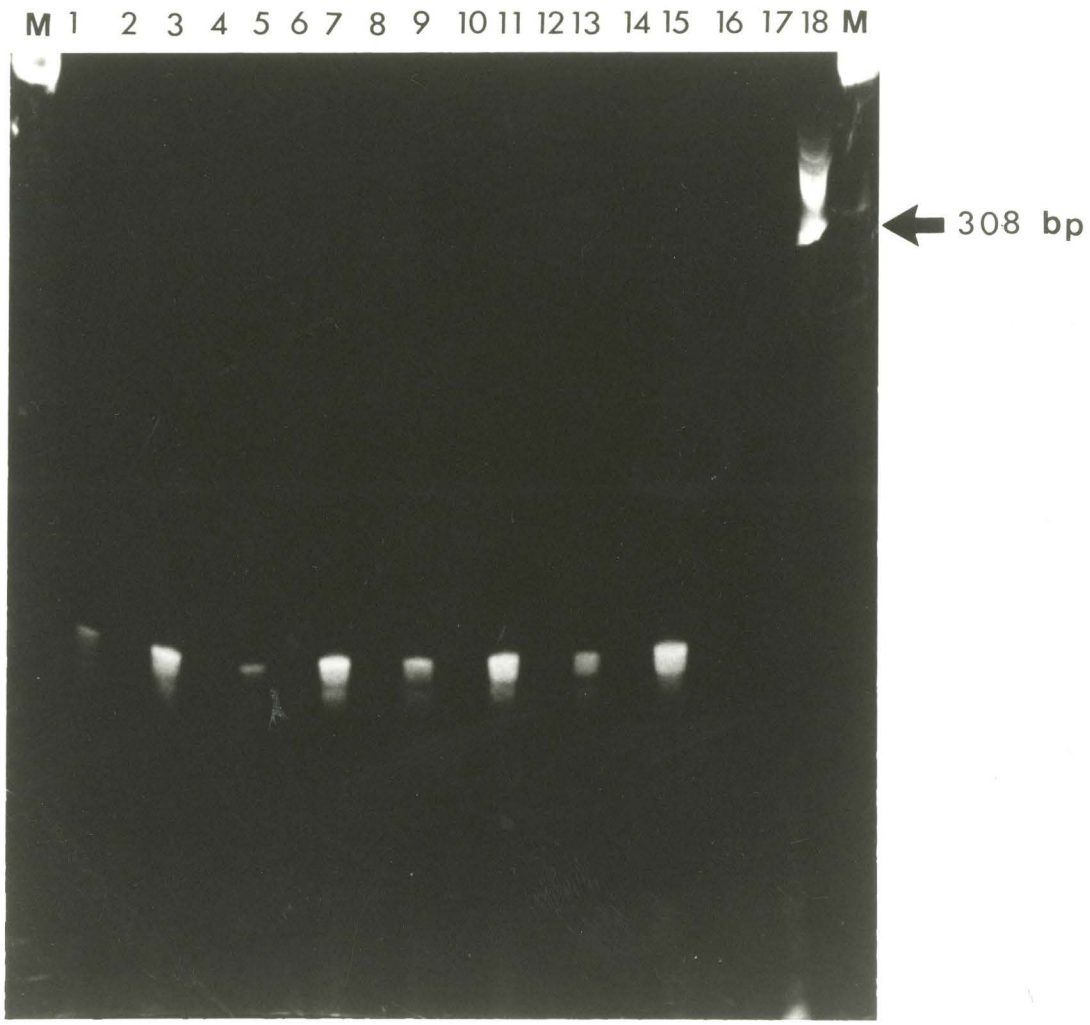
III.2.2. Search for negative control:

In the search for a more appropriate negative control, we used DNA extracted from different EBV transformed human cell lines in the PCR assay and subjected it to 20 cycles and 40 cycles of amplification using the PCR reagent kit. The PCR reagent kit was used in order to rule out possibility of introducing contamination through reagents and utmost care was taken in handling DNA samples. The amplified product was electrophoresed on an 8% polyacrylamide gel, stained with ethidium bromide and photographed. No 308 bp band was seen in any of the wells including the water (No DNA) control, as can be seen in figure 7, which confirmed that our reagents were contaminated.

This suggested the need for extreme care in preparation and handling of reagents, primers, DNA and any material required for PCR assay. Having pinpointed the problem to molecular contamination, it was sought to working in the hood, autoclaving all reagents and minimizing the number of additions by making reagent buffer and PCR mix. SA(DR1) which is an EBV transformed human cell line was chosen as one of the negative controls in addition to water (No DNA) which also served as a control for contamination.

Figure 7: Gel after 20 and 40 cycles of PCR on negative control DNA using reagent kit.

Different negative control DNA were amplified for 20 and 40 cycles and run on an 8% polyacrylamide gel, and visualized on EtBr staining. Lanes marked with an M are lambda DNA cut with Hind III used as a size marker. Lanes 1, 2, 3, & 4 represent control DNA A done in duplicates with and without reagent kit (lanes with odd numbers represent control DNA amplified 40 cycles and even numbered lanes represent control DNA amplified 20 cycles). Lanes 5, 6, 7, & 8 represent control DNA B done in duplicates, lanes 9, 10, 11, & 12 represent control DNA C done in duplicates, and lanes 13, 14, 15, & 16 represent control D (No DNA) done in duplicates. Lane 17 is control DNA C amplified for 40 cycles in a previous experiment and lane 18 is HPV 16 plasmid DNA amplified for 40 cycles in a previous experiment. Control DNA C which is SA(DR1) and control D (No DNA) were chosen as the negative control DNA.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
308 bp

III.2.3. Limits of detection by PCR assay using E7 primers:

To determine sensitivity of assay using E7 primers, varying dilutions of HPV 16 plasmid DNA (0.01 pg - 100 ng) was amplified for 45 cycles including water (No DNA) as a negative control. 10 μl aliquots were taken out at the end of 20, 25, 30, 35, 40 and 45 cycles and this was electrophoresed on an 8% polyacrylamide gel, stained with ethidium bromide and photographed. pBR322 cut with Hae III was used as a size marker. The results are presented in figure 8a. The detection limit by polyacrylamide gel electrophoresis [PAGE] was 0.1 pg (100 fg) of HPV16 at 30 cycles or more of amplification, which represents approximately 7000 molecules of HPV16. This calculation of approximate number of molecules of HPV 16 is based on molecular weight of a single molecule of HPV 16 which is about 1.32×10^{-5} pg and this was in turn calculated based on the approximate molecular weight of each base as being equal to 660.

Molecular weight of HPV 16 = $660 \times 12{,}000 = 8 \times 10^{6}$

Weight/ Molecule = 8×10^{6} gm/ $6 \times 10^{23} = 1.32 \times 10^{-17}$gm

$= 1.32 \times 10^{-5}$pg

Number of molecules in 0.1 pg = $0.1/1.32 \times 10^{-5}$

$= 7.6 \times 10^{-2} \times 10^{5}$

$= 7.6 \times 10^{3}$ molecules

A reconstruction experiment where varying concentrations of HPV 16 plasmid DNA was added to 100 ng of

SA(DR1) and amplified up to 45 cycles, suggested that there was a 10 fold decrease in sensitivity on reconstruction, which can be overcome by replenishing dNTP's and primers after about 20 cycles [figure 8b]. The apparent depletion of reactants may explain the levelling off of the curve after exponential increase in concentration of the amplification product.

III.2.4. Specificity of PCR assay using E7 primers:

In an experiment to determine the specificity of the PCR assay, 100 ng each of HPV types 6, 11, 16, 18 and 33 plasmid DNA, and SA(DR1), water (No DNA) were amplified for 20 cycles and were run on a polyacrylamide gel. Figure 9 shows that a 308 bp band was seen with HPV 16, a faint band was seen with HPV 11 and a greater than expected size band was seen with HPV 18. This suggests that the assay is fairly specific for HPV 16. The faint band seen with HPV 11 could be either due to cross reactivity of the probe with HPV 11 or could also be due to prior contamination of HPV 11 DNA with HPV 16 DNA.

III.2.5. Determination of number of cycles of amplification required:

To determine the number of cycles of amplification required for clinical specimens without decreasing sensitivity, SA(DR1), water (No DNA), HPV16 plasmid DNA and

5 clinical specimens picked at random were amplified up to 40 cycles and aliquots taken out at 30, 35 and 40 cycles. This was electrophoresed on an 8% polyacrylamide gel, O/N at 50 volts. The results, presented in figure 10, indicate that 30 or 40 cycles yielded essentially similar results, and the negative controls did not give a 308 bp band. Although 30 cycles appeared sufficient, it was decided to use 40 cycles with the hope that it increases sensitivity.

III.2.6: PCR assay for clinical specimens using E7 primers:

The specimens of DNA from 163 cervical cancer cases were assayed for HPV16 DNA sequences by PCR using E7 primers and 40 cycles of amplification. 100 ng of each DNA sample was used per assay. The expected size of the amplification product was 308 bp. Figure 11 shows a representative batch of clinical specimens showing a 308 bp band on amplification after 40 cycles.

A few of the specimens yielded bands which were greater than the expected size as can be seen in figure 10. These were suspected as being a variant of HPV16 with an insertion and/or substitution. To investigate this further and also to determine whether or not these are HPV16 related E7 sequences, 10μl of these amplified samples were run on 3% Nusieve-agarose gels and photographed as shown in figure 12a. The DNA was subsequently blotted onto nitrocellulose filters by standard Southern blotting

Figure 8a: Gel after amplification of HPV 16 plasmid DNA to test for sensitivity of the PCR assay using E7 primers.

