Screening and Characterization of Human Papillomavirus (subtypes) in a Specific Pakistani Population

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(2014-2016)

Human genome

Prevalence virus.

بسم الله الرحمن الرحيم

Dated: _____

FINAL APPROVAL

It is certified that we have read the thesis submitted by Huma Iqbal and it is our judgement that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for MS degree in Biotechnology.

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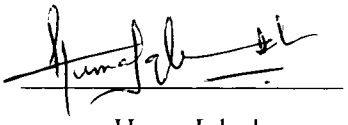
Declaration

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date: _____



Huma Iqbal

Dedicated to

My Parents

Whose Love and Prayers accompanied me forever

And

My Brothers and Sister

For their unwavering support

A thesis submitted to Department of Biotechnology & Bioinformatics,

FBAS,

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as a partial fulfilment of requirement for the award of the degree of MS Biotechnology

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All praises to Allah Almighty who says in Holy Quran, "And your Lord is the most gracious Who taught you by the pen. Taught man (those things) which he did not know". Countless Darood on the Prophet of mercy Hazrat Mohammad and his pious progeny (Sall-Allah-o Alaihay-wa-Aalihi-Wassalam) who showed the path of knowledge to mankind.

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LIST OF ABBREVIATIONS

HPV	Human Papillomavirus
EV	Epidermodysplasia verruciformis
H-R	High Risk
L-R	Low Risk
PCR	Polymerase chain reaction
LI	Late region 1
E	Early region
ORF	Open reading frame
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
IARC	International association for research on cancer
STD	Sexually transmitted disease
OC	Oral contraceptives
IUD	Intra-uterine devices
HIV/AIDS	Human immunodeficiency virus/ Acquired immune deficiency syndrome
BSLII	Biosafety level II
TBE	Tris/Borate/EDTA
PAGE	Polyacrylamide gel electrophoresis

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ABSTRACT

Human papillomaviruses (HPVs) are major pathogens associated with the development of cancer of the uterine cervix which is the most common malignant tumour of women worldwide. Human Papillomaviruses infection is a very common sexually transmittable infection having more than 100 subtypes distinguished by genetic variability. Among the HPV subtypes that infect the anogenital tract, subtypes 16, 18, have been defined as high-risk for cervical carcinoma. Reliable diagnosis of HPV infection, particularly the 'high-risk' types (16/18), may facilitate early identification of 'high-risk' populations for developing cervical cancer and may augment the sensitivity and specificity of primary cervical cancer screening programmes by complementing the conventional Pap test. Thus, baseline information on human papillomavirus (HPV) prevalence and type distribution is highly desirable to evaluate the impact of prophylactic HPV vaccines in the near future. It will encourage scientists to study in detail multi-stage cervical carcinogenesis and take preventive strategies related to HPV.

In the current study, general consensus primers were used to screen 100 samples (*n=100*) obtained from patients attending the outpatient department of Mother and Child Hospital, PIMS and Sheikh Zayed Hospital Rahim Yar Khan were screened for the presence of Human Papillomavirus and its subtypes. All the patients enrolled were married and above 20 years of age. In order to determine the prevalence of HPV in the given population, PCR was performed using consensus primers (F1/R1) and genotyping of each positive sample was determined using restriction fragment length polymorphism (RFLP) technique followed by standard sequencing.

Out of 100 patients (n=100), only 10 (n=10) were found to be positive for Human Papillomavirus, indicating a prevalence of 10% on our general population. Out of these 10

HPV positive patients, 1 (1%) patient was positive for HPV 16, 2 (2%) found to be HPV 18 and 1 (1%) each was HPV 33 and HPV 61. The remaining 5 (5%) population samples contained low risk types HPV. An association of the obtained results with different risk factors associated as also established. These results indicate that Human Papillomavirus is present in the general population patients of our population, thus proper screening methods and tests should be devised for timely detection and severity of infection.

Chapter

Introduction

Cervical carcinoma is considered to be the third most common cancer in women in the world. The prime risk factor in development of cervical cancer is the infection caused by various human papillomavirus (HPV) types (Kjaer *et al.*, 1995; Schiffmen *et al.*, 1993). HPV are a group of more than 150 related viruses. 40 such types can be easily transmitted through direct skin-to-skin contact during vaginal, anal, and oral sex. HPV DNA has been found in more than 99% of cervical cancer biopsies. HPV-16 and -18 account for about 70% of cancers of the cervix, vagina, and anus and for about 30–40% of cancers of the vulva, penis, and Oropharynx (Munoz *et al.*, 2006). Among these, HPV type 16 (HPV-16) is the most common, followed by HPV 18, -45, -31, and -33 (Bosch *et al.*, 1995; Walboomers *et al.*, 1999). In Western countries, HPV vaccines are being widely introduced (Markowitz *et al.*, 2007), while new potential broad-spectrum HPV vaccines are under development (Jagu *et al.*, 2009,). Primary cervical screening using novel strategies based on the HPV DNA assays is highly recommended (Sankaranarayanan *et al.*, 2009, Munoz *et al.*, 2008). HPV infections are the most common infection that is transmitted through sexual contact. In fact, at some point in their lives, a large part of sexually active population is infected with one or more of its types. Recent studies show that 42.5 percent of women suffer from genital HPV infections at some point in their life, while around 7 percent of adults suffer from oral HPV infections (Hariri *et al.*, 2011; Gillison *et al.*, 2012). HPV infections have an association with most genital benign and malignant neoplastic lesions. The human Papillomaviruses are grouped into mucosal and cutaneous types, as well as epidermodysplasia verruciformis (EV)-associated types. Moreover, according to their association with malignant lesions HPV types are also classified as low- and high-risk types (Bosch *et al.*, 1995; Van Ranst *et al.*, 1996).

HPV is so common that more than half of all sexually active adults will be infected in their lifetime, although young, sexually active women bear the brunt of both infection and clinical complications. Though most of these infections are cleared by host immune system, some women will develop eruptive diseases including genital warts, cervical dysplasia, and invasive cervical cancer. The present options for treatment are not curative; and thus,

preventative vaccines for lowering the incidence of HPV infection and its associated diseases may offer a promising substitute to current therapies (Ault, 2006).

The cervical cancer patients normally come with the complains of abnormal vaginal bleeding, spotting, unusually long or heavy periods, or blood-stained discharge from the vagina between periods, bleeding or bloody discharge from the vagina after menopause, bleeding after douching, post coital pain and bleeding, watery discharge from the vagina, increased amount of discharge from the vagina, foul-smelling discharge from the vagina (Devita *et al.*, 2008). The natural history of cervical cancer is thought to be characterized by a well-defined, and what was once believed to be a lengthy, pre-malignant phase. In the cervix, high-risk HPV is one necessary factor in the development of cervical squamous cell carcinoma. Normally, the pre-invasive lesions are asymptomatic; however incidental symptoms may be produced by concomitant symptoms (Souhami 2005). However, further maturation of cervical epithelial cells develops into dysplastic lesions. Lesions containing morphological changes, without involvement of the full thickness of the epithelium by basal-type neoplastic cells and resulting in malignancy (atypia) are referred as dysplasia. Most of these low-grade dysplastic lesions will revert without intervention, while others may expande to cover the full thickness of the cervical epithelium thus becoming high-grade which might further progress through invasive stage to develop into tumor (Pytynia *et al.*, 2014).

1.1 Human Papillomavirus Genome and Proteins

In 1983 scientists first isolated Human Papillomavirus HPV type 16 in cervical cancer cells and within 12 years it was confirmed as a human carcinogen necessary for the development of cervical cancer (Gillison *et al.*, 2014). The Papillomaviruses are a heterogeneous, circular double-stranded DNA viruses having genome size of around 8 kb. These infect humans as well as numerous and diverse animal species. To date, more than 120 types of Human Papillomaviruses (HPVs) have been classified, out of which 84 have been cloned and sequenced completely, partial sequence analysis of a polymerase chain reaction (PCR) product of others have been identified (Terai *et al.*, 2001). To recognize a Papillomavirus into a distinct type, the

complete genome should be cloned and L1 gene sequence should be at least 90% similar to previously typed HPVs (Delius *et al.*, 1998).

All HPVs are circular, non-enveloped, double stranded genome which are approximately 8 kilo base pair in size. Majority of HPV encode eight major proteins, six of these are located in the 'early (E)' region while two in the 'late (L)' region (Table 1). The 'early' proteins are regulatory proteins. They play roles in HPV genome cell cycle, replication and transcription, apoptotic regulation, cell signaling, structural modification of the infected cell and, immune modulation. Majority of these proteins are expressed throughout the infectious cycle with possibly reduced expression at late stages. E4 accompanied by E5 are expressed late in infection (Doorbar *et al.*, 1997). E2 contains an N-terminal domain mediating protein–protein interactions, a C-terminal DNA binding domain and a flexible hinge region. The L1 and L2 proteins subsequently follow the E proteins and are particularly expressed in the granular layer of the epithelium. These comprise the virus capsid which is necessary for transmission of virus, spread and survival in the environment. (Graham *et al.*, 2010).