Different concentrations of HPV 16 plasmid DNA (0.01 pg - 100 ng) were amplified up to 45 cycles using No DNA as a negative control, and were electrophoresed on an 8% polyacrylamide gel and visualized on EtBr staining. M represents the size marker (pBR322 cut with Hae III). Each set of lanes represented by numbers 1-9 consist of 7 lanes which represents 0, 20, 25, 30, 35, 40 & 45 cycles of amplification. Lane set 1 represents 100 ng of HPV 16 plasmid DNA, lane set 2 is 10 ng, lane set 3 is 1 ng, lane set 4 is 0.1 ng (100 pg), lane set 5 is 10 pg, lane set 6 is 1 pg, lane set 7 is 0.1 pg, lane set 8 is 0.01 pg of HPV 16 and lane set 9 is water (No DNA).

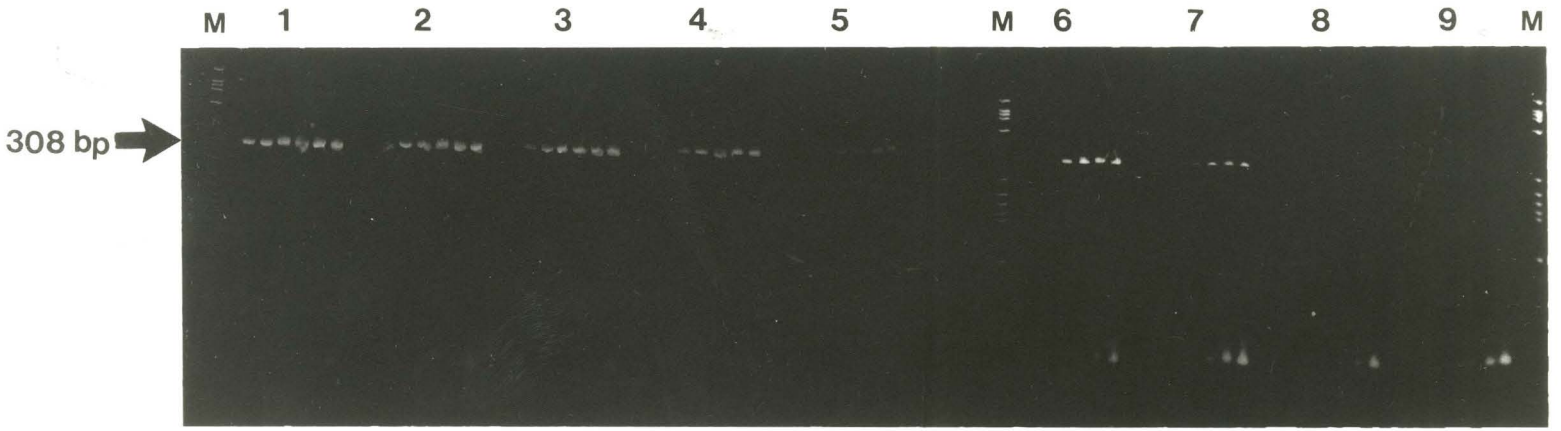

M
1
2
3
4
5
M
6
7
8
9
M
308 bp

Figure 8b: Gel showing reconstruction experiment of HPV 16 plasmid DNA in negative control DNA to test for sensitivity on reconstruction.

Different concentrations of HPV 16 plasmid DNA was reconstructed in 100 ng of SA(DR1) and amplified upto 35 cycles, and aliquots taken out at the end of 20, 25, 30 & 35 cycles and run on a polyacrylamide gel. Lanes 1, 2, 3 & 4 are after 20, 25, 30 & 35 cycles of amplification in each lane set. All lane sets (A - E) have 100 ng of SA(DR1) in addition to which lane set A has 10 pg of HPV 16 plasmid DNA, lane set B has 1 pg of HPV 16, lane set C has 0.1 pg of HPV 16, lane set D has 0.01 pg of HPV 16, lane set E has no HPV 16 and has only 100 ng of SA(DR1), and lane set F has No DNA (water). M represents the size marker (pBR322 cut with Hae III).

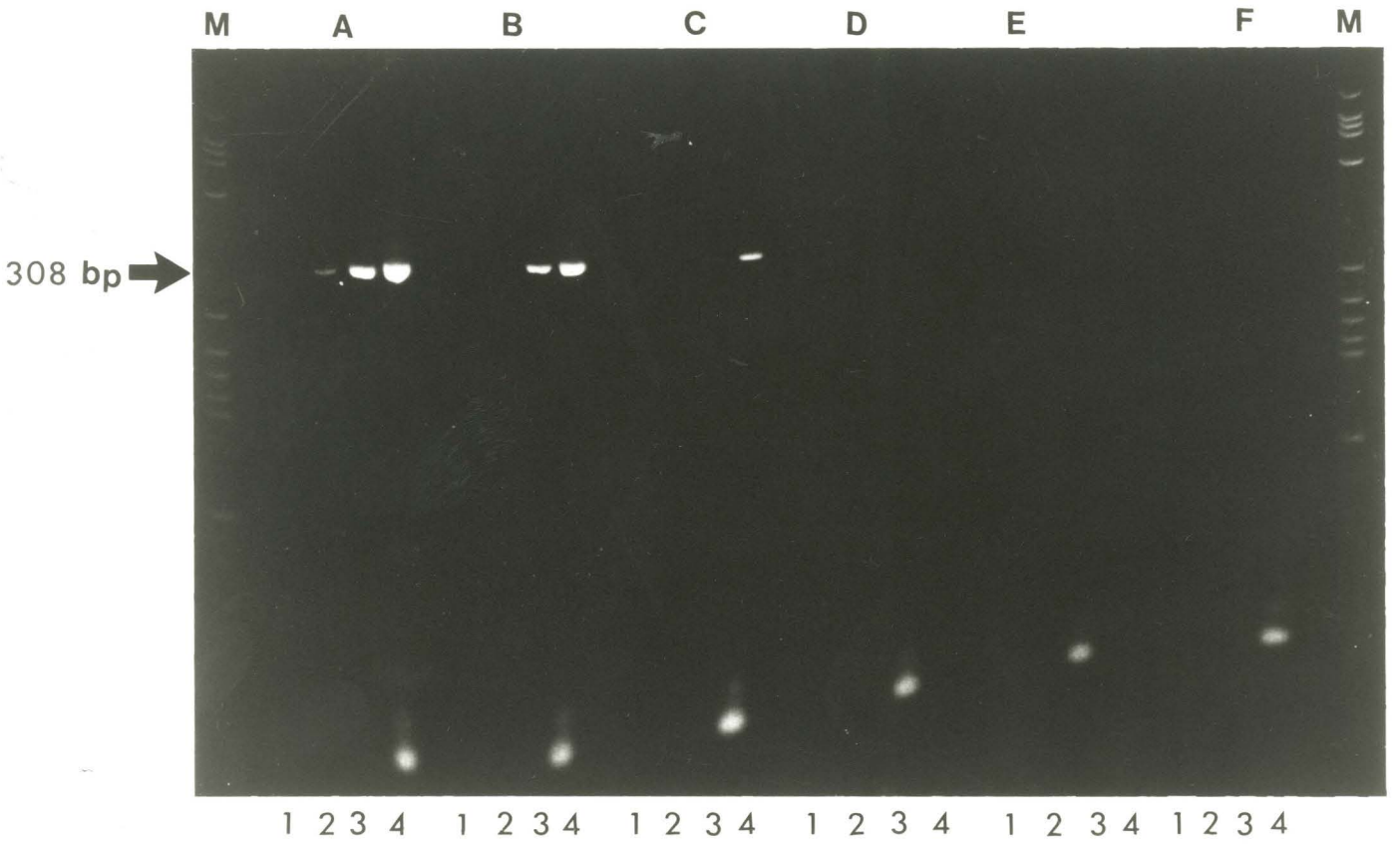

M
A
B
C
D
E
F
M
308 bp
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

Figure 9: Gel to test for specificity of PCR assay using E7 primers.

Different HPV types (100 ng) and two negative control DNA were amplified for 20 cycles and run on a polyacrylamide gel. M represents the size marker (pBR322 cut with Hae III). All odd numbered lanes represent amplified DNA and the even numbered lanes represent corresponding unamplified DNA. Lanes 1 & 2 represent HPV 33, lanes 3 & 4 represent HPV 18, lanes 5 & 6 represent HPV 16, lanes 7 & 8 represent HPV 11, lanes 9 & 10 represent HPV 6, lanes 11 & 12 represent SA(DR1) and lanes 13 & 14 represent No DNA (water).

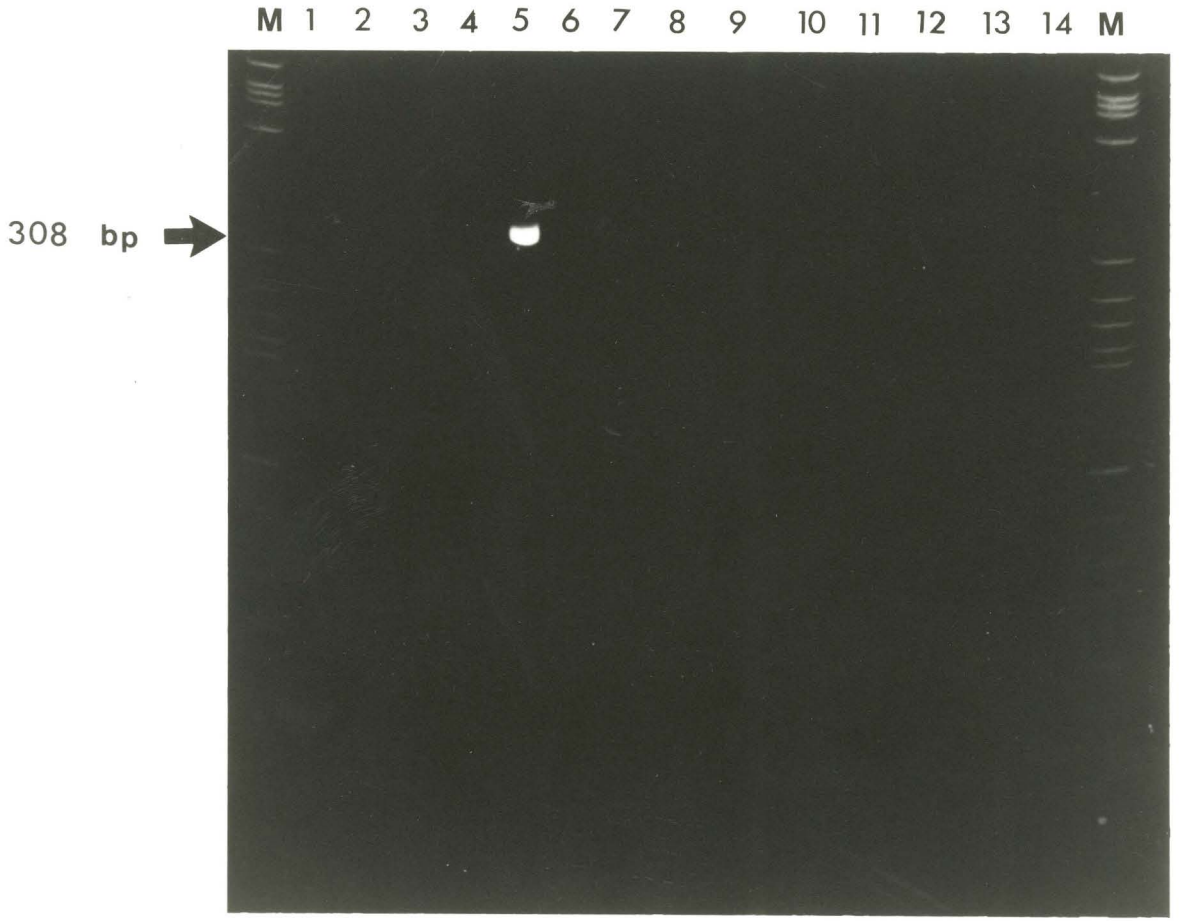
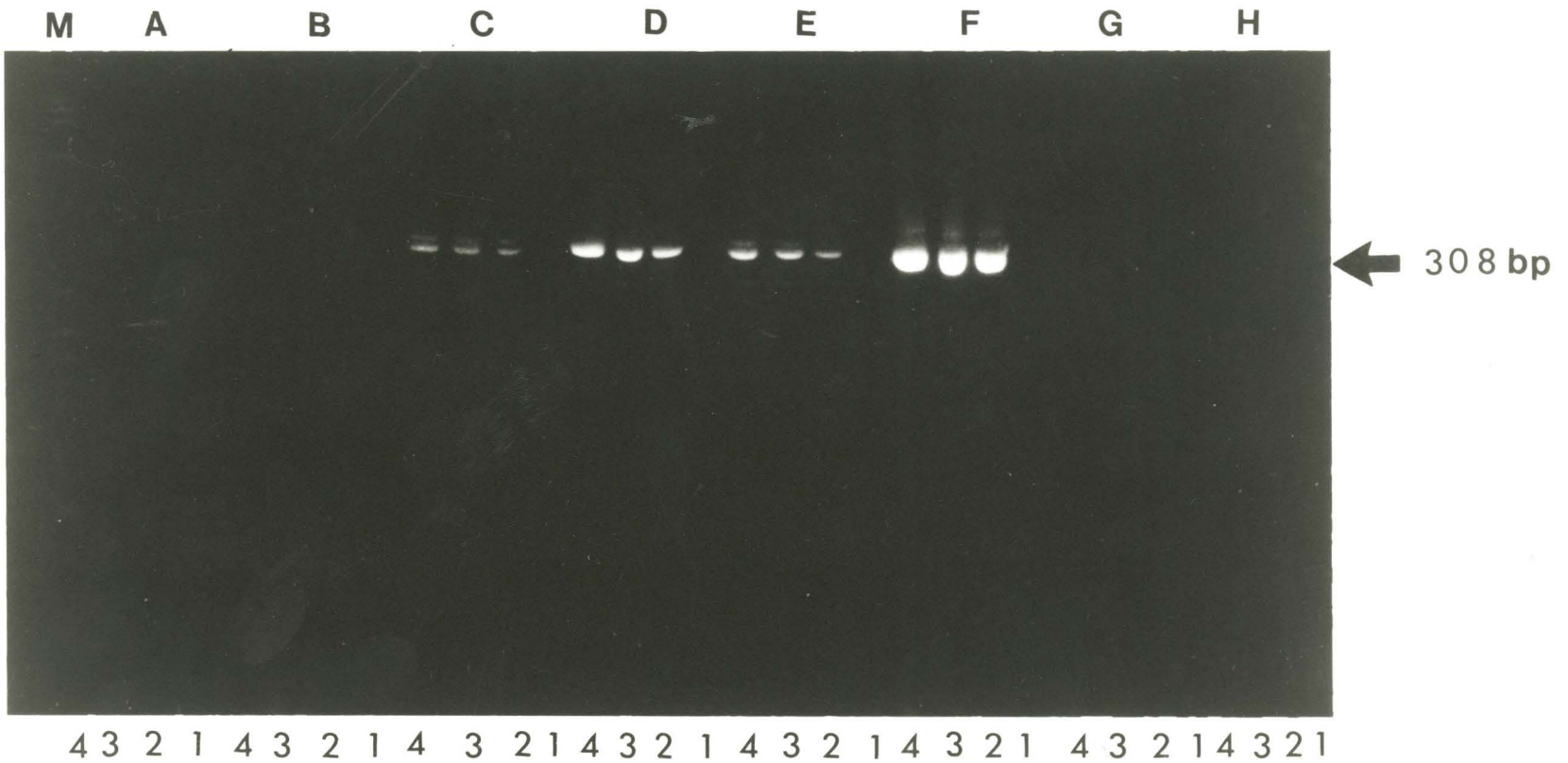

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
308 bp

Figure 10: Gel to determine number of cycles of PCR amplification required.

To determine number of cycles of amplification required for clinical specimens, SA(DR1), No DNA, HPV 16 plasmid DNA and 5 clinical specimens were amplified up to 40 cycles, aliquots taken out at 30, 35 & 40 cycles, and electrophoresed on a polyacrylamide gel. M represents the size marker (pBR322 cut with Hae III), Lane sets A -E represents clinical specimens C18, C12, C10, C9, & C8 respectively, lane set F is HPV 16 plasmid DNA, lane set G is SA(DR1) and lane set H is No DNA (water). Each set has 4 lanes, 1 is unamplified control, 2 is 30 cycles amplified sample, 3 is 35 cycles amplified sample & 4 is 40 cycles amplified sample.


M A B C D E F G H
308 bp
4 3 2 1 4 3 2 1 4 3 2 1 4 3 2 1 4 3 2 1 4 3 2 1 4 3 2 1 4 3 2 1

procedures, baked at 80°C for 2 hours and probed with radiolabelled oligonucleotide probe (20mer) specific for E7 region of HPV 16. The filter was then washed, air dried and exposed to autoradiography. The results are shown in figure 12b. Those specimens which yielded signals on probing were considered positive for HPV 16 related sequences.

III.3. Parameters for PCR using primers representing L1 sequences of HPV 16:

L1 open reading frame [ORF] codes for the major capsid protein of HPV16 and is expressed only in highly differentiated keratinocytes. Primers representing the L1 sequences were used for the PCR assay and tests were done on 112 specimens of cervical cancer, using 100 ng of eachspecimen per assay, carried out exactly as was the assay for E7 region. Ten μl of the amplified product was electrophoresed on an 8% polyacrylamide gel, stained with ethidium bromide and photographed under an ultraviolet transilluminator. Water (No DNA) was used as negative control and HPV 16 plasmid DNA and DNA extracted from Caski cells were used as positive DNA controls in each assay. Figure 13 shows a representative batch of specimens after 40 cycles of amplification using L1 primers.

Figure 11: Gel showing amplification of a batch of clinical specimens using PCR and E7 primers after 40 cycles.

Gel showing representative batch of specimens after 40 cycles of PCR amplfication. M represents pBR322 cut with Hae III which was used as a size marker. Lane 1 is SA(DR1), lane 2 is No DNA control, lane 3 is HPV 16 plasmid DNA, and all other lanes represent 100 ng of DNA from clinical specimens after 40 cycles of amplification.