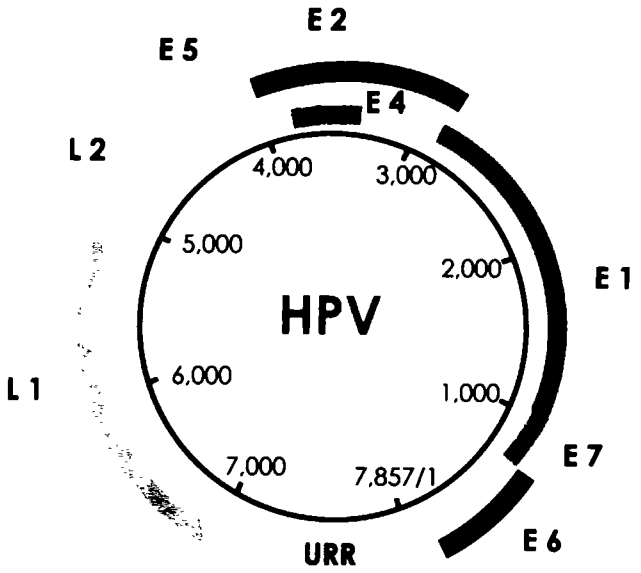


Fig.1.1 Schematic presentation of HPV genome showing the arrangement of the early E and non-structural genes, the capsid genes (L1 and L2) and the upstream regulatory region.

Adapted from: Munoz *et al.*, 2006

Gene Category	Gene Function
E1	Viral replication
E2	Modulation of transcription and replication
E4	Productive viral infection
E5	Transforming properties
E6	Oncoprotein: interaction with p53 protein
E7	Oncoprotein: interaction with pRb protein
L1	Major capsid protein
L2	Major capsid protein

Table 1.1 Human Papillomaviruses gene functions. Adapted from: Anderson 2002

1.1.1 Open reading frame of HPV:

The Papillomavirus genome is divided into an early region (E), encoding six (E1, E2, E4, E5, E6, and E7) open reading frames (ORF) which are immediately expressed after initial infection of a host cell, and a late region (L) that encodes a major capsid protein L1 and a minor capsid protein L2. A single DNA strand encodes all viral ORFs

After the infection of host cell, early promoter of HPV16 is activated and a polycistronic primary RNA containing all six early ORFs is transcribed. This polycistronic RNA contains three exons and two introns and undergoes active RNA splicing to generate multiple isoforms of mRNAs (Zheng *et al.*, 2006).

In some cervical carcinoma cell lines, a minor, non-spliced transcript is detected which encompasses the full-length reading frames of the E6 and E7 proteins (Smotkin *et al.*, 1989, Smotkin *et al.*, 1986). If RNA preparations are contaminated with viral genomic DNA, it can produce an amplified product which is identical to that derived from the full-length transcript. However, the existence of this transcript in cervical premalignant lesions and carcinomas remain controversial (Cornelissen *et al.*, 1990; Falcinelli *et al.*, 1993). Moreover, the close proximity of the E6 protein translation termination codon to the initiation codon for E7 in the full-length transcript makes it unlikely that the E7 protein is efficiently translated (Kozak *et al.*, 1987). (Fig.3)

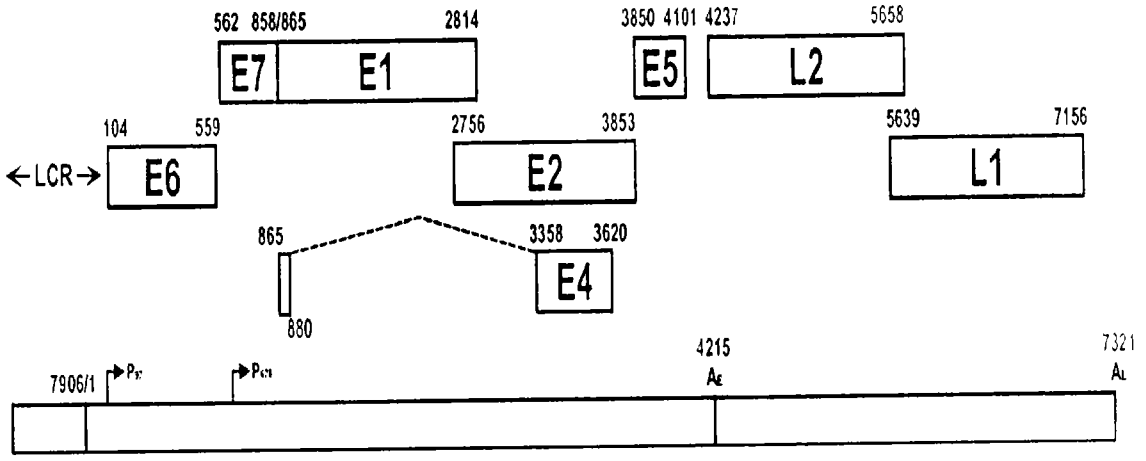


Fig. 1.2. Genome structure and transcription map of HPV-16.

In the middle of the panel, the bracket line represents a linear form of the virus genome presenting a better form of head-to-tail junction, promoters (arrows), and early (AE) and late (AL) polyadenylation sites. The open reading frames (ORFs) (open boxes) are drawn above the bracket. The numbers over each ORF are the nucleotide positions of the first nucleotide of the start codon and last nucleotide of the stop codons in the HPV-16 genome. The E4 ORF spans two exons and formation of the intact E4 ORF requires RNA splicing (dashed lines). LCR indicates a long control region.

1.2 Transmission and Integration

HPV type, anatomical location, and the nature and timing of local cellular and tissue influences defines the outcomes and progress of HPV infection (Chang *et al.*, 1995; Stoler *et al.*, 2000). Virions enter basal and para basal cells in areas of erosion whereas viral DNA in the cell nuclei. The viral infection can become widely dispersed when extensive tissue repair is going on. Viral persistence in keratinocytes is capricious and depends on its different types (Ho *et al* 1995). Finally, integration of viral DNA may take place which can result in lifelong persistence of some viral genes in the cell. In the cervix, low risk HPV types' infection is of relatively short duration, whereas infection by highest risk types remains for longer time period. At times, these infections may become persistent and lasts years or even decades; increasing the risk of cervical cancer. (Bosch *et al.*, 2002). The infection can become widely dispersed and persistent when HPV enters immature, actively dividing metaplastic basal stem cells. On contrary, if HPV infect only in the para basal transit amplifying cells the infection may become transient or quasi-persistent.

A primary event in carcinogenesis of cervix uteri involves viral E6 and E7 genes deregulated activity, which target, p53 and pRB, respectively along with others. The E7 gene deregulated activity in proliferating cells resultantly increases the expression of p16INK4a (Gillison *et al.*, 2014) which is a feature of cervical carcinomas and their true precursor lesions. At first, HPV replicates to reach about 25–50 genomes/cell (Steenbergen *et al.*, 1998: Cheng *et al.*, 1995). Four multifunctional viral proteins E1, E2, E6, and E7 activities are involved in the process. One important activity of E7 is that it overcomes the pRB tumor suppressor block. E2F transcription factors is liberated as a result of binding of E7 to pRB and its related members', which play key roles in promoting host cell and viral DNA synthesis. Moreover E7 binds to and resultantly activates cyclin complexes, like p33–cyclin dependent kinase 2, controlling progression through the cell cycle. The p53 protective control pathways can be overcome by E6 protein (Werness *et al.*, 1990), which prevents the genetic damage that may possibly lead to carcinogenesis. HPV genome uses E2 protein to attach to host chromatin via and replicate at a steady state (Chow and Broker, 1994; Dollard *et al.*, 1992, Flores and Lambart, 1997).

This tethered theta mode of replication is considered to be beneficial as it minimizes the loss of HPV DNA from cells by non-disjunction and the presence of low amounts of HPV DNA in cells have less chances of detection by intracellular interference mechanisms which might trigger apoptosis. While the differentiation of cells and their movement to surface, a normal differentiation and maturation process leads to pyknotic condensed cells that swamp from the tissue. In virally infected tissues, however, unscheduled DNA replication is activated in some spinous cells, along with a switch in viral DNA replication to the rolling circle mode, leading to the production of viral progeny (Cheng *et al.*, 1995). This DNA synthesis reactivation can be detected by the HPV virions presence in upper layer subset of cells as well as the presence of punctate proliferating cell nuclear antigen tissue staining (a protein with a key role in DNA replication).

E6*I which is one of the spliced isoform RNAs, serves as an E7 mRNA and translate E7 oncoprotein. Contrarily, for translation of E6 oncoprotein an intron that remains intact without splicing in the E6 ORF is necessary (Tang *et al.*, 2006). However, viral E2 regulation is triggered by viral early transcription and high levels of E2 repress the transcription. The disruption of E2 ORF causes HPV genomes to integrate into host genome by preventing E2 repression on E6 and E7. This way, viral genome integration into host DNA genome increasing E6 and E7 expression and promote cellular proliferation and the chance of malignancy.

1.2.1 Integration into the host chromosome

Cancer is a multigenic disease and is mostly somatic (Kinzler and Vogelstein, 1996, 1998; Lengauer et al, 1998). The genes related to cancer can fall in two major categories: oncogenes that have a dominant effect and tumor suppressor genes with a recessive phenotype. Majority of tumour types are a result of recurrent chromosomal alterations that act as markers for the presence of an oncogenesis-related gene (Rabbitts, 1994, 1997).

Table. 1.2 Recent Lack of Homologies in cervical carcinomas

Adapted: PA Lazo, 1999

Chromosome region	No. of cases (positive/informative)	%	References
3p14.1–p22	166/391	48	Yokota et al, 1989, Jones and Nakamura, 1992 Kohno et al, 1993, Karlsen et al, 1994, Mitra et al, 1994a, Mullokandov et al, 1996 Ku et al, 1997 Larson et al 1997 Chu et al, 1998 Huettner et al, 1998 Kersemaekers et al, 1998b
4p16	27/66	40	Hampton et al 1996 Mullokandov et al 1996 Kersemaeke s et a 1998/
4q21–35	23/72	32	Mitra et al, 1994a, Mullokandov et al 1996 Kersemaekers et al 1998b
5p13–15	20/117	17	Ku et al, 1977 Mitra et al 1994a Mullokandov et al 1996 Kersemaekers et al 1998b
6p21.3–22	60/147	41	Mitra et al, 1994a, Mullokandov et al 1996 Huettner et al 1998 Kersemaekers et al 1998b
11p15	12/43	28	Mitra et al, 1994a Mullokandov et al 1996
11q	7/		Mullokandov et al, 1996 Huettner et al 1998 Kersemaekers et al 1998a 1998b
17p13.3	50/208	24	Fujita et al, 1992 Kaelbing et al 1992 Mitra et al, 1994a, Park et al, 1995 Mullokandov et al 1996 K et al Kersemaekers et al 1998
18q12.2–22	15/75	24	Mitra et al, 1994a Mullokandov et al, 1996 Kersemaekers et al 1998

1.3 Worldwide Human Papillomavirus Prevalence

Epidemiologic studies have shown that the association of genital Human Papillomaviruses (HPV) with cervical cancer is strong, independent of other risk factors, and consistent in several countries. HPV DNA was detected in 93% of the tumors, with no significant variation in HPV positivity among countries. (Bosch et al, 1995). In two different meta-analysis performed between 1995 to 2009, the global HPV prevalence has been estimated between 10-12%. The corresponding estimates by region were 20.9-23.4% in Africa, 11.3% in Northern America 16.1% in Latin America while 20.4% in Central America and Mexico. In different regions of Europe HPV prevalence lies between 8-21%, varying with age groups. A strikingly low prevalence percentage has been found in population of Asia where it ranges from 7.5-8.4 % (Bruni *et al.*, 2010; de Sanjose *et al.*, 2007).

Presently, 118 HPV genotypes have been classified on the basis of their biological oncogenic potential, niche and phylogenetic position (de Villiers *et al.*, 2004). The cutaneous (skin) and mucosal (anogenital and oral) types of HPV are distinguished and are defined as high-risk and low-risk (LR) genotypes, based on their association with cervical neoplasia or associated lesions. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 are considered oncogenic or high-risk types, with types 26, 53, and 66 being probably oncogenic (Munoz *et al.*, 2003). Type-specific HPV data, among the women of different age groups showed 5 most common types worldwide. These were HPV-16 (3.2%), HPV-18 (1.4%), HPV-52 (0.9%), HPV-31 (0.8%), and HPV-58 (0.7%) (Bruni et al, 2010). Types 16 and 18 which are also the vaccine targeted types are most frequently found worldwide. HPV-16 is the most common type everywhere while HPV-18 and other high-risk types, such as types 52, 31, 58, 39, 56, and 51 are among the most common HPV types after HPV-16 and share similar prevalence. HPV-31 is found to be very common in Europe and Latin America but in Northern America or Asia, it is surpassed by HPV-52. However, HPV-18 is top-ranked in most regions. (Bruni *et al.*,). The most prevalent genotypes in East Asia were HPV 16 (23.9%), 18 (11.0%), 58 (9.4%), 56 (6.3%) and 52 (5.3%), while in South-east Asia, they were HPV 52 (12.9%), 16 (8.5%), 58 (5.2%), 18 (5.0%) and 66 (4.9%). HPV-31, 33 and 35 were less common in both sub-regions (Peng et al, 2012).

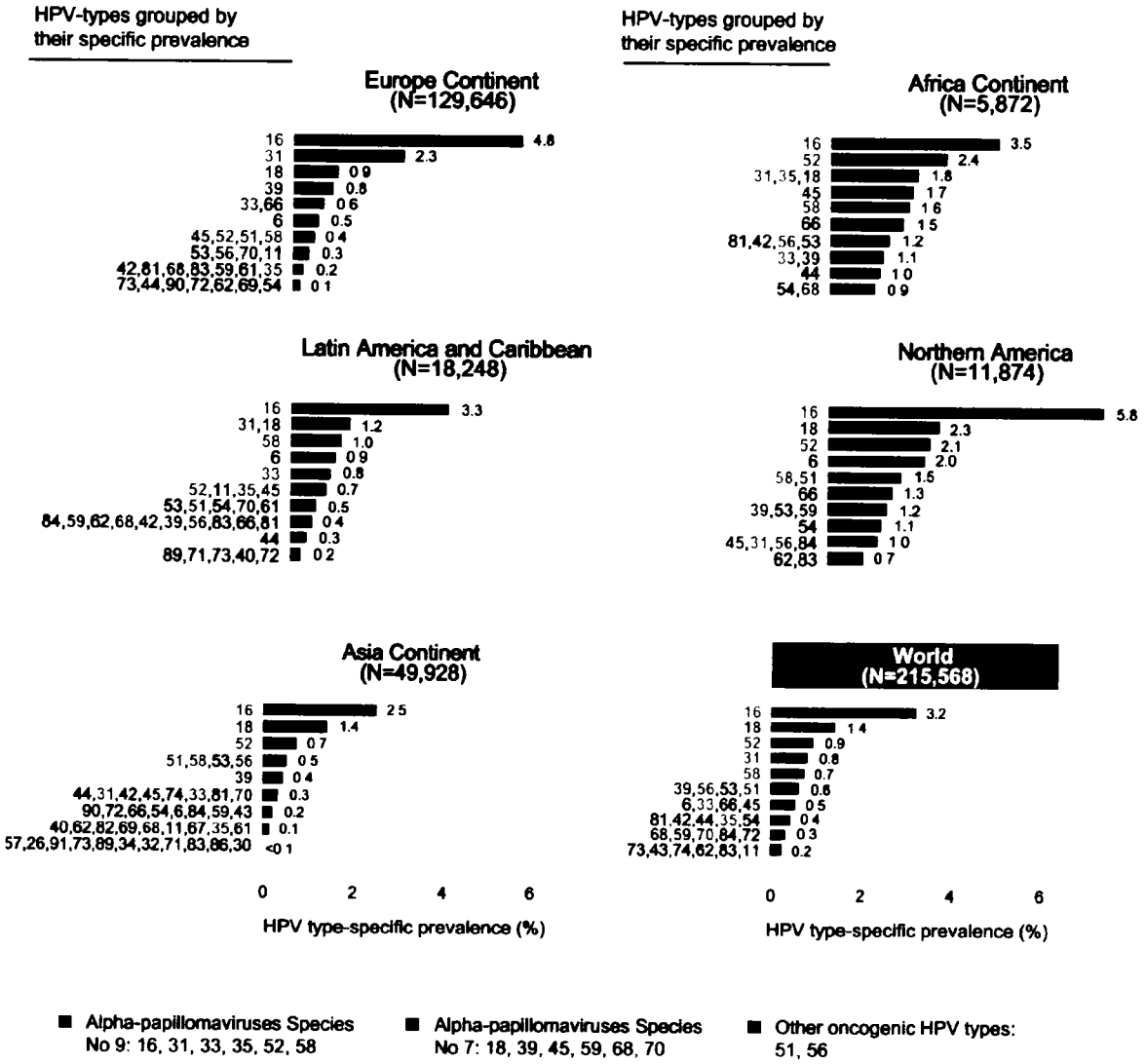


Fig. 1.3 Geographical distribution of different Human Papillomavirus genotypes.

Adapted from: Bruni *et al.*, 2010

1.4 Risk Factors

Cervical cancer can also be caused by factors other than HPV. These risk factors are briefly discussed as under:

1.4.1 Parity

In the IARC multicentre study, the risk of cervical cancer increased up to four folds in patients who had seven or more full term pregnancies as compared to the women who were HPV positive but were nulliparous. When HPV positive women reporting seven or more pregnancies were compared with women who reported one to two full term pregnancies, it still showed a twofold increased risk (Bosch *et al.*, 2002). In cohort studies conducted in the countries like Manchester and Denmark- the states with low parity, showed less visible effects of cancer (Deacon *et al.*, 2000; Kjaer *et al.*, 1988).

1.4.2 Age

A strong determinant of the diagnosis of a carcinogenic viral infection in relation to cancer progression is shown to be the age at exposure. The risk of cervical cancer is related to age at first sexual intercourse. More recent studies that included HPV measurements have shown age at first HPV exposure is actually a measure of age at first intercourse. It has been anticipated that the developing cervix (at the age of peri-menarche) or the healing cervix (as a result of cervical trauma, deliveries, or any other STD infection) are high risk situations when HPV infection may reach the basal layer and set up as a persistent infection (Bosch *et al.*, 2002)

1.4.3 Smoking

There has been evidence since 1970's that tobacco is a causative agent of numerous cancer types (Winkelstein *et al.*, 1977). Personal cigarette smoking was found to increase the risk of cervical cancer however; the study showed that the women who smoked were at a lower risk of infection as compared to women who were passive smokers (Martha *et al.*, 1989).