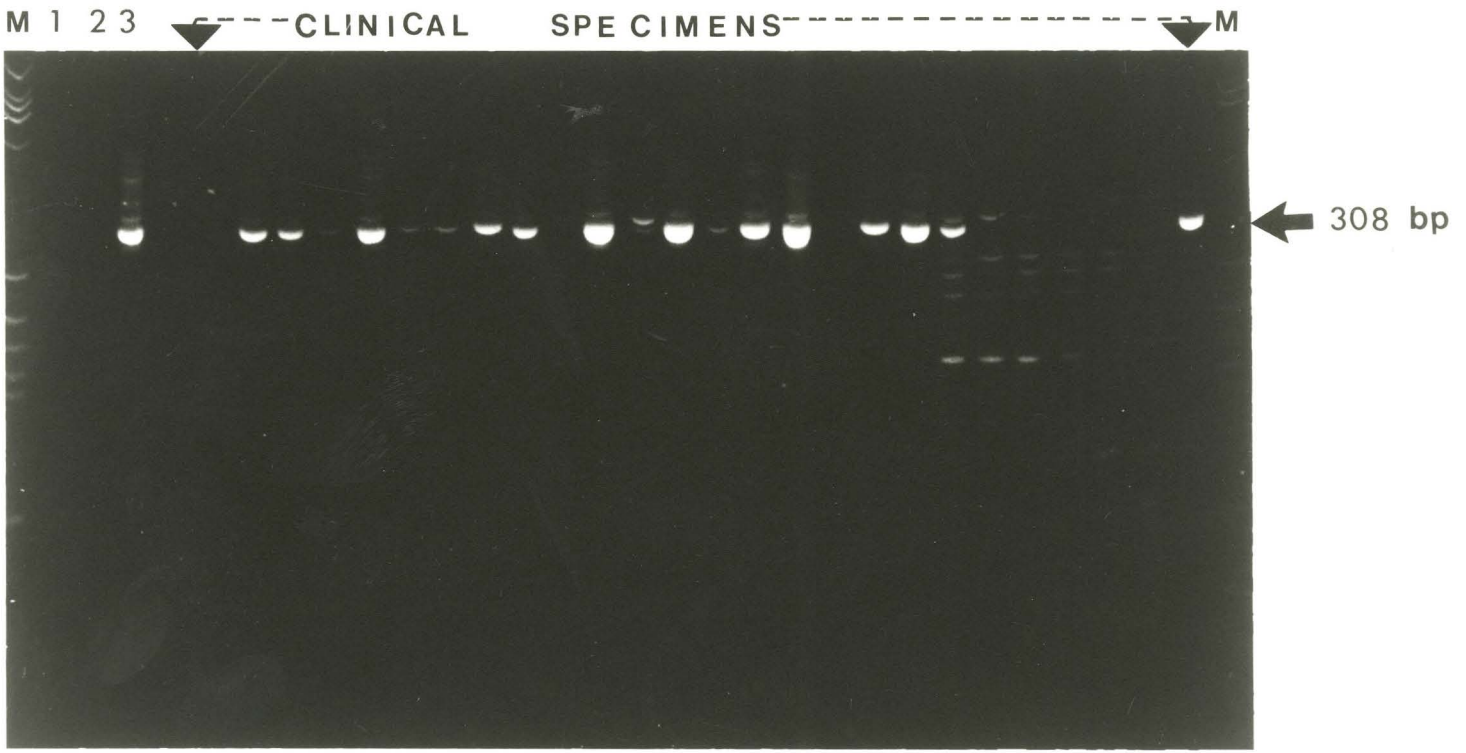

M 1 2 3
CLINICAL SPECIMENS
M
308 bp

Figure 12a: Nusieve Agarose gel showing clinical specimens which gave off-sized bands after 40 cycles of PCR amplification using E7 primers.

Few of the samples which gave off sized bands on amplifying 40 cycles using E7 primers were electrophoresed on 3% Nusieve agarose gels, visualized on EtBr staining under an ultraviolet transilluminator. 1 represents pBR322 cut with Hae III used as a size marker. Lane 2 is SA(DR1), lane 3 is HPV 16 plasmid DNA and lanes 4 to 20 represent clinical specimens.

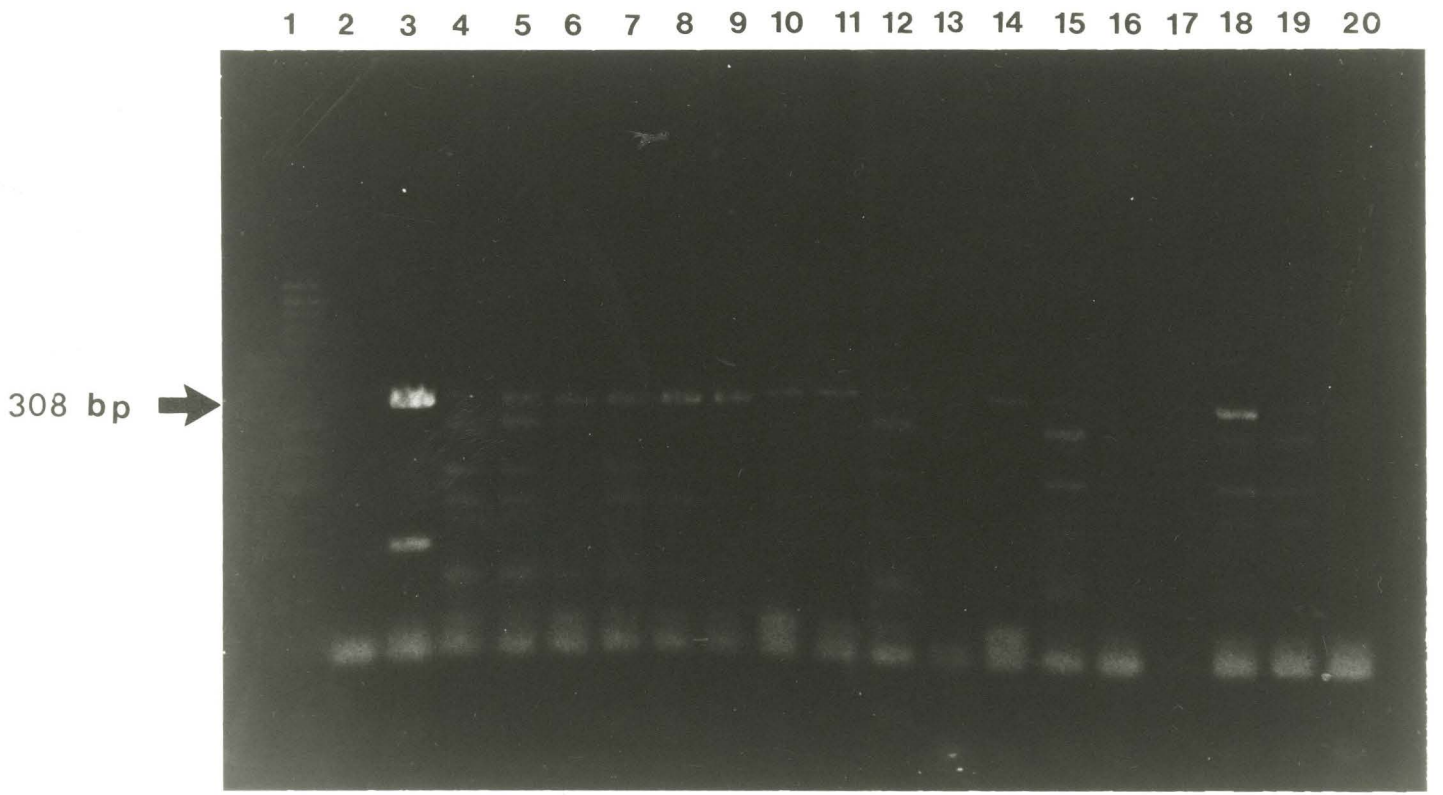

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
308 bp

Figure 12b: Autoradiograph obtained on Southern blotting of off-sized bands from previous gel (12a) onto nitrocellulose.

The Nusieve agarose gel of figure 12a was Southern blotted onto nitrocellulose, probed with radiolabelled oligonucleotide probe (AB596) specific for E7 region of HPV 16 and exposed to autoradiography. Lanes 3, 13 & 15 of gel 12a gave a signal on Southern blotting whereas all other lanes showed no signal. Lane 3 represents HPV 16 plasmid DNA which was used as a positive control. Lanes 13 & 15 represent clinical specimens which gave a positive signal on Southern blotting and probing with an E7 specific oligonucleotide probe. All other lanes were negative on blotting and probing.