1.4.4 Oral Contraceptives

The relationship between use of oral contraceptive (OC) and cervical cancer is complicated by possible confusions with sexual behavior (Zondervan *et al.*, 1996). Use of OCs for less than five years was not related to cervical cancer. The risk increased for five to nine years of use and for more than 10 years. The results of this analytical study indicates that the using OCs for five or more years is a cofactor that increases up to fourfold the risk of cervical cancer among women who are carriers of HPV DNA (Moreno *et al.*, 2002).

1.4.5 Other factors

A current meta-analysis showed that women who have HIV/AIDS have a six-fold increased risk of cervical cancer and women who have under-gone organ transplant have more than double the risk of developing cervical cancer, strongly indicating that immune-suppression plays a role (Grulich *et al.*, 2007). The International Agency for Research on Cancer (IARC) states that HIV is a causal agent of cervical cancer (Cogliano *et al.*, 2011).

1.5 Diagnosis

1.5.1 Assays for the detection and typing of HPVs

HPV cannot be propagated in tissue culture; in most cases its accurate identification relies on molecular biology techniques. Hybridization procedures such as Southern and Northern blots, dot blots, in-situ hybridization, signal-amplification molecular technology (Hybrid Capture assay, version hc2; Digene, Gaithersburg, MD, USA), and DNA sequencing are used for direct detection of HPV genome as well as for achieving its trancripts (Iftner and Villa, 2003). mass screening of HPV is performed using a group specific HPV test, such as the Amplicor HPV test or HC2 which identifies the infection with one or more of 13 high-risk types but cannot provide a type-specific result (Stefanie *et al.*, 2008). DNA sequencing of the viral genome is the only procedure possibly capable of recognizing all HPV types and variants present in a biologic specimen. However, at present this methology is labor intensive and requires expensive

equipment. Furthermore, further development is required in direct sequencing of specimens containing multiple HPV types (Villa and Denny, 2006).

1.5.2 PCR-based assays for HPV detection and typing

Theoretically, PCR can produce one billion copies 30 amplification cycles, from a single double-stranded DNA molecule. The most widely used PCR-based protocols utilize consensus primers directed to a highly conserved L1 gene region of the DNA, and are potentially capable of detecting all mucosal HPV types (Bernard *et al.*, 1994). A number of primers are used as consensus primers including the single pair of consensus primers the MY09/11 degenerate primers [Manos *et al.*, 1989], GP5þ/6þ (Jacobs *et al.*, 1997) and their multi-primer derivatives, the PGMY09/11 primers (Gravitt *et al.*, 2000). Additionally, in order to amplify highly conserved regions of the L1 or E1 gene a number of consensus primers have been developed which are followed by cycle sequencing for type identification (E1-PCR). The latter method has considerable limitations for detecting multiple HPV types in a single sample.

Both the choice of adequate high-risk types for a screening test for cervical cancer and for the development and production of a prophylactic vaccine are dependent on the accessibility to large-sized epidemiological studies investigating the risk for cervical cancer associated with various HPV types.

1.6 Objectives:

The aim of this study is:

- Screening of Human Papillomavirus to assess its prevalence in the specific non-cancerous population under study.
- Genotyping of HPV in the positive samples using molecular techniques technique.
- To evaluate the risk factors associated with Human Papillomavirus in the HPV positive population.

Chapter

Materials and Methods

2.1 Patient selection

This study was approved by the Board of Ethical Review Committee, International Islamic University, Islamabad. In this study 100 samples were selected from the female patients who came with general gynecological problems and underwent per speculum observation. Samples were taken from married women, with informed consent with the patient and a questionnaire was administered.

2.2 Sample collection

The samples were collected from patients attending the outpatient department of Pakistan Institute of Medical Sciences (PIMS), Islamabad and Sheikh Zayed Hospital, Rahim Yar Khan, from March 2014 till Jan 2016. Cervical scrap was taken using Cell Path cytobrushes. The spatula and the rest of the scraped cell materials were transferred to a 2 ml collection vial containing 1 ml of normal saline on ice and then stored at -86°C.

2.3 DNA extraction

The whole process of DNA extraction was carried out in a controlled environment of Biosafety Level II cabinet (BSLII) at the Molecular Diagnostic Labs, NORI to avoid contamination of samples and to minimize the exposure laboratory personals and the laboratory environment to viral DNA. The proteinase K method for DNA was used with slight modifications as per requirements:

1. The samples were thawed to bring them to room temperature.
2. Then they were vigorously stirred and centrifuged at 4000 rpm for 10 minutes at room temperature. The supernatant was discarded and pellet was obtained. The cell pallet was re-suspended in 500 µl of detergent buffer (***Appendix I***) and 100µg/ml Proteinase K (Steinke *et al.*, 1995; Shikova *et al.*, 2009).

3. The sample was vortexed to dissolve the pellet completely and incubated for 2 h at 56°C in a water bath for lysis and later at 95oC for 10 minutes to deactivate proteinase K.
4. An equal volume of phenol (equilibrated with Tris, pH 8) was then added in the eppendorfand mixed well for 1 min by inverting several times.
5. Then the sample was centrifuged at 8000 rpm for 10 min. (temp: 4°C).
6. The upper aqueous layer was shifted to a new tube and an equal volume of (1:1) of phenol and chloroform: isoamyl alcohol (24:1) was added. The sample was mixed several times by inverting the tube and centrifuged at 8000 rpm for 10 minutes.
7. Supernatant was collected in a fresh tube and double volume of absolute alcohol (Merck) was added. The tube was gently inverted several times and chilled at −20°C, followed by centrifugation at 8000r pm for 20 min (4oC).
8. Supernatent was discarded and the pellet was washed by 250ul of 70% ethanol and centrifuged for 1 minute at 8000 rpm.
9. The pellet was air dried and eluted in 55ul Elution buffer (thermovycler) and stored at -21oC.

2.4 Polymerase Chain Reaction Amplification

General PCR primers can be used to amplify a broad spectrum of HPV genotypes (Hildesheim *et al.*, 1994). L1 degenerate primers F1/R1 targeting a 450-bp region in the L1 open reading frame of HPVs were used in single amplification reactions of 50 ml volume (Randolph *et al.*, 2000).

Each reaction mixture tube contained

- 50ul template DNA,
- 0.25µl Taq DNA polymerase,
- 2.0mM dNTPs (dATP, dCTP, dGTP, dTTP),
- 1X Taq DNA polymerase buffer (Mg2+ plus) and
- 20µM primer.

Table. 2.1 Sequence of consensus primers. F1/R1 (Shikova *et al.*, 2009)

Primers	Sequence
F1	CGTCC(AC)A(AG)(AG)GGA(T)ACTGATC
R1	GC(AC)CAGGG(AT)CATAA(CT)AATGG

Table. 2.2 PCR cycling parameters. (F1/R1 Primer set)

Steps	Temperature °C	Time
Denaturation	95	1 min
Annealing	55	1 min
Elongation	72	1 min
Final extension	72	10min

2.5 Agarose Gel Electrophoresis

2.5.1 Qualitative Analysis

The quality of the genomic DNA was examined by gel electrophoresis using 0.7 % agarose gel. 2µl of each DNA sample was mixed with 1µl of 1x DNA loading dye (*Appendix II*) and was loaded in the gel. Electric current was applied at 20 volts until DNA enters into the gel and was raised to 50 volts for rest of the run. Electrophoresis was stopped when the dye had travelled nearly 2/3rd of the gel. Gel was visualized by a Gel doc system (BioRad) under UV light and picture was captured by using BioRad Gel Doc system.

2.6 Restriction Fragment Length Polymorphism (RFLP)

Cervical scrape DNA samples that were positive for HPV DNA by F1/R1 were further subjected to restriction fragment length polymorphism (RFLP) to determine different subtypes of HPV present. PCR product amplified using F1/R1 primers were digested in NlaIII (CATG/NEB) enzyme. In a 20-µl mixture, 6 µl of F1/R1-derived PCR products, 2 µl of 10_ NE Buffer 4, 2 µg of bovine serum albumin (BSA), and 1 U of NlaIII were mixed, and the solution was incubated at 37°C for 16 hr. Theoretical restriction pattern for each high risk HPV sub-type was found using NEB Cutter Tool V2.0.

2.7 Polyacrylamide Gel Electrophoresis (PAGE)

The digested products (10 ml) were loaded onto an 8% polyacrylamide gel (*Appendix III*) in 1x TBE buffer prepared by diluting 10x TBE buffer in 1:9 ratios (*Appendix IV*). After electrophoresis, the gel was stained with EtBr (0.5 mg/ml), and the fragments were visualized using a Bio-Rad Gel Doc system. DNA sequences of HPV L1 genes were obtained from the GenBank Database. Theoretical digestion patterns were obtained using the NEB cutter V2.0 tool.

2.8 Sequencing

2.8.1 Elution of DNA from the Gel for DNA sequencing

Gene Jet Gel Extraction Kit was used for extraction of amplified product from the gel, following the manufacturer's protocol. First, the PCR amplified product was run by 2% agarose gel and the bands were visualized under UV light of gel doc system. A sharp blade was used to excise the DNA bands from the gel. The gel slice was transferred into pre-weighted Eppendorf tube and again the weighed along with the gel slice and the weight difference was determined. Equal volume (100ul of Binding Buffer for every 100 mg of agarose gel) was added to the tube containing gel slice. For 10 minutes, the tube was incubated at 60oC. During incubation the tube was continuously inverted to facilitate the melting process of the gel. When the gel completely dissolved,100ul of 100% isopropanol was added to every 200ul solution mixture and mixed thoroughly. In the next step the sample was transferred to the Gene Jet purification column and centrifuged for the 1-2 minute at 13,000 rpm. The mixture passed through the column was discarded and the column was placed back. The previous set was repeated by adding 700ul of Wash Buffer diluted with Ethanol to Gene Jet purification column. To completely remove the Wash Buffer residue, the empty column was centrifuged for extra one minute. The Gene Jet column was then transferred into a recovery tube and then 50ul of pre-thawed (60°C) of Elution Buffer was applied to the center of purification column membrane and incubated for 3 minutes, then centrifuged at 14000 rpm. The purified sample was used for sequencing.

2.8.2 Phylogenetic analysis of sequencing results

ABI automated sequencer was used to carry out the sequencing of the selected samples. Then the Chromas software version 2.0 was used to view the nucleotide sequences and the resulting sequence were compared with the similar sequencings in the NCBI Gene Bank. CCL software was used to draw the phylogenetic tree.

2.9 Statistical analysis

The associations between the different demographic factors and the HPV infection were assessed for statistical significance using frequency and percentages and plotting them on bar charts and pie charts. Mean values and standard deviations for different clinical parameters among HPV positive patients were also evaluated. All analysis was performed using the statistical package SPSS 24.

Chapter

Results

This study was conducted over 100 numbers of samples collected from the patients coming in the General Outdoor Patient Department of gynaecology in Pakistan Institute of Medical Sciences (PIMS) Islamabad, Shaikh Zayed Hospital Rahim Yar Khan. The patients selected were of different age groups, marital status, ethnical groups and parity. In this section, results are presented as cross tabulation tables to illustrate the number and frequencies of the subjects according to different factors and characteristics along with the means and standard deviations. Results are analysed with the help of percentage, mean, standard deviation through pie charts, bar graphs and tables.

3.1 Screening of HPV DNA

The presence of HPV infection in cervical samples was detected by amplification of HPV DNA by PCR using F1/R1 primer set (Table 2.2) used to amplify 450 base pair fragment of viral DNA. The reaction conditions and PCR program used for amplification is described in methodology section in detail. The amplified product was analysed by running 2% agarose gel electrophoresis. The gel picture of the amplified product showed band of size approximately 450bp, depicting the sample is positive for HPV (Fig. 3.1). In a total of 100 samples that were tested for Human Papillomavirus, 10 samples showed bands after PCR amplification. Thus, prevalence of HPV in the selected population was found to be 10/100 (10%).

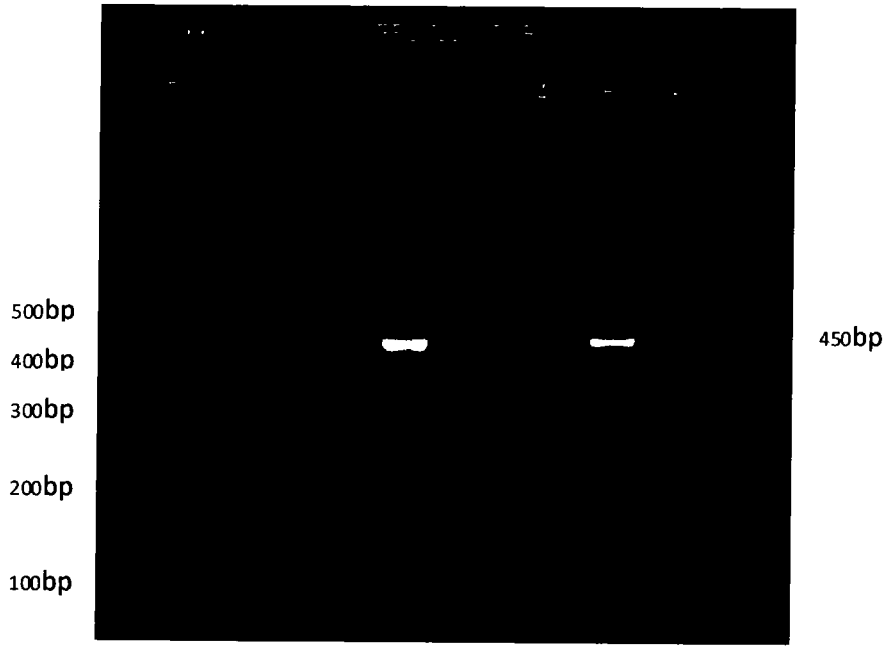
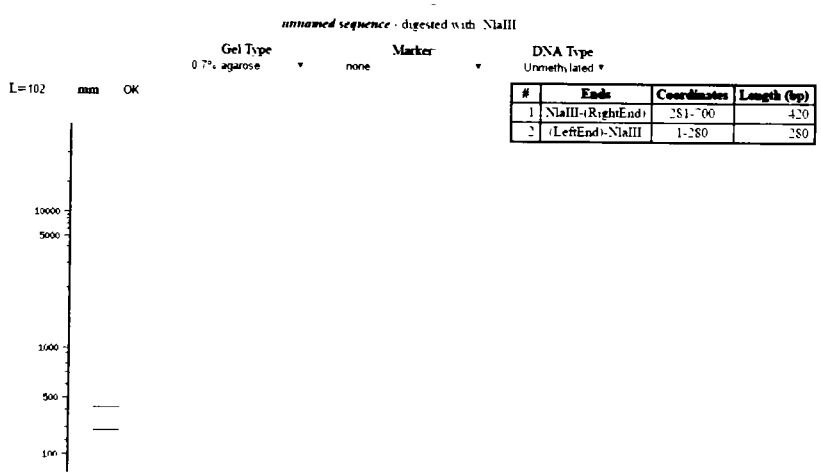


Figure 3.1 Amplified product of F1/R1: Lane 1 shows negative control (NC). Lane 2-6 shows positive HPV samples with band size 450 bp. Lane 7 was loaded with a positive control. Lane M shows ladder of 100bp.

3.2 Restriction Fragment Length Polymorphism (RFLP) Analysis for Detection of HPV Genotypes

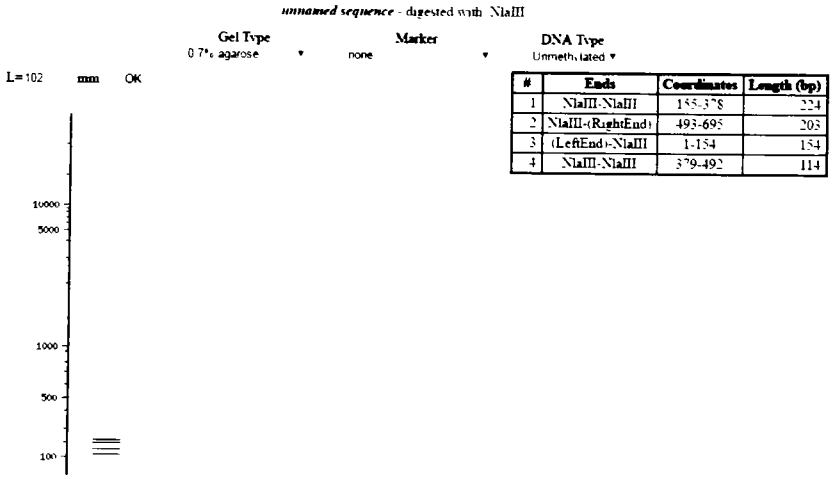
To distinguish HPV genotypes into high risk and low risk amplified product was digested with *NlaIII* enzyme. The theoretical restriction pattern of each genotype were found out using NEB cutter which shows different band sizes for high risk HPV type 16, 18 and 33 (Fig. 3.2, 3.3, 3.4 respectively). Restriction pattern of HPV genotype was analysed on polyacrylamide gel electrophoresis (PAGE). PAGE showed bands of different sizes (Figure 3.5).

Out of 10 HPV positive samples, 1/100 (1%) were found to be high risk HPV 16. 2/100 (2%) were high risk HPV 18. 1/100 (1%) was HPV type 33 HPV type 61. The remaining 5 out of 100 (5%) were low risk HPV subtypes. Thus the prevalence of high risk HPV subtypes in our specified population were 1% HPV 16, 2% HPV 18, 1% HPV 33 and 61 respectively according to present study. The results are significant and have p value equal to 0.347 which is less than 0.5.