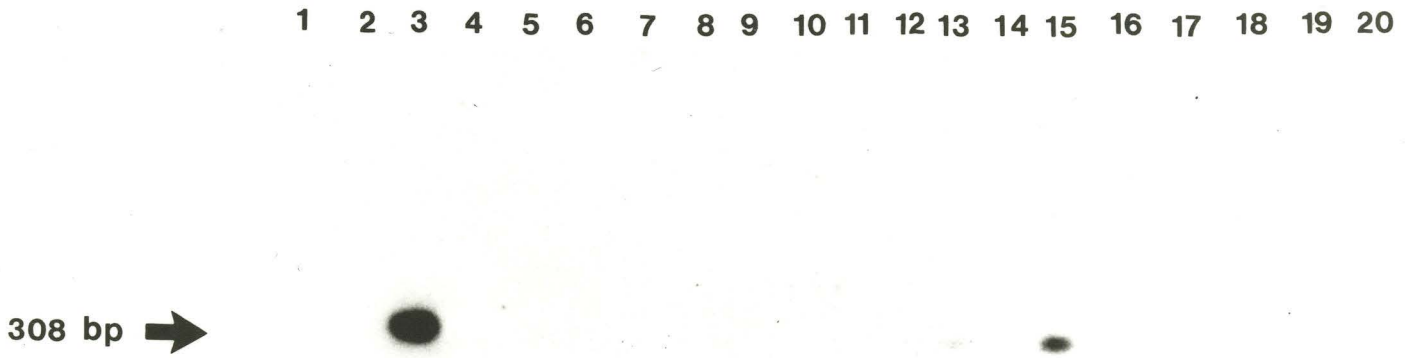
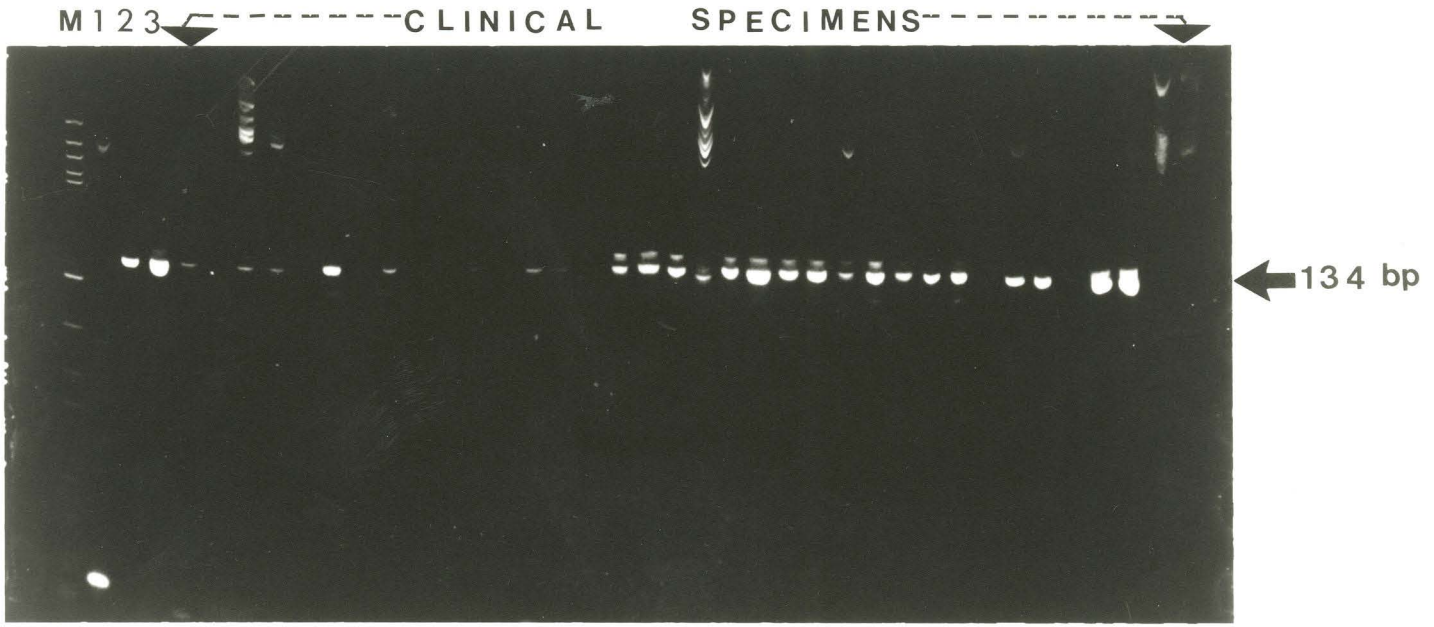

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
308 bp

Figure 13: Gel showing representative batch of clinical specimens after 40 cycles of PCR amplification using L1 primers.

Different clinical specimens including positive and negative controls were amplified for 40 cycles and were electrophoresed on 8% polyacrylamide gels, stained with EtBr and visualized under an ultraviolet transilluminator. M represents the size marker (pBR322 cut with Hae III) and lane 1 represents negative control (No DNA), lane 2 represents 100 ng of DNA extracted from Caski cells and lane 3 represents 100 ng of HPV 16 plasmid DNA and all other lanes represent 100 ng of DNA from clinical specimens.


M 1 2 3
CLINICAL SPECIMENS
134 bp

III.4. Analysis of clinical specimens by PCR:

The DNA samples of 163 cervical cancer cases were assayed for HPV 16 DNA sequences using PCR and E6 primers, including a positive (HPV 16 plasmid DNA) and a negative control (Carl's DNA) in each assay. Using the E6 primers, the negative control DNA (Carl's DNA) also gave a signal on amplifying 20 cycles and probing with an E6 specific radiolabelled oligonucleotide probe. From the evidence presented above, it was concluded that the signal seen was due to molecular contamination. Since the oligonucleotide probe detected a product in the control DNA, viral DNA could only be detected in clinical specimens if it produced a signal stronger than that found in controls. This necessitated quantitating the product by densitometric measurements and establishing a cut off point, as described in the definition of positivity. A ratio of reactivity was established by dividing the density of the specimen product by the density of the control DNA product. A large proportion of the samples yielded ratios of 1 or less. It was reasoned that ratios of 1 represented reaction product that could have arisen from contamination of the reagents used in these assays and densities of greater than 20% of control would be attributable to viral DNA in the samples. Thus, a ratio of 1.2 or greater was arbitrarily selected as the criterion of positivity.

The distribution of the results of the PCR assays on 163 clinical specimens using E6 primers are shown in table 1. Any value of ≥1.2 was considered clearly positive. Using E6 primers, 95 (58.3%) of 163 samples were positive for HPV 16 DNA. The quantities of E6 amplified product detected in relation to Southern blot positivity are shown in figure 14. The proportion of samples positive by Southern blot increased as the amount of reaction product increased for E6 amplification product.

The distribution of results on testing 163 clinical specimens with E7 primers is shown in table 1, and the values assigned ranged from 0 - 5, wherein 0 represented a negative and 1-5 represented the degree of intensity of the band. In case of E7, negative DNA's did not give a 308 bp band. So, all clinical samples assigned values of ≥1 were considered clear positives and a value of 1.0 was decided as the cutoff point for both E7 and L1. Samples assigned values less than 1.0 were considered negative. 103 (63,2%) of 163 specimens assayed with E7 region primers had values of ≥1.0.

Few of the samples yielded bands which were greater than the expected size. These were given values of 0.1 or 0.2 based on band intensity. Off-sized products were observed for 29 of the 163 samples. The off-sized bands were suspected as being variants of HPV 16 with an insertion and/or substitution. These 29 samples were

Table 1

Distribution of PCR results in relation to Southern Blot

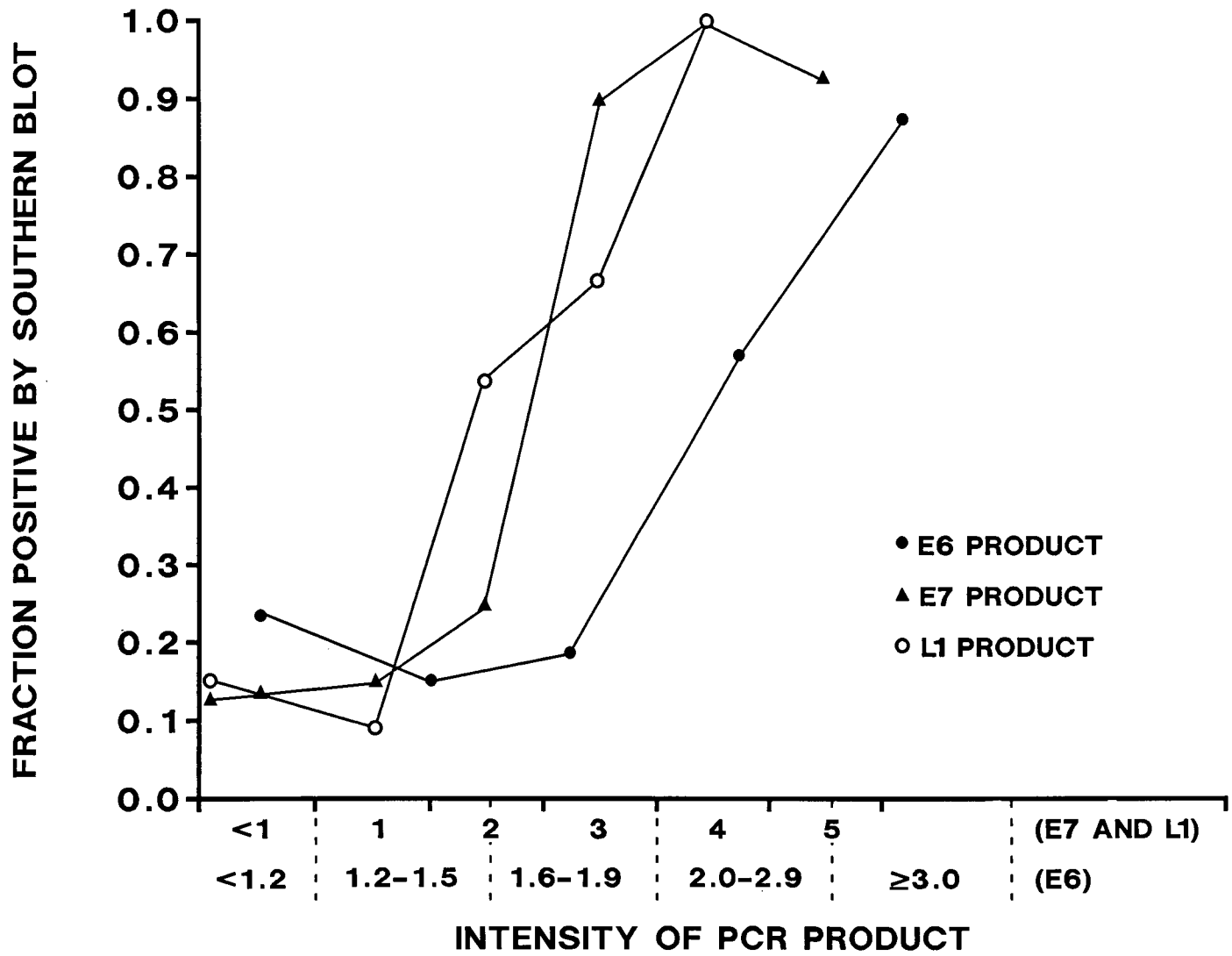
Southern blot	Number of samples yielding density ratios with E6 primers				
	<1.2	1.2-1.5	1.6-1.9	2-2.9	≥3
+	16	5	3	8	28
-	52	28	13	6	4
Total	68	33	16	14	32
% +	23.5	15.2	18.7	57.1	87.5

Southern blot	Number of samples yielding scores with E7 primers						
	0	<1	1	2	3	4	5
+	4	4	5	7	18	8	13
-	27	25	28	21	2	0	1
Total	31	29	33	28	20	8	14
% +	12.9	13.8	15.2	25	90	100	92.9

Southern blot	Number of samples yielding scores with L1 primers				
	0	1	2	3	4
+	5	2	21	8	6
-	28	20	18	4	0
Total	33	22	39	12	6
% +	15.2	9.1	53.8	66.7	100

Figure 14: Graph showing distribution of PCR results in relation to Southern blot results.

Graph showing distribution of PCR results using E6, E7 and L1 primers, in relation to Southern blot results. (●) is E6 distribution, (▲) is E7 distribution and (o) is L1 distribution.


FRACTION POSITIVE BY SOUTHERN BLOT
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
E6 PRODUCT
E7 PRODUCT
L1 PRODUCT
<1
1
2
3
4
5
(E7 AND L1)
<1.2
1.2-1.5
1.6-1.9
2.0-2.9
≥3.0
(E6)
INTENSITY OF PCR PRODUCT

Southern blotted and probed for E7 specific sequences. Positive bands on autoradiographs, seen in figure 12b, indicated that 7 samples had HPV 16 related sequences. Thus, the total number positive was 110 (67.5%) out of 163 samples assayed. Interestingly, 2 of the 7 samples which were positive on blotting and probing were Southern positive, indicating that these represented false negatives of the E7 PCR assay, and the other 5 samples which were positive on blotting and probing were either positive by one or both of the PCR assays done on other regions of HPV 16 sequence.

The DNA samples of 112 cervical cancer cases were assayed for HPV 16 DNA sequences using PCR and L1 primers. The distribution of the L1 results are shown in table 1, and the values assigned were based on degree of intensity of the 122 bp band, on a scale of 0-4 where 0 represented a negative. The values assigned were on visual inspection, and were not based on densitometric measurements for reasons described in the methods section. All specimens $\geq$1.0 were considered positive. Out of 112 cervical cancer cases tested sucessfully using the L1 primers, 79 of them were positive (70.5%).

The quantities of the PCR amplification products detected in relation to Southern blot positivity are shown in figure 14. The proportion of samples positive by Southern blot increased as the amount of reaction product increased for all three PCR assays.

III.5. Comparison of different assays:

The specimens of DNA from the cervical cancers were assayed for HPV DNA using 6 methods. The specimens were tested by the Southern blot method (1), slot blot method using HPV 16 L1 area probe (2), slot blot method using HPV 33 probe (3), PCR using E6 primers (4), E7 primers (5) and L1 primers (6). Southern and slot blot methods are hybridization methods of detecting HPV DNA. The HPV 33 probe measures a sequence heterologous to the HPV 16 sequence and therefore serves as an internal specificity control. PCR methods of amplifying specific sequences of HPV 16 are compared to the Southern and slot blot methods in terms of percent positivity, sensitivity and specificity.

The sensitivity of the method is the proportion of true positive individuals which the method classifies "+". Its complement is the false negative rate (denoted by β). The specificity of the method is the proportion of true negative individuals who are actually classified "-". Its complement is the false positive rate (denoted by α). Both these proportions are conditional on the true state of the individual, and are independant of the prevalence of true positives in the population (Walter. 1984). The method utilizing the EM-algorithm makes use of an important assumption of independant errors.

The specimens were tested by the Southern blot method which has been considered the 'gold standard'. This assay yields qualitative data based upon the presence or absence of typical bands on an autoradiograph. DNA from 70 (37.2%) of 188 samples tested by this method were positive.

The 188 samples were also tested by slot blot using an L1 fragment of HPV 16 DNA and HPV 33 plasmid DNA as probes under relatively low stringency conditions of hybridization. The degree of hybridization in the latter 2 assays was estimated by densitometric mesurement of the resulting autoradiographs, and values were assigned. The distribution of the values obtained from these assays is shown in Table 2. Using the definition of positivity described in materials and methods, 95 (50.5%) of 188 samples were positive with the L1 probe while 84 (44.7%) were positive with the HPV 33 probe.

The relation between the intensity of the signals obtained with the slot blot assays and positivity by Southern blot are shown in figure 15. The proportion of samples positive by Southern increased dramatically as density values for L1 area increased, reaching 95% for samples with density values exceeding 9. This pattern suggests that the L1 probe detected similar sequences as the Southern blot but detected smaller amounts of viral DNA. In contrast, greater proportions of Southern blot positive samples were found at low density values of the

Table 2.

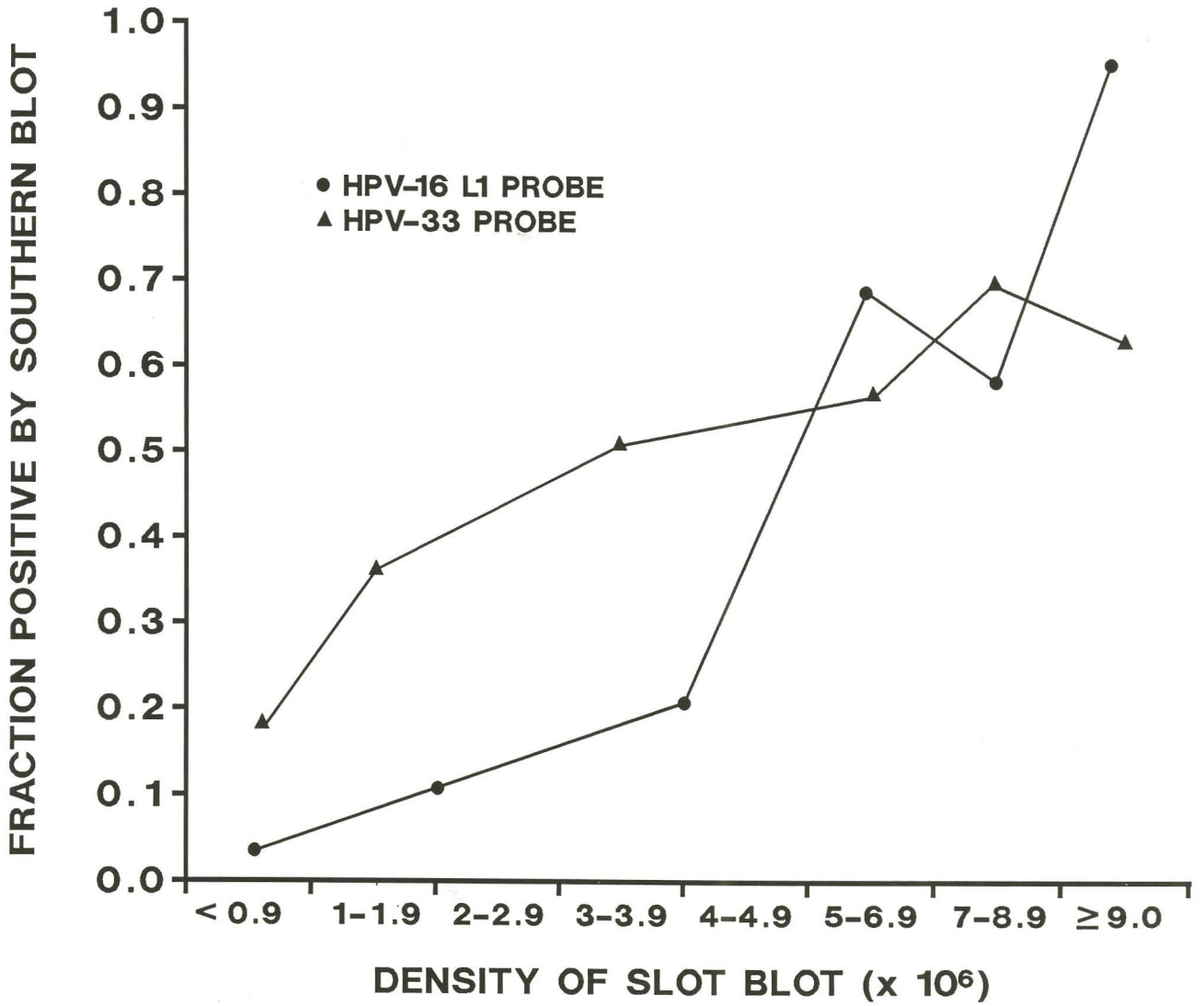
Distribution of slot blot assays in relation to Southern blot.