#	Ends	Coordinates	Length (bp)
1	NlaIII-(RightEnd)	281-700	420
2	(LeftEnd)-NlaIII	1-280	280

Figure 3.2. NEB Cutter tool gel image for HPV type 16



#	Ends	Coordinates	Length (bp)
1	NlaIII-NlaIII	155-378	224
2	NlaIII-(RightEnd)	493-695	203
3	(LeftEnd)-NlaIII	1-154	154
4	NlaIII-NlaIII	379-492	114

Figure 3.3. NEB Cutter tool gel image for HPV type 18

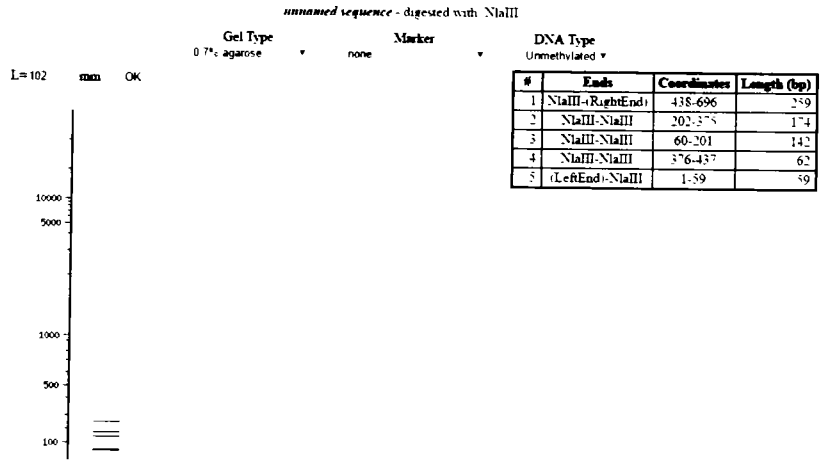


Figure 3.4. NEB Cutter tool gel image for HPV type 45

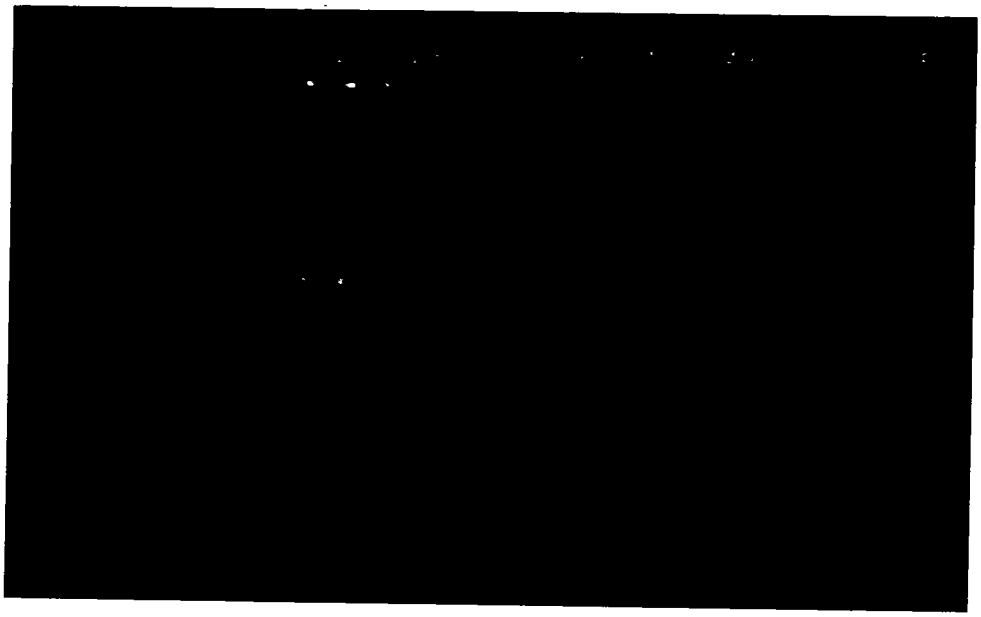


Figure 3.5. Restriction fragments digested using NlaIII enzyme:

Lane 1, 2, 3, 4 and NC show the uncut PCR products of 450bp, Lane 1R shows the restricted product at around 224, 203, 154 and 114 bp showing high risk HPV type 18.

Lane 2R show restricted product at 259, 174 and 143 showing high risk HPV type 45.

Lane 3R show restriction product of 420 and 280 bp showing high risk HPV type 16.

Lane 4R shows restriction product of 239 and 171bp that shows low risk HPV type 61.

Table. 3.1. Physical and Clinical Parameters

Variables	Means values with Standard Deviations (SD)
Age	44.20±18.36
Number of years married for	20.85±14.0792
No. of pregnancies completed	4.60±2.22
No. of abortions	1.50±1.90

Table. 3.2. Some other physical and clinical Parameters noted

Variables	Findings	Calculated Percentages
Vaginal discharge	Yes	80%
	No	20%
Dyspareunia	Yes	50%
	No	50%
Post-coital bleeding	Yes	30%
	No	70%
Fever	Yes	20%
	No	80%
Methods of Contraception used	Intrauterine Devices (IUD)	20%
	Oral means	30%
	None	50%

3.3 STATISTICAL ANALYSIS

3.3.1 Distribution of HPV According to Age Groups.

Patients belonging to different age groups were taken. They were all married and older than 10 years of age. Out of the 10 HPV positive patients, 6 (60%) lied between the age group of 21-35 years, 3 (30%) lied between the age group of 36-50 years while 1 (10%) were older than 50 years. The highest percentage of infection was found in females between the age of 21-35 year (Fig. 3.7). The mean age was found to be 44.20 with 18.36 standard deviation (Table. 3.1).

3.3.2. Distribution of HPV in Females According to Number of Years They Are Married for

Among the total 10 HPV positive patients, 3 (27%) were married for 1.5 to 10 years, 4 (37%) were married for 11-20 years, 2 (18%) for 21-30 years while 1 out of 10 each (9%) were married lie in groups married from 31-40 years and 41-50 years (Fig. 3.8).

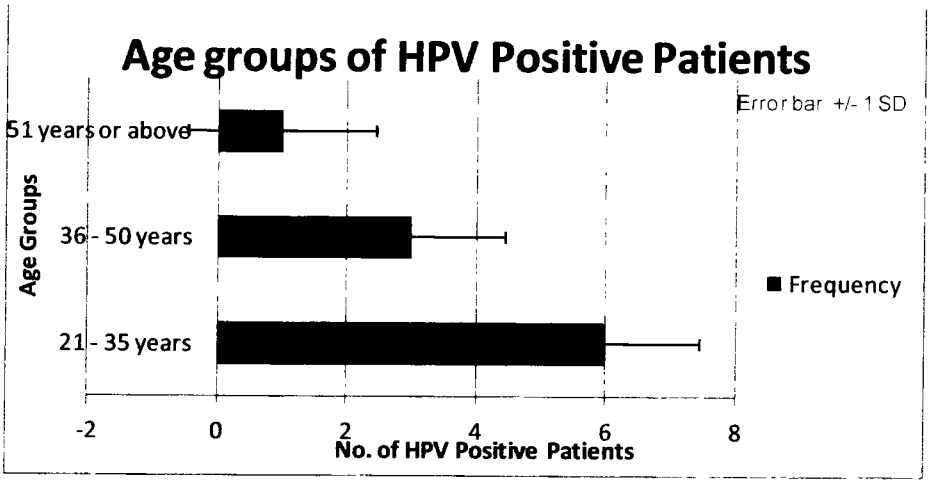


Fig 3.6. Comparison between Age Groups of HPV Positive Population.

HPV was highest in young women who lie between 21-35 years of age. Mean age = 44.20 ± 18.36 SD.

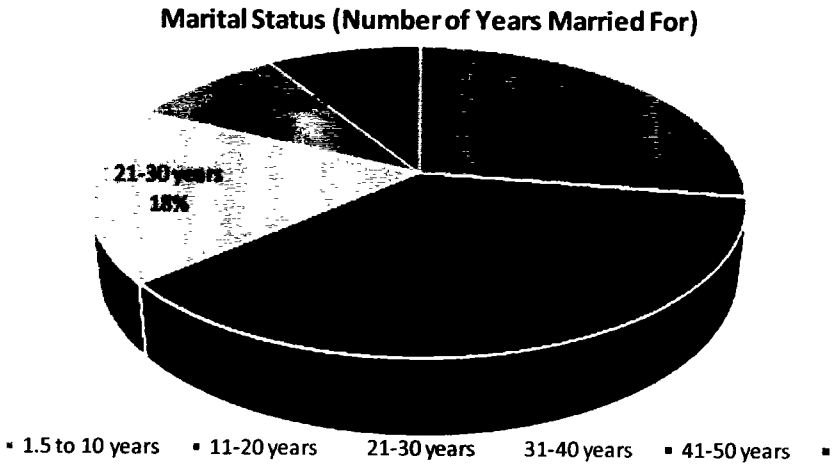


Fig. 3.7 Marital Status (number of years HPV positive patients are married for).

The highest incidence of HPV was found in women married for last 20 years or less. It has been suggested that such women are sexually active and thus have higher chances of acquiring virus from their partner. Mean = 20.85 ±18.07 SD

3.3.3. Distribution of HPV in Females According to Number of Full Time Pregnancies (Parity)

Mean value for number of pregnancies that HPV positive patients completed was found to be 4.60 with a standard deviation of 2.22 (Table 3.1). Out of 10 HPV positive patients, 1(10%) patients were those who had completed 2 full term pregnancy, 2 (20.0%) patients had undergone 3 full term pregnancies, 4 (40%) patients completed 6 full time pregnancies while 3(30%) patients completed 8 full term pregnancies each. The trend followed by the given results is given in (Fig.3.9).

3.3.4. Distribution of HPV in Females According to Methods of Contraception

Out of 10 positive patients, 5 had never used any means for contraception, while 2(30%) used oral contraceptives and 3 (30%) were using intra-uterine devices (fig.12).

The highest incidence of HPV was found among patients using Intra-uterine devices as a method for contraception followed by those who used oral contraception. The prevalence of HPV is relatively low in patients who were not using any contraceptives.