Southern blot	Number of samples yielding density values with L1 probe					
	<1	1-2.9	3-4.9	5-6.9	7-8.9	≥9
+	2	4	5	11	7	41
-	54	33	19	5	5	2
Total	56	37	24	16	12	43
% +	3.6	10.8	20.8	68.8	58.3	95.3

Southern blot	Number of samples yielding density values with HPV 33 DNA probe					
	<1	1-1.9	2-4.9	5-6.9	7-8.9	≥9
+	18	4	21	8	7	12
-	75	7	20	6	3	7
Total	93	11	41	14	10	19
% +	19.4	36.4	51.2	57.1	70	63.3

Figure 15: Graph showing distribution of slot blot assays in relation to Southern blot results.

Graph showing distribution of L1 area results and HPV 33 results in relation to Southern blot results. (▲) is HPV-33 probe, (●) is HPV-16 L1 probe.


FRACTION POSITIVE BY SOUTHERN BLOT
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
● HPV-16 L1 PROBE
▲ HPV-33 PROBE
< 0.9
1-1.9
2-2.9
3-3.9
4-4.9
5-6.9
7-8.9
≥ 9.0
DENSITY OF SLOT BLOT (x 10⁶)

HPV 33 probe than with the L1 probe. In addition, fewer specimens with high density values for HPV 33 were positive by Southern blot than for high density of the L1 probe. This pattern suggests that the HPV 33 probe detected sequences not homologous with the L1 fragment of HPV 16. To analyze the distribution of L1 area results and the HPV 33 results in relation to Southern results, a weighted linear regression was attempted. It was possible to fit a straight line to the L1 area results but attempts to fit a straight line to the HPV 33 curve (refer to figure 15) were unsuccessful, in spite of attempting several transformations of the data. Therefore, the distribution of L1 area results which are Southern positive and the distribution of HPV 33 results which are Southern positive were compared using a Chi-square test. Results indicated that the two distributions were statistically significantly different (Chi square = 10.05, $p < 0.01$). Also the distribution of L1 area results and HPV 33 results which are Southern negative were compared by a chisquare test. Results indicated that the distribution of L1 area results and HPV 33 results which are Southern negative are not significantly different (Chi square = 0.33, $p > 0.1$). This suggests that the two probes are measuring heterologous sequences. HPV 33 therefore, can be considered as an internal specificity control.

The relationship between the distribution of HPV 16 L1 area results and HPV 33 results were quantitated by the correlation coefficient which was 0.612. This represents either the extent of homology between the two sequences (cross reactivity) or concentration of viral DNA sequences in clinical samples. Cross reactivity of HPV 33 represents increased concentrations of HPV 16 DNA.

Sufficient DNA was available from 163 biopsies for testing by PCR and primers were used to amplify sequences in the E6 and E7 open reading frames of HPV 16 (this subsample contained 59 (36.2%) positive by Southern, 85 (52.2%) positive by L1 area probe and 78 (47.9%) positive by HPV 33 probe). The distribution of the results of the PCR assays in relation to Southern blot results are shown in table 1. Using E6 primers, 95 (58.3%) of 163 samples were positive for HPV 16 DNA while 110 (67.5%) were positive for HPV 16 DNA when the E7 primers were used. Of the 112 samples tested sucessfully using the L1 primers with PCR, 79 (70.5%) were positive for HPV16 DNA sequences. This subset of 112 samples had 59 (52.7%) positive by L1 area probe, 47 (42%) positive by HPV 33 probe, 69 (61.6%) positive for the E6 region and had 78 (69.6%) positive for the E7 region which increased to 81 (72.3%) on adding the samples which gave positive signals on Southern blotting. The distribution of L1 results are shown in table 1. The quantities of PCR products detected in relation to Southern blot positivity are shown in figure 14.

The proportion of samples positive by Southern blot increased steadily as the amount of reaction product increased for all three PCR assays as can be seen in figure 14. Since this pattern is similar to the distribution of L1 area probe results in relation to Southern blot results, this suggests that PCR assays using E6, E7 and L1 primers and slot blot method using L1 area probe detect similar sequences. This also indirectly suggests that these sequences detected by PCR assays are different from that detected by HPV 33 probe. This is substantiated by correlation coefficients which quantitate the correlation between PCR results and L1 area results, and the correlation between PCR results and HPV 33 results. The results listed in table 3 suggest a greater correlation between PCR results and L1 area results than between PCR results and HPV 33 results. This also indicates that PCR and L1 area probe are detecting similar sequences, and that these sequences are different from the HPV 33 sequences.

III.6. Statistical analysis of data:

Of the 6 methods used, slot blot using HPV 33 probe measures a different sequence and PCR using L1 primers were done only on 112 specimens. Therefore, the other four methods, Southern blot method, slot blot method using L1 area probe, PCR using E6 primers and PCR using E7 primers were compared in terms of percent positivity, sensitivity

Table 3

	Correlation coefficients	
	L1 area	HPV 33
E6 (PCR)	0.682	0.412
E7 (PCR)	0.626	0.399
L1 (PCR)	0.427	0.082

and specificity of each method in relation to each other.

The percent positivity of slot blot method using L1 area probe was significantly different from that of Southern blot method ($p = 0.005$), thereby suggesting that slot blot method is more sensitive than Southern. The percentage positivity of PCR results by E6 primers and by E7 primers are significantly different from that of Southern blot results ($p = 0.0001$). Although PCR (E7) results were significantly different from L1 area results ($p = 0.0067$), PCR (E6) results was not significantly different ($p = 0.32$). This suggests that PCR using E7 primers pick up significantly more positives than the Southern or slot blot assays.

The direct estimation of sensitivity and specificity of the four methods were computed using a program utilizing the E-M algorithm. The results shown in table 4 indicate that overall, PCR is not a superior method. Although PCR appears more sensitive in that it picks up lower copy numbers of the virus than the Southern blot method, the specificity decreases.

Table 4.

Sensitivity and specificity of different methods

	Southern	L1(Slot blot)	E6(PCR)	E7(PCR)
Number positive	59	85	95	110
Percent positivity	36.2	52.2	58.3	67.5
Sensitivity ($1 - \beta$)	83	95 (91)*	77 (74)*	91 (90)*
Specificity ($1 - \alpha$)	98	79 (74)*	56 (51)*	50 (46)*

* The values in parenthesis indicate estimates of sensitivity and specificity based on Southern blot which is considered the gold standard.

IV. DISCUSSION

Cervical cancer ranks second only to breast cancer as a documented cause of death from cancer in women (Peto et al., 1986). Cervical intraepithelial neoplasia progresses from mild to severe CIN and then to invasive disease over a period of time. There are several risk factors for CIN and invasive cancer, such as socioeconomic variables, sexual behavior,infection with sexually transmitted agents, smoking, diet, reproductive history, and use of hormonal contraceptives. Human genital papillomaviruses are currently regarded as the most likely sexually transmitted agents involved in cervical oncogenesis (Reeves et al., 1989). The fact that there are various known risk factors for cervical cancer suggests a multifactorial etiology.

Human papillomavirus type 16 (HPV 16) has been associated with cervical carcinoma and is believed to be one of several risk factors which possibly has a role in the initiation of the disease. Histological evidence, and the evidence that cervical cancers and cervical cancer cell lines have HPV DNA, and that E6 & E7 ORF's are actively transcribed, lends support to the hypothesis that HPV is an

etiologic factor of cervical cancer. Demonstration of viral DNA sequences in the tumor biopsy material is therefore a good way of establishing an association between the virus and the disease. This is especially true because of the lack of a suitable tissue culture system which supports viral replication.

Several methods of detecting viral DNA sequences such as Southern blot method, slot blot method, filter and tissue in situ hybridization methods have been used to study the prevalence of HPV in cervical cancer cases. HPV 16 DNA has been found in some cancer cases but not in all of them. The reported prevalence of HPV 16 in cervical cancer cases is varied. Whether or not this means that HPV 16 is present in some and not in all cases or whether HPV 16 DNA is present but we are unable to detect it due to the lack of a more sensitive method is not known. With the hypothesis that HPV 16 is present in more cases than can be detected by other methods, and since in theory, in vitro enzymatic amplification of specific sequences could lead to increased sensitivity of DNA detection, we chose to utilize the PCR technology to test the prevalence of HPV type 16 in cervical cancer biopsy material.

Polymerase chain reaction (PCR) involves two oligomers that flank the region to be amplified and are used to amplify specific sequences. After repeated cycles of denaturation, primer annealing and primer extension, it

results in the exponential accumulation of the sequence of interest, defined by the primers. The probe is complementary to the target sequence which is amplified (Saiki et al., 1985) and is not complementary to the primers. Since this method relies on amplification of discrete fragments of DNA, it becomes very easy to detect DNA fragments that are initially present in the sample in picogram quantities. The increased specificity, sensitivity, yield and simplicity of using Taq polymerase makes it suitable for automation (Saiki et al., 1988). The increased specificity of Taq polymerase over Klenow fragment of DNA pol I, is because of the temperature range optimum (65°C-75°C) whereas for Klenow the optimum temperature range is 25°C-37°C. This high temperature eliminates spurious extensions and amplification of only perfect hybrids is favoured. Also, it is likely that high temperature may reduce extent of secondary structure in the template therefore potentially minimizing extent of spurious extensions, thereby further improving sensitivity (Saiki et al., 1988).

The advantages of PCR are that, it requires picogram quantities of DNA, is rapid and easy to perform, the assay in theory is highly sensitive and specific, and with Taq polymerase is suitable for automation. This makes PCR a feasible diagnostic or screening tool.