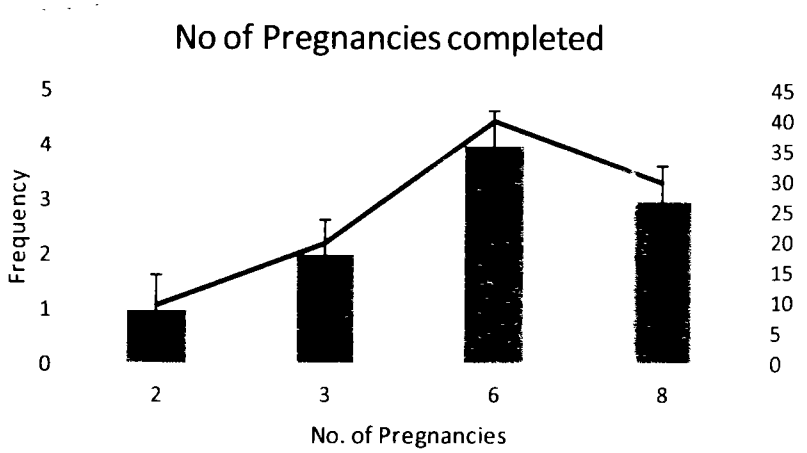


Figure 3.8. Bar chart showing the number of pregnancies each HPV positive woman completed.

Mean = 4.60 ± 2.22 SD

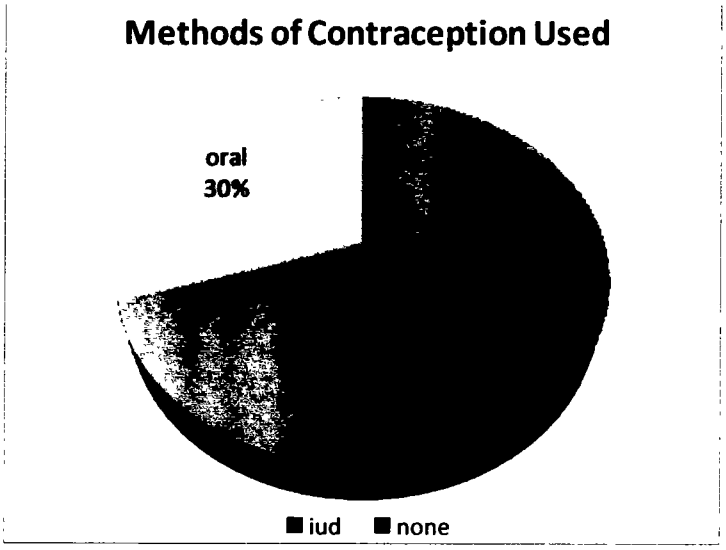


Fig 3.9 Comparison between HPV positive females using different contraception methods.

The highest incidence of HPV was found among females who were not using any means of contraception, followed by the female group who were using intra-uterine devices as a method for contraception. The females using oral contraception had the lowest prevalence of Human Papillomavirus.

3.4 Elution of DNA from the Gel for DNA sequencing

The amplified product was eluted from the gel by using Gene JET^{TM} Gel Extraction Kit, according to the manufacturer's protocol. The amplified product was run on 2% agarose gel and the required band pattern was obtained. The bands were excised from the gel by using a sharp blade and the DNA was eluted from the gel by using Gene JET^{TM} Gel Extraction Kit. After elusion 5µl of eluted DNA was again analysed by 2% agarose gel (Figure 3.6). The sequences results were compared with similar sequences from different regions of the world as submitted in NCBI. A phylogenetic map was drawn using CLCC software to further confirm the sequence analogy with similar sequences (Fig. 3.11)

3.5 Phylogenetic Analysis:

Four different HPV gene sequences of HPV are reported in this study. Fifteen sequence of HPV genotype 16, 18, 45, 11 and 33 were obtained from NCBI. All these were used for construction of phylogenetic tree using CLCC software as shown in figure 3.11. All sequence is grouped into five clusters. Phylogenetic analysis of HPV sequences revealed that NORI 18 sequence was linked with other four sequences of HPV 18 reported in different regions of the world and lie in cluster 1. Similarly, HPV 16 was linked in the cluster with HPV sequences from different regions of the world, they all lie in cluster 2. 2 sequences of NORI HPV 45 have shown close association and found in separate cluster 3. Sequence of HPV 33 and HPV 6 were closely related.

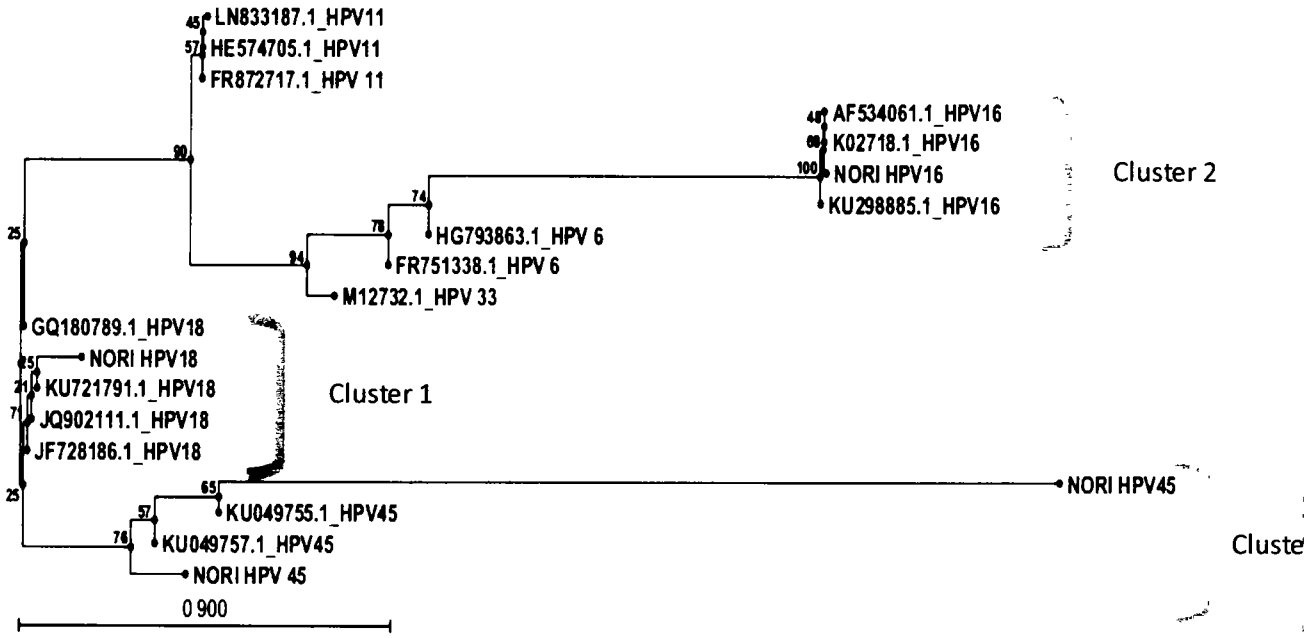


Fig. 3.10. Phylogenetic Tree of Core Sequence of HPV:

Three sequences (HPV 16, HPV 18, and HPV 45) compared with fifteen sequences from NCBI of different genotype and origin.

Chapter

Discussion

Human Papillomavirus is the principal cause of death in females due to cancer. It is genetically very diverse group of viruses that is evolving very rapidly. The infections of HPV are more prevalent in developing countries. Pakistan being a developing country bears a significant portion of infectious diseases, but like many other sexually transmitted diseases. HPV screening is not a common practice. This study intended to get a clear picture about molecular characterization of Human Papillomavirus in Pakistan. Being a dense populated area Pakistan contributes significantly toward world data base of infectious diseases. But unfortunately, no authentic data is available about types and nature of HPV prevalent in Pakistan.

Human papillomavirus (HPV) is a chief source of death and morbidity as it causes cancer of cervix. The aim of this study was to assess the prevalence of HPV in general infectious patients visiting gynaecology outpatient departments of PIMS, Islamabad and Sheikh Zayed Hospital, Rahim Yar Khan. The results presented offer a first insight into awareness about HPV and the need to establish effective screening technology for it. Data analysis showed 10% (10/100) HPV infected patients in the population assessed. This prevalence is lower than global HPV prevalence (11.7%) reported by Bruni *et al.*, 2010. It has also been reported previously that the prevalence of HPV varies from 10-30% (Dunne *et al.*, 2007; Franco *et al.*, 1999; Shin *et al.*, 2003; Pham *et al.*, 2003; Thomas *et al.*, 2004; Stamataki *et al.*,2010). The reason for the relatively low prevalence is likely due to monogamous behaviour in our society where number of partners is relatively limited. HPV infection is considered to be very transient and many studies have shown the disappearance of this infection within a few years (Evander *et al.*, 1995).

Incidence of high risk HPV genotype 16 was found in 1% and HPV 18 was found 2% (1/100 and 2/100 respectively). These values are also lower than those reported 5.2% high risk HPV type in Europe by Clifford *et al.*, In addition, the results are also against author's large age-range, namely 15–74 years (Clifford et al *et al.*, 2006).