The increased sensitivity of PCR method is also its disadvantage because of problems related to contamination, which can result in false positives. Therefore, extreme care needs to be exercised in preparation and handling of all reagents and material used in the assay. Cross contamination is a serious problem because of the inherent sensitivity of the assay method. All reagents have to be autoclaved, pipette tips plugged and aseptic conditions maintained.

In order to test the feasibility of using PCR technique to detect low copy numbers of the virus in cervical carcinoma, we used this method to detect HPV 16 sequences in 163 cases of invasive cervical cancer. The sensitivity limit exceeds that of Southern blot hybridization. Therefore, this method has the potential to identify latent viral infections which indicate subclinical viral infection or disease, which might play a role in initiation and progression of cervical cancer.

The prevalence of HPV in cancer of the cervix using other methods could reflect the low sensitivity of the detection systems used, therefore underestimating the HPV prevalence. De Villiers, et al (1987) detected HPV in only 35% of scrapes from women with invasive carcinoma of the cervix and in 3% of scrapes from controls. If HPV is a major risk factor in the pathogenesis of cervical cancer, then it is expected to occur in greater number of cervical

cancer cases. The prevalence rate of HPV in cervical scrapes or cervical biopsies with positive cytology varies from 17% to 80% using other methods (Campion et al., 1986; De Villiers et al., 1987; Henderson et al., 1987; Wagner et al., 1984). Whether or not this means, HPV is involved in initiating the disease and not in progression of the disease is not known and could be the reason for the low prevalence rate of HPV. Besides, not all cytological abnormalities lead to cervical carcinoma. The presence of HPV may indicate progression to cervical cancer, although follow up studies on these cases have not been done.

Melchers, et al (1989) have used PCR, FISH and Southern blot methods to detect different HPV types in cases and controls. They found 70% of cases with abnormal cytology to be positive for HPV DNA, and only 5% of controls were positive for HPV DNA using PCR whereas 46% of cases and 2% of controls were positive for HPV DNA using Southern and FISH. PCR therefore picks up more samples as being positive for HPV, thereby increasing sensitivity of PCR in comparison to other methods. The relatively high occurrence of HPV in the group with cytologically abnormal smears strongly suggests that HPV is an important agent in the pathogenesis of cervical cancer. The oncogenic role of these HPV types should be confirmed in follow-up studies to clarify the role of HPV in cancer progression (Melchers et al., 1989).

Young, et al.(1989) found 95% of cases with cytological abnormality to be positive for HPV 16 or 11 or both, and 70% of controls were positive for HPV 16 or 11 using PCR. This suggests that the prevalence of HPV infection in normal cervical tissue is greater than previously suspected.

Tidy,et al (1989) found 84% of normal cervical samples contained HPV 16 DNA, 67% of dyskaryotic smears and 100% of carcinomas to be positive for HPV DNA using PCR.

Shibata, et al (1988) detected either HPV 16 or 18 in paraffin embedded tissues using the PCR in all 10 cases. Shibata, et al (1988b) also used PCR on formalin fixed, paraffin-embedded tissue in normal and dysplastic cervical tissue. Presence of HPV 16 or 18 in 20 out of 21 dysplastic and 3 out of 10 normal cervical sections was demonstrated. Kiyabu, et al (1989) used PCR to detect HPV in invasive squamous carcinoma from formalin fixed tissue sections. 50% of squamous carcinomas contained HPV related DNA sequences. HPV 16 or 18 were detected in 70% anogenital carcinomas.

Since HPV 16 DNA has been demonstrated in cervical cancer cases, and since there has been evidence suggesting that E6 and E7 open reading frames of HPV 16 are expressed in cervical carcinoma, these two regions are thought to play a role in transformation. Hence, these two regions of HPV 16 were chosen as the target sequences for enzymatic amplification using PCR in our study. HPV type specific

synthetic oligonucleotide primers representing the E6 and the E7 regions were synthesized based on published sequences of HPV 16 (Seedorf et al., 1985). Synthetic oligonucleotide linker - primers modified near their 5' ends to produce convenient restriction sites (linkers) for cloning directly into sequencing vectors were designed for the E7 region of HPV 16 based on Scharf, et al's (1986) paper. One primer had an Eco RI site and another primer had a Bam HI site near the 5' ends. HPV type common primers to amplify L1 sequences of HPV 16 were also chosen, based on published sequences of HPV 16.

The choice of the primers dictated the size of the amplified product for all three regions of HPV 16. The amplification products were 122 bp, 308 bp and 134 bp long for E6, E7 and L1 regions of HPV 16, respectively. The amplified products were detected either by probing with specific oligonucleotide probes (complementary to a region within the amplified product) or by direct visualization by ethidium bromide staining of the gel.

The limits of detection by PCR technique using E6 primers was 1 pg of HPV 16 plasmid DNA and with E7 primers was 0.1 pg of HPV 16 plasmid DNA. The PCR assay was found to be sensitive and specific in that it amplified HPV 16 sequences specifically, and results in selective enrichment of specific viral DNA sequences. The PCR technique was applied to clinical specimens of DNA extracted from biopsy

tissue obtained from invasive cases of cervical carcinoma.

DNA extracted from 163 clinical specimens were analyzed by PCR using oligonucleotide primers specific for E6 and E7 regions of HPV 16. 112 of these specimens were also analyzed using primers specific for the L1 ORF. For reasons described in the methods section under the definition of positivity, a cutoff point had to be established for PCR using E6 primers. All specimens yielding values ≥1.2 was considered positive. For E7 and L1, all specimens yielding values ≥1.0 were considered positive. Also, the specimens which gave off-sized bands with E7 primers, which gave a positive signal on Southern blotting and probing with an E7 specific oligo probe, were also considered positive.

Of the 163 specimens analyzed by PCR using E6 and E7 primers, 95 (58.3%) were positive for the E6 region of HPV 16, and 110 (67.5%) were positive for the E7 region of HPV 16. Of the 112 specimens that were analyzed by PCR using L1 primers, 79 (70.5%) were positive for the L1 region of HPV 16. This subsample had 69 (61.6%) positive for E6, and 81 (72.3%) positive for E7 region of HPV 16.

PCR results were compared with results using other methods of detection of HPV 16 DNA, such as Southern blot and slot blot methods of hybridization. 188 specimens were analyzed by Southern blot method and slot blot method using L1 area probe and HPV 33 probe. Using the definition of

positivity described in the materials and methods, 37.2% were positive by Southern blot method, 50.5% were positive by the L1 area probe while 44.7% were positive by the HPV 33 probe.

The difference in distribution between L1 area results and HPV 33 results in relation to Southern positives (refer to graph 1) was estimated by a chisquare test (Chisquare = 10.05, $p < 0.01$), which indicates that the two distributions are significantly different. The distribution of the L1 area results and the HPV 33 results in relation to Southern negatives was also computed (Chisquare = 0.33, $p > 0.1$), which indicates that the two distributions are not significantly different. This suggests that HPV33 measures a sequence which is heterologous to that measured by the HPV 16 L1 probe. Since HPV 33 is measuring a heterologous sequence and since PCR using L1 primers was performed only on 112 specimens, the other 4 methods were compared.

The group of 163 specimens which were analyzed by Southern blot, slot blot using L1 area probe, PCR using E6 and E7 primers was used to compare the percent positivity, sensitivity and specificity in relation to each other. This group had 59 (36.2%) positive by Southern, 85 (52.2%) positive by L1 area probe, 95 (58.3%) positive by PCR using E6 primers and 110 (67.5%) positive by PCR using E7 primers as can be seen in table 4. This indicates that the percent

positivity of PCR method using E7 primers shows a significant increase over the Southern and Slot blot methods.

The sensitivity and specificity comparing these 4 methods were computed using the E-M algorithm. The results presented in table 4 suggests that, there is a marginal increase in sensitivity of PCR (using E7 primers) over the Southern blot method. Although, the sensitivity of PCR is not significantly different from the slot blot method, there appears to be a significant reduction in specificity. This decrease in specificity could be explained as being due to the increased percent positivity of the PCR assay which results in lesser agreement with other methods. The explanation for this reasoning is that the program utilizing the E-M algorithm takes into consideration, the information based on two or more tests as being the truth. The assumption of independant errors made in this analysis may not be appropriate, because the true state of the attribute being observed is not a dichotomy (as assumed here) but a continuum. If a true continuum has been dichotomised it is likely that the misclassification rates are higher for subjects near the +\- boundary than for more extreme individuals. This could lead to positive correlation of the observer errors, thereby leading to the overestimation of sensitivity and specificity (Walter. 1984).

The advantages of PCR technique are that, it is easy to perform, requires only picogram quantities of DNA, is highly sensitive, can be automated, and therefore seems to be a more feasible diagnostic tool compared to other DNA detection methods in screening the population at large and can be used in epidemiological studies. The disadvantage is that it suffers in specificity compared to other methods of DNA detection. Although the advantages of Southern and slot blot methods are its sensitivity and specificity, their disadvantages are that a large quantity of DNA is required, involves radioactive material, is time consuming and cumbersome which makes it unsuitable for screening large populations.

In conclusion, although the advantages of using PCR as a sensitive diagnostic or screening tool appears attractive, statistical analysis suggests its decreased specificity compared to other methods is a disadvantage. Whether or not this decreased specificity represents decreased agreement with other methods is not known. The assumption of independant errors in the analysis used might not be appropriate as discussed. If it is not appropriate, then the sensitivity and specificity would be overestimated due to the fact that the misclassification rate of the borderline individuals is higher. The role of HPV 16 in cervical cancer might help interpret the data more meaningfully.

VI. REFERENCES

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