In the present study, the study population comprised only married women and hence the prevalence of HPV infection in the premarital period was not studied. It was against medical practitioner's ethics to invite unmarried women to participate in the study and usually cervical smears were not taken from them unless they presented with a severe

complaint at a hospital. The exclusion of unmarried women can possibly be a reason for the lack of age related decline of HPV in this study. In our study we observed that incidence of HPV is higher 6 (60%) in age group lied between of21-35 years as compare to 36-50 years 3 (30%) and >50years 1(10%). These findings are inconsistence with Manhart *et al.*, 2006 that higher incidence of HPV observed in sexually active females.

In our study, HPV positive women who reported 3 or more full term pregnancies were at a twofold increased risk of HPV infection compared nulliparous (having zero parity) women. This statement is consistent with previous studies reported that, there was still a twofold increased risk when women reporting seven or more pregnancies were compared with HPV positive women who reported one to two full term pregnancies (Muñoz *et al.*,2002, Deacon *et al.*, 2000, Kjaer *et al.*,1988). In the results obtained in our study, out of 10 HPV positive patients, 1(10%) patients were those who had completed 2 full term pregnancy, 2 (20.0%) patients had undergone 3 full term pregnancies, 4 (40%) patients completed 6 full time pregnancies while 3(30%) patients completed 8 full term pregnancies each. The graph indicates a direct proportionality of HPV infection with increasing parity.

However, the prevalence of HPV was not influenced by the time period between menarche and age at marriage. This is a window period for premarital sexual activation and possibly the HPV infection in the present study population was mostly attained after marriage. If it is true, then the infection is likely to persevere as constant sexual transmission from the husband takes place, as in our study the highest incidence of HPV was found in population who was married for 20 years or less.

We observed difference in the use of contraceptive and risk of acquiring HPV infection but the difference is not statistically significant. These results are supported by previous studies (Bosch *et al.*, 2002). Out of 10 positive patients, 5 had never used any means for contraception, while 2 (20%) used oral contraceptives and 3 (30%) were using intra-uterine devices.

Type HPV 16 is most prevalent types globally. The 90% of invasive cervical cancers are due to persistent infection with high-risk HPV worldwide Two third of these cases are due to HPV 16 and up to 90% of HPV-associated extra-cervical tumours (Burk *et al.*,2011). All HPV 16 isolates are closely related, previous studies inferred five distinct phylogenetic branches among HPV 16 variants: Kuwait (KU), Korea (KO), and African-1 (Af-1)

corresponding to the geographic locations from which the samples were obtained (Chen *et al.*, 2005). Our NORI HPV16 lie in the same cluster as all other HPV variants showing high sequence similarity.

Our sequence NORI HPV18 lied in the cluster with sequences obtained from Thailand (GQ), Netherland (KU), China (JQ) and Italy (JF) showing close homology with the HPV 18 sequences obtained in the Asian and European regions. Two sequences of HPV 45 from United Kingdom (KU) were selected and two sequences NORI HPV 45 obtained in our study were closely linked in a cluster.

Our HPV phylogenetic analysis showed that 21 Nucleotides are substituted with respect to reference sequence. This finding points to the possibility of functional differences between variants within each type, which is of relevance for epidemiological, etiological, pharmaceutical, and vaccination research.

The sequences from HPV downloaded from NCBI and accession numbers with regional details and their years of isolation given in Appendix 2. The phylogenetic analysis was carried out by Mega 5 shows in (Fig. 3.11). The NJ tree of HPV PAK (1) with other all HPV reference sequences was constructed. Resulted NJ tree showed three clusters numbered as 1, 2 and 3 with our obtained sequences. Two clusters showed HPV subtypes that were not found in our study.

The study provides some new data on the genetic diversity of HPV subtypes which may help to understand the oncogenic potential of the virus and to improve management of patients as this knowledge is widely used for research in vaccine development. Genomic sequence analysis followed by phylogenetic analysis for assigning genotype is time-consuming and inconvenient for clinical use (Verbeeck *et al.*, 2005) and not adapted to clinical studies on large-scale routine use.

Chapter

CONCLUSION FUTURE RECOMMENDATIONS

The proportion of women infected with human papillomavirus (HPV) varies greatly across populations, as might the distribution of HPV types. In the present study, we detected different HPV subtypes, especially HPV 16 and 18 the two types that cause the most HPV-associated cancers, are prevalent in non-cancerous population in Pakistan. Furthermore, we have found an association of HPV infection with increasing parity; it has been established to be prevalent in population which is sexually active.

Reliable diagnosis of HPV infection, particularly the 'high-risk' types (16/18), may facilitate early identification of 'high-risk' populations for developing cervical cancer and may augment the sensitivity and specificity of primary cervical cancer screening programmes by complementing the conventional Pap test.

In a pooled analysis, the population-based sampling and centralized HPV-testing protocols from the International Agency for Research on Cancer (IARC) HPV prevalence survey to compare HPV-type distribution in representative samples of women from 13 areas in 11 countries across sub-Saharan Africa, Asia, South America, and Europe (14). HPV-screening assays identify a large group of women without cytological abnormalities, many of whom harbour HPV types with very different progressive potential. Thus, testing for only a subset of high-risk types might improve cost-effectiveness.

Population-based data for HPV-type distribution is prerequisite to development of new HPV-screening tests and to assessment of the effect of future vaccination on HPV infections of differing severity, but these data are limited or missing for many world regions. Furthermore, relevant studies are difficult to combine or compare because of varying study designs, especially in enrolment of women and HPV-testing protocols.

Serious efforts are being made towards developing different effective prophylactic vaccine strategies to control HPV infection. The ultimate therapeutic goal for all HPV-associated malignancies would be development of HPV-specific therapeutics, including small molecules targeted to viral oncoproteins and therapeutic HPV vaccines designed to augment cellular immune responses to viral oncoproteins. The Food and Drug Administration (FDA) has recently approved three vaccines to prevent HPV infection: Gardasil®, Gardasil® 9,

and Cervarix®. These vaccines provide strong protection against new HPV infections, but they are not effective at treating established HPV infections or disease caused by HPV (Hildesheim *et al.* 2007; Schiller *et al.* 2012).

Chapter

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Appendix I

Detergent buffer for DNA extraction:

TRIS HCL (Trisaminomethane hydrochloride) pH 8.3	10mM
KCl	50mM
$MgCl_2$	2.5mM
Triton X100	0.45%
Tween (Polysorbate) 20	0.45%

All ingredients were dissolved in 100 ml of water and volume was make upto 500ml.

Appendix II

1x loading dye (Per ml Water):

Bromophenol Blue	4.16 mg
Xylene Cyanol	4.16 mg
Sucrose	0.66 g

Mix all ingredients and volume was make up to 1 ml.

Appendix III

8% Polyacrylamide Gel:

30% acrylamide	2.7ml
10x TBE buffer	1ml
Ammonium persulfate (APS)	70uL
TEMED	3.6uL
Water	6.194 ml

All ingredients were mixed and immediately poured in casting tray.

Appendix IV

10x TBE (Tris- Borate- Ethylene diamine tetra acetic acid) Buffer pH 8.3 (500ml):

89 mM Tris	54g
89 mM Boric acid	27.5g
2 mM EDTA	4.65g

All the above materials are mixed and volume was make up to 500 ml by adding water.

CONSENT FORM

Please tick as appropriate:

- *I am the patient*
- *I am the patient's relative/parent*

Relation to the patient: ----------

I give this researcher/student my consent to use or disclose my protected health information to carry out research for screening and characterization of Human papillomavirus in Pakistani population.

I have been informed that I may review complete description of uses and disclosures before signing this consent.

I understand that I have the right to request a restriction of how my protected health information is used. However, I also understand that the researcher/student is not required to agree to the request. If the researcher/student agrees to my requested restriction, they must follow the restriction (s).

I understand the following:

1. The material will be published without my/the patient's name attached and every attempt will be made to ensure my/the patient's anonymity. I understand, however, that complete anonymity cannot be guaranteed.
2. The material will be published in Journal/Book/any other mode.
3. The material will not be used for advertising or packaging.

Signed: _____

Name: _____

Date: __/__/__.

Sample no. _____

Patient Information

Name:			
Age:	DOB:	Sex: M	F
Marital status:	Spouse's name:		
No. of Children:	Names:		
Address:			
City:	Phone # (home):		
Phone#(mobile):			
Hospital:			
Physician's name:			

Which of the following conditions are you currently being treated or have been treated for in the past(please tick):

☐ Heart disease/Murmur/Angina	☐ Shortness of breath	☐ Eye disorder
☐ Diabetes	☐ High cholesterol	☐ Asthma
☐ Seizures	☐ Kidney/Bladder problems	
☐ High blood pressure	☐ Lung problems/cough	☐ Stroke
☐ Liver problems/Hepatitis	☐ Low blood pressure	☐ Arthritis
☐ Sinus problem	☐ Headaches/Migranes	☐ Seasonal allergies
☐ Neurological problems	☐ Cancer	☐ Tonsillitis
☐ Anemia or blood problems	☐ Depression/Anxiety	☐ Ulcers/colitis
☐ Swollen ankles	☐ Ear problems	
☐ Psychiatric care	☐ Thyroid problems	

Please describe any current or past medical treatment not listed above:

_____

Please list your past surgeries:

_____

_____

Clinical Presentation:

Vaginal discharge: Yes/No

Color of discharge: _____ Frequncy: _____

Abdominal pain: Yes/No Fever: _____

Other signs: _____